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DEVELOPMENT OF A SCALABLE PROCESS FOR LENTIVIRAL VECTOR MASS PRODUCTION BY TRANSIENT TRANSFECTION

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DEDICATION

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RÉSUMÉ

Les vecteurs lentiviraux (LVs) sont considérés comme des véhicules de transfert prometteurs pour des applications en thérapie génique. Cependant, il y a un manque évident de stratégies de production efficaces et transposables à grande échelle. En effet, les techniques actuelles de production ne pourront pas suffire à la génération du nombre de LVs nécessaires pour les évaluations en phases cliniques et éventuellement pour leur commercialisation en cas de succès des essais.

Dans ce travail, nous avons tout d'abord fait le point sur les méthodes actuelles de production des LVs et fait ressortir leurs contraintes intrinsèques. Jusqu'à ce jour, la production routinière de LVs se fait presqu'exclusivement à petite échelle avec des cellules adhérentes. Il est clair que ce mode de production ne sera pas suffisant pour répondre aux demandes futures en LVs. Afin de faciliter les essais cliniques et la production de LVs à des fins thérapeutiques après certification par les autorités de règlementation, il apparaît important de mettre au point de nouvelles stratégies de production à grande échelle et permettant l'obtention de vecteurs à hautes concentrations.

Une technologie de production de LVs par transfection transitoire de cultures en suspension de la lignée cellulaire HEK293 a été développée. Dans ce procédé transposable à grande échelle, l'opération en mode perfusion a permis de résoudre la problématique associée à la faible stabilité fonctionnelle des LVs. La combinaison de plusieurs approches, incluant la transfection à haute densité cellulaire, la sélection de formulations avancées de milieux de culture et l'ajout de butyrate de sodium en tant qu'additif activateur d'expression, a permis d'augmenter significativement les productivités volumétriques et spécifiques. Ainsi, il a été possible d'augmenter de 100 fois le taux de LVs fonctionnels, que ce soit à petite ou à grande échelle, permettant d'atteindre des titres fonctionnels maximaux en LVs avoisinant les 10^8 unités de transduction (tu)/mL.

Les cinétiques de production ont été évaluées en utilisant plusieurs méthodes de quantification des LVs. Ainsi, on a pu observer que les titres maximaux en LVs fonctionnels sont atteints deux jours après transfection. Ces résultats ont également démontré que la qualité des LVs produits (la qualité étant définie ici comme le rapport entre le nombre de LVs fonctionnels et le nombre de LVs totaux) était généralement faible : de l'ordre de 1 à 4 % du nombre de particules virales

totales (mesurées comme le nombre de génomes viraux associés aux particules virales) étaient fonctionnelles. Une variation de ce pourcentage au cours du temps a été observée suite à l'ajout de butyrate de sodium après transfection. Des expériences complémentaires ont permis de démontrer que la qualité des LVs pouvait être améliorée en réduisant le temps de séjour des particules virales dans le milieu de culture et donc en opérant à plus hauts débits de perfusion. Enfin, nos résultats ont mis en évidence que la cytotoxicité des protéines membranaires VSV-G pouvait être une limitation possible aux rendements de production dans ce système.

Les cinétiques de production des LVs en bioréacteur ont été caractérisées grâce à l'utilisation d'un suivi en ligne du signal de permittivité. Les mesures de permittivité ont permis de suivre des évènements tels que le bourgeonnement des vecteurs viraux ainsi que la variation des propriétés diélectriques des cellules productrices suite au relargage des particules virales. L'évolution de la permittivité a été analysée et reliée à la cinétique de production des LVs afin d'identifier quatre phases caractéristiques post-transfection.

La caractérisation détaillée du système des LVs, effectuée avec des méthodes de quantification hors-ligne et des outils de suivi en ligne, a mis en lumière plusieurs stratégies permettant un meilleur suivi du procédé ainsi que l'optimisation de celui-ci.

La stratégie de production qui a été établie fut dès lors basée sur la transfection à des concentrations cellulaires élevées couplé à un mode de perfusion à haut débit. Cette approche, qui a permis d'obtenir des LVs fonctionnels à de hautes concentrations, pourrait facilement être adaptée à la production d'autres constructions de LVs.

Les stratégies de production développées dans ce travail ont permis de générer des titres viraux avoisinant les 10^{10}-10^{11} tu/L, ce qui correspond à des titres totaux de l'ordre de 10^{12}-10^{13} génomes viraux (vg)/L dans les surnageants de production. Les cinétiques de production et l'évolution du procédé sont bien documentées, ce qui permettra de guider les optimisations futures. En plus de faciliter la production de LVs, ce procédé pourrait être transférable à des installations manufacturières, afin de permettre l'évaluation et l'utilisation de ces vecteurs dans des essais cliniques.

ABSTRACT

Lentiviral vectors (LVs) are promising delivery vehicles for applications in gene therapy. Yet, the field still relies on inefficient and non-scalable production strategies. Current production methods will become a limitation in later clinical evaluation phases and for commercialization when large amounts of LVs need to be generated.

In this work, we first reviewed current LV production methods and point out the constraints intrinsic to these protocols. To date, routine LV production is almost exclusively performed in small scale systems using adherent cells. These protocols will not be able to satisfy the anticipated future demands in LVs. For their widespread testing in clinical settings and for the production of LV-based therapeutics after their approval, novel scalable production strategies are needed to robustly produce these vectors at high yield.

An optimized protocol for LV production was developed using a HEK293 cell line grown in suspension cultures. In this scalable process, production in perfusion mode addressed the low stability of functional LVs. Several strategies such as transfection at high cell density, selection of advanced medium formulations and addition of the expression-enhancing additive sodium butyrate resulted in significant improvements of volumetric and specific productivity. The overall yield in functional LVs was increased by 100-fold and similar results were obtained for small and bioreactor scale cultures, reaching maximum functional LV titers in the range of 10^8 transducing units (tu)/mL.

The production kinetics under improved conditions was then analyzed employing several LV quantification methods. After transfection, highest functional LV titers were reproducibly found 2 days post-transfection. The results also showed that LV quality, as the ratio of functional to total LV particles was generally low with only 1-4 % of the total viral particles (measured as the number of viral genomes) being functional, i.e. having the ability to transfer genetic information. This ratio was not constant over time when sodium butyrate was added after transfection. LV quality was also increased at higher harvest rates. Our results also indicate that the cytotoxic effects of VSV-G might be limiting for further yield improvements of the current LV production system.

LV production kinetics was also characterized using online monitoring of the permittivity signal in bioreactor productions. Permittivity measurements are valuable for identification of events such as budding of viral vectors as dielectric properties of the producing cells are affected during the viral release process. The evolution of the permittivity signal was analyzed and linked to the LV production kinetics to identify four key process transition phases after transfection which are characteristic of LV production.

The in depth process characterization of the LV system with offline LV quantification methods and online tools provided several avenues for process monitoring and further process optimization.

The developed LV production strategy is based on high cell density transfection. The approach delivers LVs at high yield in a timely manner and should be easily adaptable to other LV constructs.

The strategies developed in this work provide unprecedented yields of 10^{10}-10^{11} tu/L, corresponding to 10^{12}-10^{13} viral genomes (vg)/L of production supernatant. Production kinetics and process evolution are well characterized, which will guide future yield optimization. As it facilitates the production of LVs, the developed process should thus enable the evaluation and use of these vectors as a therapeutic and should be an attractive option to generate LV for future clinical trials.

CONDENSÉ EN FRANÇAIS

La thérapie cellulaire et la thérapie génique sont des secteurs très prometteurs pour le traitement de plusieurs maladies potentiellement mortelles et pour lesquelles peu de traitements alternatifs existent. Le traitement des anomalies génétiques, du cancer et certaines stratégies de vaccination font partie des applications les plus importantes. La thérapie génique a été développée sur la base de progrès considérables accomplis au cours des vingt dernières années lors de recherches menées sur les maladies génétiques (Rosenzweig 1999). Le principe général repose sur l'introduction d'une copie fonctionnelle d'un gène défectueux ou absent dans une cellule, afin d'éliminer la maladie engendrée par ce dernier (McTaggart et Al-Rubeai 2002).

Pour assurer le transfert de l'information génétique, des véhicules permettant de livrer des transgènes de façon efficace et sécuritaire au sein des cellules cibles sont requis. Ces véhicules (ou vecteurs) peuvent être d'origine virale ou non. Des vecteurs viraux ont été développés à partir de différentes souches virales naturelles.

L'essor de la thérapie génique a été considérablement ralenti à cause d'incidents survenus durant les programmes d'évaluation clinique. Malgrè tout, près de 1500 essais cliniques ont à ce jour été effectués avec des résultats expérimentaux démontrant un avenir prometteur pour les vecteurs viraux. Ainsi, la première commercialisation d'un vecteur viral en tant qu'agent thérapeutique a été autorisée en Chine en 2007.

Les vecteurs lentiviraux (LVs) sont considérés comme l'un des véhicules de transfert les plus prometteurs pour les applications en thérapie génique. Les LVs sont la plus récente des classes de vecteurs et sont conçus à partir du virus d'immunodéficience humaine de type 1 (VIH-1). Ces vecteurs sont néanmoins dépourvus des propriétés pathogéniques du VIH-1, ne peuvent ni se répliquer ni transférer des gènes viraux, et ne sont pas reconnus par le système immunitaire des patients. Ils conservent par contre leur capacité de transférer des transgènes (Throm, Ouma et al. 2009). En comparaison avec d'autres vecteurs viraux, ils offrent divers avantages, tels que la possibilité d'accueillir une longue séquence de nucléotides, la capacité à transduire des cellules

ne se divisant pas, ainsi qu'une expression longue et stable du transgène (Cockrell et Kafri, 2007).

Jusqu'à present, les LVs ont démontré leur potentiel en thérapie génique et sont utilisés dans au moins 25 études cliniques (D'Costa, Mansfield et al. 2009). Le premier essai clinique utilisant des LVs a été approuvé en 2002. Plusieurs autres traitements contre des maladies infectieuses et génétiques utilisant des LVs sont actuellement en cours, ont été approuvés ou sont en préparation (D'Costa, Mansfield et al. 2009). Les LVs sont donc un outil important pour le developpement d'avancées significatives dans le traitement de nombreuses pathologies. Celles-ci regroupent les maladies neurodégénératives, telles que l'adrénoleucodystrophie (ALD) et la maladie de Parkinson ainsi que la β-thalassémie et sa forme plus sévère, la drépanocytose. Enfin, le traitement de certains cancers, après transduction de cellules ayant des fonctions immunologiques manquantes chez le patient, est également une approche thérapeutique pour laquelle des essais cliniques sont développés (D'Costa, Mansfield et al. 2009).

L'utilisation des LVs est jusqu'à présent limitée exclusivement à des applications *ex vivo* sur les cellules des patients. Les approches i*n vivo* doivent tenir compte du large tropisme cellulaire des LVs pseudotypés avec la glycoprotéine G du virus de la stomatite vésiculaire (VSV-G). Ce large tropisme empêche le ciblage du vecteur à un type cellulaire bien défini, suscitant des inquiétudes quant à la possibilité de transduction non-spécifique de cellules telles que les cellules dendritiques, souches et germinales.

Bien que les stratégies basées sur les LVs présentent un large potentiel pour de nombreuses cibles thérapeutiques, leur utilisation intensive est fortement tributaire de la capacité de produire ces vecteurs en grandes quantités. Actuellement, la production des LVs à haut rendement demeure une contrainte à l'application clinique des stratégies thérapeutiques élaborées experimentalement. Les méthodes actuelles de production couramment employées présentent de faibles rendements et de nombreuses limitations en terme de mise à l'échelle puisqu'elles reposent sur la culture de cellules adhérentes. Les protocoles de production des LVs demeurent largement empiriques; les

procédés ne sont généralement pas bien caractérisés au niveau de la productivité cellulaire, des titres maximums pouvant être atteints, des cinétiques de production, du temps de récolte optimal et de la stabilité du vecteur. Il semble donc évident que ces méthodes de production rudimentaires ne soient pas directement adaptables à une production à grande échelle, car la robustesse et la reproductibilité de ces procédés est insuffisante pour la fabrication industrielle des LVs. En effet, les techniques actuelles de production ne pourront pas suffire à la génération du nombre de LVs nécessaires pour les évaluations en phases cliniques et éventuellement pour leur commercialisation.

L'objectif principal de cette thèse est l'identification des paramètres critiques pour la production de LVs dans des cultures de cellules HEK293 en suspension, permettant ainsi le développement d'un procédé transposable à plus grande échelle et la production de LVs à hautes concentrations. Les méthodes actuelles de production de LVs souffrent de faibles rendements et il reste plusieurs questionnements au niveau de la productivité et des étapes de production. La caractérisation de ces différentes étapes reste à faire. Il est, par ailleurs, attendu que le rendement fonctionnel des LVs avec ce système peut être amélioré de façon significative à l'aide d'un suivi en ligne et hors ligne et d'un contrôle des paramètres critiques du procédé.

Le but ultime de cette recherche est d'identifier, de suivre et de contrôler des paramètres critiques qui déterminent la production efficace des LVs et qui permettent le développement d'un procédé robuste et transposable à grande échelle. Plus spécifiquement, ces travaux visent:

1) L'identification des conditions du procédé qui ameliorent la productivité des LVs et le développement d'une stratégie d'optimisation détaillée en vue d'améliorer les techniques actuellement utilisées pour la production de LVs
2) La caractérisation des cinétiques de production pour guider l'identification de stratégies permettant une succession d'opérations robustes et optimisées
3) La mise en évidence de liens entre les cinétiques de production virale et des mesures effectuées en ligne afin de permettre le suivi en temps réel du procédé

Dans ce travail, nous présenterons tout d'abord une revue de la littérature approfondie, qui couvre les propriétés des systèmes LVs actuels, ainsi que leur quantification et qui fait le point sur les méthodes actuelles de production, tout en soulignant les contraintes intrinsèques de celles-ci.

Jusqu'à ce jour, la production routinière de LVs se fait presqu'exclusivement à petite échelle avec des cellules cultivées en adhérence. Néanmoins, des améliorations significatives ont été obtenues à ce niveau. En effet, bien qu'ils soient longs, fastidieux et sujets à une grande variabilité, les protocoles actuels de production utilisant des flacons statiques (T-flasks) permettent tout de même d'obtenir des concentrations suffisantes pour supporter des recherches à petite échelle. Toutefois, en raison de la large gamme de protocoles de production et de méthodes de quantification retrouvés dans la littérature, il s'avère difficile de comparer les résultats provenant des différents groupes de recherche. De plus, les approches d'optimisation s'appuyant sur des principes de génie biochimique sont rarement retrouvées dans la littérature couvrant le sujet. Les procédés existants ne sont donc pas adaptés pour répondre aux demandes futures en LVs. Afin de permettre des essais cliniques et la production de LVs à des fins thérapeutiques, il apparaît primordial de mettre au point de nouvelles stratégies efficaces de production à grande échelle et permettant avec obtention de vecteurs à hautes concentrations.

Dans ce travail, une stratégie de production de LVs par transfection transitoire a tout d'abord été développée avec des cultures de la lignée cellulaire HEK293 en suspension. Dans ce procédé facilement transposable à grande échelle, il est apparu que la production en mode perfusion permettait de résoudre la problématique associée à l'instabilité fonctionnelle des LVs.

Par la suite, plusieurs stratégies comme la transfection à haute densité cellulaire, la sélection de formulations avancées de milieu de culture et l'ajout de butyrate de sodium en tant qu'additif stimulant l'expression ont permis d'augmenter significativement les productivités volumique et spécifique.

Plus spécifiquement, l'utilisation d'une approche de transfection à haute densité cellulaire a permis d'augmenter la productivité volumique par un facteur cinq. Par la suite, une optimisation

des conditions de transfection transitoire au polyéthylènimine (PEI) dans un milieu plus performant a permis de doubler le rendement de la méthode. Les meilleurs résultats ont été obtenus avec un ratio massique en PEI:DNA de 2:1 et 0.4-0.6 µg d'ADN total/10^6 cellules utilisées pour la transfection dans le milieu de culture HyClone SFM4Transfx-293 (HyQ) medium. Cette technique a permis de réduire les effets cytotoxiques du PEI et d'augmenter le rendement de la méthode. Il est à noter que de toutes les conditions évaluées, l'ajout de butyrate de sodium a eu l'effet le plus significatif sur le titre fonctionnel, en augmentant de quinze fois le rendement total. Ainsi, il a été possible d'augmenter de plus de 100 fois le taux de LVs fonctionnels, que ce soit à petite ou à grande échelle, permettant ainsi d'atteindre des titres maximums avoisinant les 10^8 unités de transduction (tu)/mL dans le surnageant non-concentré. Il a également été démontré que le procédé est transposable à la culture en bioréacteur tout en conservant un rendement similaire, et ce jusqu'à une échelle de 3 L. Les résultats suggèrent qu'avec ce procédé à haut rendement, une seule production dans une unité pilote permettrait de produire la quantité de LVs requises pour la réalisation d'essais cliniques de phase I.

Dans le cas du système modèle étudié, pour obtenir un rendement total de l'ordre de 0.5 à $1x10^{10}$ tu à petite échelle, la meilleure stratégie consisterait à effectuer la production à une densité cellulaire d'environ 5 à $10x10^6$ c/mL en effectuant 2 changements de milieu par jour. Ces conditions permettraient ainsi d'obtenir de hauts rendements totaux, tout en préservant la qualité des LVs. Les surnageants cellulaires pourraient ensuite être congelés pour de futures étapes de purification ou être directement concentrés par ultracentrifugation.

À l'échelle de bioréacteur et pour l'obtention de rendements supérieurs à $1x10^{11}$ tu, le choix d'opérer avec changement(s) de milieu dépend fortement de la possibilité d'effectuer adequatement les étapes de purification subséquentes, ainsi que de l'importance accordée à la qualité des LVs.

Changer l'équivalent de deux volumes de réacteur par jour (2 VVD) permet d'obtenir des LVs de qualité supérieure par rapport à une production réalisée à 1 VVD. Des LVs en quantité deux fois

plus importantes sont retrouvés dans le surnageant à 2 VVD, mais la période de conservation à 4°C est limitante en raison de l'instabilité des vecteurs. En outre, la congélation de larges volumes est difficilement réalisable. Ainsi, c'est la stratégie de purification qui doit dicter si la production avec de nombreux échanges de milieu est avantageuse ou non.

Il est important de noter que les rendements totaux en LVs sont fonction du système d'expression et du transgène d'intérêt; les rendements absolus peuvent dès lors significativement différer des valeurs observées pour le modèle utilisé dans ce travail. Nous avons décrit une démarche générique de production de LVs pouvant s'appliquer pour l'amélioration des constructions de LVs et leur évaluation à petite et à grande échelle. La stratégie de perfusion à plus grande échelle peut également faciliter le développement des technologies reliées, telles que le passage à grande échelle des procédés de purification qui était initialement limité par l'incapacité de générer de grands volumes de surnageant contenant des LVs concentrés.

Toutefois, même en utilisant des conditions améliorées de production, la productivité cellulaire spécifique en vecteurs viraux fonctionnels demeure faible (60-120 tu/cellule). Nos résultats ont d'ailleurs permis de mettre en évidence que les rendements de production dans ce système sont limités par les effets cytotoxiques de VSV-G. Si ces observations sont confirmées avec d'autres systèmes d'expression, le développement et l'utilisation de lignées cellulaires stables serait alors questionnable, puisque la production des LVs par ces systèmes pourrait également être limitée par les effets toxiques de VSV-G.

La production de LVs est également fonction de la disponibilité des nutriments dans la culture. Toutefois, un travail plus approfondi est requis pour comprendre l'influence de l'état métabolique des cellules sur les rendements de production. Des résultats préliminaires ne permettaient pas de déterminer si le procédé actuel était limité par des contraintes d'ordre métabolique ou par une efficacité de transfection à haute densité cellulaire.

Les cinétiques de production à petite échelle et en bioreacteur ont été évaluées initialement en utilisant les méthodes de quantification des LVs. Les titres maximaux en LVs fonctionnels ont été observés deux jours après transfection. Ces résultats ont également montré que la qualité des LVs, définie comme étant le rapport entre le nombre de LVs fonctionnels et le nombre de LVs totaux, était généralement faible, soit à peine 1 à 4 % du nombre de particules virales totales (mesurées comme le nombre de génomes viraux). Une variation de ce pourcentage au cours du temps a été remarquée suite à l'ajout de butyrate de sodium après transfection. La qualité des LVs a pu être également améliorée via des taux de récoltes plus élevés.

Les méthodes actuelles de quantification représentent une contrainte importante pour le développement du procédé, puisqu'elles sont pour la plupart longues, fastidieuses et génèrent des résultats très variables. Il y a un urgent besoin de standardisation et de nouvelles méthodes de quantification pour les vecteurs lentiviraux sont requises. Pour ce faire, la précision et la rapidité des différentes méthodes sont des paramètres critiques à considérer. Actuellement, la quantification du titre des LVs par la RT-PCR semble être l'approche directe la plus efficace afin d'obtenir un nombre total de particules. Au niveau des méthodes indirectes, nos résultats ont révélé l'importance de contrôler le nombre de passages cellulaires afin de diminuer significativement la variabilité des mesures de titres de LVs fonctionnels.

Afin de permettre le suivi en temps réel du procédé de transfection transitoire, il est nécessaire d'identifier des outils d'analyse en ligne capables de détecter le relargage et les cinétiques de production des vecteurs viraux. De tels outils facilitent grandement le développement des procédés, supportent leur optimisation et l'implantation de la démarche PAT (« Process Analytical Technologies »). Cette dernière vise à soutenir l'innovation dans les procédés pharmaceutiques, en vue d'améliorer les critères de production et d'assurance de qualité. Cette démarche vise donc à améliorer la robustesse des procédés et à faciliter le contrôle en ligne de la qualité du produit. Les cinétiques de production en bioréacteur ont pu être suivies en cours de culture grâce à une mesure en ligne de la permittivité. L'évolution de la permittivité a ainsi permis d'identifier quatre phases de transition caractéristiques associées à la production de LVs après transfection. Les résultats du suivi en ligne ont reflété d'une manière reproductible les

cinétiques de production des LVs pour plusieurs productions en bioréacteur, utilisant des conditions d'opération différentes. Des changements relatifs pendant la phase de production ont montré une corrélation avec le rendement total en termes de génomes viraux de ces productions en bioréacteur. Les mesures de permittivité ont permis de suivre des évènements tels que le bourgeonnement des vecteurs viraux ainsi que la variation des propriétés diélectriques des cellules productrices suite au relargage de particules virales. Cette technologie constitue donc un bon outil pour le criblage des conditions d'opération et la caractérisation du procédé.

La caractérisation détaillée du système des LVs, effectuée avec des méthodes de quantification hors-ligne et des outils de suivi en ligne, a mis en lumière plusieurs stratégies permettant un meilleur suivi du procédé ainsi que l'optimisation de celui-ci. Les améliorations les plus significatives peuvent être espérées au niveau moléculaire, telles qu'une augmentation de la stabilité des LVs fonctionnels, une diminution de la cytotoxicité de l'enveloppe ou encore des stratégies de régulation de VSV-G. Des enveloppes de LVs alternatives ont été décrites dans le but de les utiliser pour l'obtention des titres viraux plus élevés (Verhoeyen et Cosset 2004; Cronin, Zhang et al. 2005). Par contre, les LVs qui ne sont pas pseudotypés avec VSV-G sont limités à des applications et à des cibles cellulaires spécifiques.

D'autres possibilités pour améliorer le procédé sont également envisageables au niveau du choix des conditions opératoires. Une meilleure caractérisation du métabolisme des cellules HEK293 dans le milieu HyQ permettrait de développer des stratégies d'alimentation permettant d'augmenter la densité cellulaire lors de la transfection, sans perte de productivité spécifique. Dès lors, il semble nécessaire d'effectuer des études métaboliques et des travaux portant sur l'optimisation du milieu afin d'augmenter les taux de LVs produits. Par exemple, des concentrations potentiellement inhibitrices en lactate ont été observées lors des cultures en perfusion. En raison du manque de méthodes simples et fiables pour quantifier les LVs, il est recommandé de réaliser cette étude en utilisant des systèmes de transfection pour lesquels la production protéique peut être plus facilement mesurée.

Des travaux portant sur la production de vecteurs rétroviraux suggèrent que le contenu lipidique cellulaire joue un rôle critique dans la production des vecteurs (Merten 2004; Coroadinha, Alves

et al. 2006; Coroadinha, Ribeiro et al. 2006; Coroadinha, Silva et al. 2006). Comme la concentration en lipides dans les vecteurs produits semble déterminer leur stabilité, il serait intéressant d'étudier spécifiquement les effets de la concentration en lipides du milieu de culture sur la production et la stabilité des LVs (Beer, Meyer et al. 2003; Carmo, Faria et al. 2006; Coroadinha, Alves et al. 2006). Aussi, d'autres études pourraient porter sur l'exploitation des signaux en temps réel pour des fins de contrôle et de réglage du procédé. Par exemple, le signal de permittivité pourrait être utilisé pour contrôler directement le taux de récolte. L'alimentation en milieu pourrait également être contrôlée en fonction du signal de permittivité avant transfection, ce qui permettrait de minimiser la consommation du milieu de culture et la formation de métabolites toxiques.

Les stratégies développées dans ce travail ont permis de générer des titres viraux avoisinant les 10^{10} à 10^{11} tu/L, ce qui correspond à des titres de l'ordre de 10^{12} à 10^{13} génomes viraux (vg)/L dans les surnageants non-concentrés de cultures. Les cinétiques de production et l'évolution du procédé sont bien documentées, ce qui permettra de guider les optimisations futures. Le procédé développé rend ainsi possible l'évaluation et l'utilisation de ces vecteurs en tant que thérapie et pourra être une option intéressante pour la production future de LVs à des fins cliniques.

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NOMENCLATURE

α:	'Cole-Cole a' empirical parameter describing the decrease in permittivity with increasing frequency
AAV:	Adeno-associated virus vectors
Ampho:	MLV 4070A envelope
AZT:	Azidothymidine
BBS:	N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffered saline
BIV:	Bovine immunodeficiency virus
BR:	Stirred tank bioreactor
*bv, bv*r:*	biovolume, biovolume multiplied by the cell radius (from *d*)
CAp24 ELISA:	Enzyme-linked immunosorbent assay targeting the LV capsid protein p24
CB5:	Medium supplement cell boost 5
CB5 mod:	Modified formulation of cell boost 5, compatible with transfection
CB5 reg:	Regular formulation of cell boost which is inhibitory for transfection
cGMP:	Current good manufacturing practice
C_M:	Capacitance per membrane area
CMV:	Cytomegalovirus
COS-1:	COS-1 african green monkey kidney cells
COS-7:	COS-7 african green monkey kidney cells
(c)PPT:	(Central) polypurine tract
CTS:	Central termination sequence
CV-1:	CV-1 african green monkey kidney cells
d:	Mean cell diameter
$\Delta\varepsilon_{FC}$, $\Delta\varepsilon_{0.6MHz}$:	Dual-frequency permittivity signal

$\Delta\varepsilon_{max}$:	Dielectric/permittivity increment
DEPC:	Diethylpyrocarbonate
DMD:	Sequential discontinuous medium exchange per day
DMEM:	Dulbecco's modified Eagle's medium
DMEM-0:	DMEM without serum
DMEM-10:	DMEM + 10 % FBS
DNA:	Deoxyriobonucleic acid
DO:	Dissolved oxygen
dox:	Doxycycline, tetracycline analog
dpt:	Day(s) post-transfection
Ecd-on:	Ecdysone/ponasterone A-response system
EIAV:	Equine infectious anaemia virus
EMEA:	European Medicines Agency
EYFP:	Enhanced yellow fluorescent protein
fc:	Characteristic frequency
FIV:	Feline immunodeficiency virus
FDA:	Food and Drug Administration
G418 :	Geneticin
Gag-Pol/*gag*:	*Gag*-encoding plasmid packaging construct/the *gag* protein
GALV:	Gibbon ape leukemia virus
GFP :	Green fluorescent protein
GFP+ :	Quantification of GFP-positive cells
GTA:	Gene transfer assay
HBS:	HEPES buffered saline
HCD:	High cell density

HEK293:	Human embryonic kidney cell line
HIV:	Human immunodeficiency virus
HOS:	Human osteosarcoma cells
hpt:	Hours post-transfection
HT1080:	Human fibrosarcome cell line
HyQ:	HyClone SFM4Transfx-293™, commercial serum-free medium formulation
HyQ+:	HyClone SFM4Transfx-293™, supplemented with 5 % (v/v) of CB5 mod, a modified formulation of cell boost that does not inhibit transfection
ICH:	International Conference on Harmonisation
IEX :	Anion exchange chromatography purification
lacZ(co):	(Codon-optimized) bacterial lacZ
LCD:	Low cell density
LCR:	Locus control region
LC-SFM L:	Low calcium containing, serum free culture medium including lipid mixture
LC-SFM GL:	Low calcium containing, serum free culture medium including lipid mixture and gelatine peptone
LTR:	Long terminal repeat
LV(s):	Lentiviral vector(s)
MT4:	Human T cell lymphotropic virus-I (HTLV-I) carrying human T cell line
N:	Cell density
n.a.:	Not available
nlacZ :	Nuclear localized β-galactosidase enzyme
PAT:	Process analytical technology

PB:	Polybrene®
PEI:	Polyethylenimine
UC:	UltraCULTURE™ , commercial serum free medium formulation
YFP+:	Quantification of YFP-positive cells
IMDM:	Iscove's modified Dulbeccos's medium
MLV:	Moloney Leukemia virus
OSR:	Oxygen sparging rate
P:	Membrane enclosed volume fraction
PCR/RT-PCR:	Polymerase chain reaction (RT = real time)
RCL(s):	Replication competent lentiviruses
RD114:	Feline endogenous virus RD114 envelope
Rev/*rev :*	*Rev*-encoding plasmid construct/ *rev* protein
(m)RNA:	(messenger) Ribo nucleic acid
RRE:	*Rev* responsive element
RRV:	Ross River virus
rT:	Reverse transcriptase
RV:	Retroviral vectors
SEC :	Size exclusion chromatography
SFV:	Semliki Forest virus
σ_i:	Conductivity of the cytoplasm/intracellular conductivity (mS cm^{-1})
SIN:	Self inactivating
SIV:	Simian immunodeficiency virus
σ_m:	Conductivity of the medium (mS cm^{-1})
spec.prod.:	Specific productivity (tu/c), based on cell density at transfection

$t^1/_2$:	Half-life of functional LV (h)
Tet:	Tetracycline
Tet-off:	Tet-inducible system: tet/dox removal required for induction/transcription
Tet-on:	Tet-inducible system: tet/dox addition required for induction/transcription
tu:	Transducing units
VG titer:	Total LV particles quantified by RT-PCR as viral genomes (vg)
vif:	Virus infectivity factor
VP titer:	Total LV particles quantified by CAp24 ELISA as the capsid protein p24
vpr:	Viral protein R
VSV-G:	Vesicular stomatitis virus glycoprotein G/its plasmid construct
VVD:	Volume(s) of medium per reactor volume per day
WHO:	World Health Organization
WPRE:	Woodchuck hepatitis virus posttranscriptional regulatory element

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INTRODUCTION

Background

Gene and cell therapy are fields holding tremendous promises as they offer therapeutic strategies for a wide variety of diseases. To date, approximately 1500 gene therapy clinical trials have been completed, are ongoing or have been approved (Edelstein, Abedi et al. 2007; http://www.wiley.co.uk/genetherapy/clinical/). A first gene therapy product has now been approved in China (Peng 2005). The treatment of genetic dysfunctions, cancer and vaccination strategies are at the forefront of possible applications. Gene therapy is based on the significant progress that has been made in understanding the genetic basis of several diseases over the last 20 years (Rosenzweig 1999). Its original principle is defined as the introduction of a normal (functional) copy for the correction of a defective or missing gene to eliminate disease symptoms (McTaggart and Al-Rubeai 2002).

All gene and many cell therapy approaches require the use of gene transfer vehicles (vectors). An ideal vector allows for the safe and efficient transfer of genetic information to the patient. Non-viral or viral vectors are used as delivery vehicles. The latter were found to be extremely efficient and have been consequently more extensively studied. The respective advantages of each viral vector are a function of the underlying biology of the virus that determines the main vector characteristics such as immunogenicity, packaging capacity, expression of the transgene, infectivity and cellular or tissue tropism.

Although conceptually straightforward, the practical development of gene therapy interventions remains challenging. A fatal incident during a gene therapy clinical trial occurred at the turn of the century and led to the death of one patient in a study conducted in 1999 (Raper, Chirmule et al. 2003). Only a few months later the results of a different trial where 3 out of 10 treated patients suffering from a severe immune deficiency (SCID-X1) developed leukemia several years after the treatment, were made public (Hacein-Bey-Abina, Von Kalle et al. 2003). However, in all treated patients, this study provided proof of principle that gene therapy can work. These incidents and other results from ineffective gene therapy clinical trials marked important setbacks for the entire field (Steinbrook 2007; Iaccino, Schiavone 2008). The scientific community also remains sceptical on the effectiveness of the first commercialized gene therapy intervention in China, partly due to the difficulty in comparing the clinical outcomes (Ma, Shimada et al. 2009).

As a consequence, research in the gene therapy area mainly shifted back from clinical applications to experimental research (Müller-Röber, Hucho et al. 2008). The main focus in the gene therapy field is now on designing better vectors with the goal to overcome the disadvantages of these early-generation gene delivery vehicles.

Lentiviral vectors (LVs) are one of the most recently developed forms of virus-derived gene delivery vectors. LVs were designed based on the biology of human immunodeficiency virus-1 (HIV-1). For that purpose, the molecular architecture of HIV-1 was dramatically altered to remove pathogenic properties, avoid replication of the vector and emergence of replication competent lentiviruses (RCLs) but conserve its natural ability for gene transfer. LVs are therefore replication-deficient, do not transfer any viral genes and recipients lack a pre-existing immune response to the vector (Throm, Ouma et al. 2009). In comparison to other viral vectors, they offer several advantages, including a large packaging capacity, the ability to transduce non-dividing cells and a stable, i.e. life-long, transgene expression (Cockrell and Kafri 2007).

To date, LVs have already demonstrated their potential for gene therapy in clinical studies and are under investigation in approximately 25 clinical trials (D'Costa, Mansfield et al. 2009). The first clinical trial using LVs was approved in 2002. Several others targeting infectious and genetic diseases are currently in progress, have been approved or are in preparation (D'Costa, Mansfield et al. 2009). Interestingly, HIV infection is to date one of the preferred targets in ongoing clinical trials using LVs, exemplifying that the system came a long way since its initial development. These therapies are based on RNA interference (RNAi) and delivery of an anti-HIV payload gene into autologous CD4+ T lymphocytes with the goal to inhibit HIV-1 replication (D'Costa, Mansfield et al. 2009).

LVs hold much promise for a wide range of applications. Disease targets that are currently under investigation comprise neurodegenerative disorders such as adrenoleukodystrophy (ALD) and Parkinson's disease, β-thalassemia and its more severe form, sickle cell anemia. Cancer therapy, after transduction of cells with immune functions, is another promising area in which clinical trials are in development (D'Costa, Mansfield et al. 2009).

To date, LVs are almost exclusively limited to *ex vivo* administration into patient cells. *In vivo* approaches face the challenge that the widely used vesicular stomatitis virus glycoprotein G (VSV-G) pseudotyped LVs have a wide tropism, which renders a targeted delivery to a certain

cell type impossible and raises concerns on transduction of off-target cell types such as dendritic, stem and germ cells.

Research Problem, Hypothesis and Objective

Although LV-based strategies hold great promise for a wide variety of targets, their extensive use highly depends on the ability to efficiently generate these vectors in large amounts. To date, LV mass production remains an important constraint for the translation of therapeutic straegies to the clinic. Current routine LV production methods have low yields and are limited in terms of their scalability. Protocols to produce LVs are largely empirical and the processes are generally not well characterized with cellular productivity, peak titers, production kinetics, optimum harvest points and vector stability being only rarely determined. It can be anticipated that these current rudimentary protocols are not readily amenable to large scale production and that they would result in insufficient process robustness to support industrial manufacturing.

The driving hypothesis of this work is that the identification of critical parameters for the production of LVs in suspension-grown HEK293 cells allows for the development of a scalable process delivering LVs at high yield. Current LV production methods suffer from low yields as several issues that determine productivity in a critical manner are not sufficiently addressed by the process strategy. Also, it is believed that the functional LV yield of this system could be improved significantly by online and offline monitoring and controlling the critical process parameters that dictate productivity.

The overall objective of this work is to identify, monitor and/or control parameters that are critical for the efficient generation of LVs and that allow for the development of a scalable and robust production process. More specifically, this work aims to:

1) identify process conditions resulting in enhanced LV productivity to propose and develop a detailed optimization strategy for the improvement of the current state of the art of LV production
2) characterize the production system and its kinetics to guide strategies that will help in defining a robust and optimized operation of the process
3) link the viral release and production kinetics to the results from online permittivity measurements to allow for monitoring and supervision of LV production in real-time

Thesis Structure

The thesis can be divided in four main parts:

1) an extensive literature review (chapter 1)
2) a methodology section (chapter 2)
3) the development of a scalable process delivering high yields (chapter 3)
4) the characterization of the LV production process (chapters 4 and 5)

The first chapter was published as a review article entitled **"Recent progress in lentiviral vector mass production"** in the ***Biochemical Engineering Journal***. In this paper, current strategies for LV production are critically assessed and compared.

The set of methods used during this research project is described in detail in chapter 2 **"Methodology for scalable LV production and process characterization"**. This chapter also comprises a short summary and error assessment of LV quantification methods.

Chapter 3 was published as a research article entitled "**Development of a scalable process for high-yield lentiviral vector production by transient transfection of HEK293 suspension cultures"** in the ***Journal of Gene Medicine***. The paper describes an optimization strategy that translated to improvements in LV yields by 100-fold under scalable operating conditions**.** In this approach, production in perfusion mode was successfully used to continuously harvest functional LV thereby addressing the critical problem of low LV vector stability.

In chapter 4, entitled **"Characterization of lentiviral vector production kinetics using offline quantification methods"** the process kinetics was assessed to further characterize the production system and to identify intrinsic limitations and further possible optimization strategies. In chapter 5, the application of permittivity measurements for real-time monitoring of LV production is presented. The chapter is entitled **"Monitoring lentiviral vector production kinetics using online permittivity measurements"** and has been submitted to the ***Journal of Biotechnology*****.**

Finally, complementary experiments are summarized in the appendices. This last section is divided in four parts. A preliminary study on LV stability in culture supernatants can be found in appendix I. Process limits and operating windows of high cell density LV production by transient transfection are presented in appendix II. Appendix III is presented as a draft manuscript entitled

“Practical applications of online permittivity signals: characterization of HEK293 batch growth and identification of metabolic shifts” and introduces permittivity measurements as real-time monitoring tool for mammalian cell cultures. Appendix IV finally summarizes additional relevant experimental observations that are not integrated in the main chapters of the thesis.

CHAPTER 1 LITERATURE REVIEW: RECENT PROGRESS IN LENTIVIRAL VECTOR MASS PRODUCTION

1.1 Article Presentation

The goal of this thesis is to improve current production methods of lentiviral vectors in order to develop strategies that will be applicable for LV mass production for clinical trials and commercialization. This chapter provides a critical review of studies reporting LV production and summarizes the progress which was made in the field since HIV-1 based LVs were first described in 1996. The performance of routine and advanced protocols is compared and it is pointed out that the LV production methods currently in place are limited in terms of their scalability. The review thus allows understanding the constraints of current LV production strategies and emphasizes the need for improved and scalable production strategies that are later presented in this thesis.

1.2 Recent Progress in Lentiviral Vector Mass Production

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1.2.1 Abstract

Lentiviral vectors (LVs) are promising tools for gene and cell therapy. They are presently used in several clinical trials as *in vivo* or *ex vivo* gene delivery vectors. However their mass production remains a challenge and might limit their potential therapeutic use. New robust and scalable processes are required for industrial production of these vectors.

In this review, we focus on the assessment of current LV production methods and evaluate the most critical limitations with a focus on scalability. The key properties of LVs are described and their inherent advantages and disadvantages discussed. A brief overview of the quantification

methods generally used to characterize vector production is also provided as well as indications on downstream processing and basic regulatory aspects.

The recent developments in the field including production in serum-free suspension cell cultures indicate that LV production is now amenable to industrial manufacturing using reliable large-scale processes.

1.2.2 Introduction

Worldwide, more than 20 clinical trials are currently investigating the use of lentiviral vectors (LVs) for gene delivery targeting a variety of diseases (http://www.wiley.co.uk/genetherapy/clinical/). In particular the ability of LVs to transduce non-dividing cells render them an important tool for the treatment of neurological and lympho-hematological disorders (Quinonez and Sutton 2002; Salmon and Trono 2002; Azzouz, Kingsman et al. 2004; Cockrell and Kafri 2007). LVs provide efficient delivery, integration and long-term expression by establishing a stable provirus in target cells (Quinonez and Sutton 2002). These properties led to a widespread use in research and clinical environments for *in vitro* and *in vivo* gene transfers (Cockrell and Kafri 2007).

With the increasing use of LVs in translational research programs, scalable, effective and robust LV production processes become of critical importance. Processes that can be swiftly transferred to manufacturing sites once LV-based vectors are ready for use in approved clinical trials are needed. As demonstrated for other viral vectors-based therapeutics, these processes are a fundamental requirement for the successful advancement of LV-based therapeutic interventions (Wright 2009). Current production methods do, however, not meet these requirements, show only low yields and are typically not easily scalable (Stewart, Leroux-Carlucci et al. 2009).

This review focuses on the mass production of LVs to provide sufficient material for clinical trials. Current production methods are compared, in particular for their scale-up potential, with respect to robust LV manufacturing. First, we briefly summarize the most important properties of lentiviruses as those determine LV functions, expression kinetics and vector stability. Then we introduce the different methods for LV quantification, compare production strategies and provide general indications on LV downstream processing methods and regulatory aspects. Since LV

systems based on human immunodeficiency virus (HIV)-1 are by far the most advanced to date, we will focus on this vector family.

LV elements are encoded by several plasmid constructs or expression cassettes (Fig 1.1). Vector particles are assembled in *trans* from constructs without most viral *cis*-acting sequences. The packaging construct codes for the core and enzymatic components of the usually HIV-1 derived virion. A second construct is responsible for expression of the viral envelope which is most commonly VSV-G (envelope construct). The viral *cis*-acting sequences are delivered by an expression cassette which encodes for the transgene of interest (transfer vector construct). These constructs need to be simultaneously expressed in a respective producer cell. We briefly summarize the most important properties and functions of these constructs and refer to extensive and more detailed reviews wherever possible.

Most recent LV production systems use 3rd generation plasmid constructs (Dull, Zufferey et al. 1998; Vigna and Naldini 2000; Quinonez and Sutton 2002) (Fig. 1.1 A). Whereas *tat*-dependent systems may result in mobilization of vectors upon transfer of *gag-pol* and *env* or HIV-1 superinfection (Bukovsky, Song et al. 1999), *tat* is removed in these constructs and its function replaced by a heterologous strong promoter (e.g. CMV). The accessory gene *nef* can be removed from LV constructs pseudotyped with the most commonly used pH-independent envelopes (Aiken 1997; Luo, Douglas et al. 1998; Chazal, Singer et al. 2001).

VSV-G is by far the most often used LV envelope (Fig. 1.1 B). This envelope protein has been already used in retroviral vector (RV) constructs to broaden vector tropism (McClure, Marsh et al. 1988; Akkina, Walton et al. 1996). Using an unknown, but ubiquitously found receptor on animal cells, the uptake of VSV-G pseudotyped LV then takes place by a receptor-mediated endocytic pathway (Coil and Miller 2004). VSV-G increases particle resistance to shear forces allowing vector particle processing by ultracentrifugation and its presence increases the thermostability of LVs produced by transient transfection (Aiken 1997; Carmo, Dias et al. 2009). However, LVs pseudotyped with VSV-G produced in human cells are inactivated by human serum complement (DePolo, Joyce D. Reed et al. 2000), and alternative envelopes for clinical *in vivo* applications of LVs have been suggested. Pseudotyping with other envelopes has been extensively reviewed elsewhere (Verhoeyen and Cosset 2004; Cronin, Zhang et al. 2005). To address the limitation of the widely used VSV-G pseudotyped LVs to transduce quiescent cells in

the G0 phase of the cell cycle, recent findings indicate that LVs pseudotyped with measles virus glycoproteins and CXCR4-tropic HIV envelope are able to transduce quiescent T cells (Ramezani and Hawley 2002; Frecha, Costa et al. 2008). Other pseudotypes allow for the targeted transduction of certain organs, subsets of cells in tissue, or impart the vector with neural retrograde transport properties (Sena-Esteves, Tebbets et al. 2004; Verhoeyen and Cosset 2004; Wong, Azzouz et al. 2004).

The third construct (transfer vector) contains the required *cis*-acting sequences (3' and 5' LTR, packaging signal (Ψ), *rev* responsive element (RRE)) and typically a reporter gene such as GFP that can be replaced by the therapeutic transgene of interest (Fig. 1.1 C). *Tat*-independent 3rd generation packaging plasmids are most often employed in combination with self inactivating (SIN) vectors (Miyoshi, Blomer et al. 1998; Zufferey, Dull et al. 1998; Iwakuma, Cui et al. 1999). These constructs render transcription of full length RNA unlikely even in the presence of viral proteins. The desired transgene is expressed of an internal, heterologous promoter. These vectors reduce the risk of RCL formation (Miyoshi, Blomer et al. 1998; Iwakuma, Cui et al. 1999). Without titer loss, it is possible to insert a transgene with a size of ~8 kb in current 3rd generation transfer vectors. Several conserved regions are included in current constructs to improve LV functionality. The woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) significantly improves transgene expression when situated in the 3' untranslated region of the coding sequence (Zufferey, Donello et al. 1999; Cockrell and Kafri 2007). The central polypurine tract (cPPT), the central termination sequence (CTS) and the polypurine tract (PPT) incorporated in central or 5' vector location and adjacent to the 3'LTR, respectively, prime and terminate plus strand proviral DNA synthesis following reverse transcription of viral RNA, resulting in elevated transduction efficiencies (Follenzi, Ailles et al. 2000; Sirven, Pflumio et al. 2000; Zennou, Petit et al. 2000; Zennou, Serguera et al. 2001; Cockrell and Kafri 2007).

1.2.3 Lentiviral Vectors

1.2.3.1 Molecular Basis of LVs

1.2.3.1.1 General Properties of Lentiviruses

Lentiviruses form a more complex subclass of retroviruses, with HIV being the most prominent and best studied member. Other viruses of this group include SIV (simian immunodeficiency virus), EIAV (equine anemia virus), FIV (feline immunodeficiency virus) and BIV (bovine immunodeficiency virus). Lentiviruses are RNA viruses in which the genome is packaged into a capsid surrounded by a membranous envelope. The capsid contains the viral RNA genome and three enzymes required for virus replication (reverse transcriptase, integrase and protease). The host-cell derived membrane envelope is protruded with an envelope glycoprotein. This envelope protein is responsible for the binding and entry into the target cell. Upon infection by membrane fusion with the host cell and viral uncoating, viral RNA and the enzymes necessary for reverse transcription of the viral RNA and for integration of the viral cDNA are released. The RNA is then reversely transcribed and integrated into the host genome, with a preference for integration within active transcription units (Schröder, Shinn et al. 2002; Wang, Ciuffi et al. 2007). At the end of the virus life cycle, the full-length viral RNA genome is transported out of the nucleus, captured by the *gag* polyprotein, subsequently packaged, and the virus buds off the cell membrane. The lentiviral provirus sequence is subsequently transferred to progeny during cell division.

Lentiviruses can infect nondividing cells, i.e. unlike oncoretroviruses, they are independent of cellular division to import their genetic information into the host cell. A preintegration complex of several proteins is formed after infection that allows translocating the genetic information into the nucleus (Amado and Chen 1999). Lentiviruses induce chronic and slowly progressive diseases (‘lenti’ means slow), typically associated with infection of macrophages, a terminally differentiated cell type (Vigna and Naldini 2000).

Mature lentiviral particles have a relatively large diameter of 90–130 nm (Higashikawa and Chang 2001; Li, Kimura et al. 2005; Segura, Kamen et al. 2006). Lentiviral particles have a mass

of $\sim 2.5 \times 10^8$ Da and present a density of 1.16 g/mL in sucrose density gradients (Vogt and Simon 1999).

An infectious wildtype lentiviral particle (e.g. HIV-1) contains a diploid single-stranded positive sense RNA genome with *cis*- and *trans*-acting sequences. *Cis*-acting are non-coding sequences that are required for viral RNA synthesis, packaging, reverse transcription and integration. The *trans*-acting sequences encode for the major structural viral components (*gag*), lentivirus-specific enzymes (*pol*), accessory proteins and the virus envelope (*env)* (Quinonez and Sutton 2002). These three open reading frames are a characteristic of all retroviruses. After translation, the polyproteins are proteolytically cleaved to form the structural and enzymatic components of the virus. Lentiviruses also contain the regulatory genes *rev* and *tat*. *Rev* acts as a virally-encoded post-transcriptional activator (Desmaris, Bosch et al.) and is critical for viral replication (Ramezani and Hawley 2002; Lever, Strappe et al. 2004). The second regulatory gene, *tat*, is a transactivator that enhances the transcriptional activity of the 5' long terminal repeat (LTR) by 100-500 fold. Furthermore, the accessory genes *vpu* and *nef* increase viral release and enhance the viral ability to escape the immune system. *Vif* (virus infectivity factor) overcomes an endogenous cellular inhibitor of viral replication and viral protein R (*vpr*) causes G2 cell-cycle arrest, the cell-cycle phase in which the expression of viral products seems to be optimal (Quinonez and Sutton 2002).

1.2.3.1.2 Development of LVs for Gene Delivery

The main motivation for the development of LVs was to complement the successful features of (onco)retroviral vectors (RV). These include the integration in the chromatin, the absence of transferred viral genes and the lack of pre-existing immunity in the recipient. These features were sought to be combined with the lentivirus-specific ability to infect nondividing cells such as T and hematopoetic stem cells (Throm, Ouma et al. 2009). Lentiviral vectors are a promising tool to treat diseases like blood-cell disorders, stem cell defects, storage and metabolic diseases (Quinonez and Sutton 2002; Kohn 2007). LVs do not transfer the genes for viral packaging proteins and have a low pro-inflammatory activity (Follenzi, Santambrogio et al. 2007). They were used in the study of neurological diseases by *in vivo* gene transfer to the central nervous system of rodents and primates (Kordower, Bloch et al. 1999; Watson, Kobinger et al. 2002; Jakobsson and Lundberg 2006). As LVs are able to efficiently transduce antigen-presenting cells

and in particular dendritic cells with tumor-associated antigens, cancer immunotherapy is another promising application (MacGregor 2001; Breckpot, Dullaers et al. 2003; Dullaers, Meirvenne et al. 2005; Wang, He et al. 2006; Breckpot, Aerts et al. 2007; Lejeune, Truran et al.). Other research applications have been extensively reviewed elsewhere (Quinonez and Sutton 2002; Cockrell and Kafri 2007).

The LV technology has already been evaluated in phase I clinical trials targeting T cells to express an anti-HIV gene and had a promising outcome (Schonely, Afable et al. 2003; Slepushkin, Chang et al. 2003; Levine, Humeau et al. 2006; Kohn 2007). To date, most of the approved clinical trials using LVs are based on the *ex vivo* gene delivery to autologous cells (http://www.wiley.co.uk/genmed/clinical/). In particular, the *in vitro* transduction of hematopoetic stem cells seems to hold great promise for gene therapy applications in the near future (Chang and Sadelain 2007).

LVs are based on the human pathogen HIV-1. Consequently, biosafety improvements were a central issue in the early development of LVs. They were originally designed as replication defective hybrid viral particles generated from the core proteins and enzymes of HIV-1 and the envelope (glycoprotein G) of a different virus, the vesicular stomatitis virus (VSV) (Naldini, Blomer et al. 1996; Naldini, Blomer et al. 1996; Vigna and Naldini 2000). The major goal in subsequent LV development was to increase biosafety by segregation of *cis*- and *trans*-acting sequences. This renders the formation of replication-competent lentiviruses (RCL) by recombination unlikely (Wu, Wakefield et al. 2000; Zufferey and Trono 2000; Ikeda, Takeuchi et al. 2003; Ni, Sun et al. 2005).

The latest 3rd-generation self-inactivating (SIN) LVs include only 10 % of viral genomic RNA which is sufficient to ensure vector functionality (Ramezani and Hawley 2002). This was achieved after removal of all genes that are not essential for gene transfer, transduction into nondividing cells and sustained transgene expression. The biosafety concerns raised over lentiviral vectors have consequently been extensively addressed through improved vector design. Current LV constructs are as safe as RV (Lever, Strappe et al. 2004), and advanced LV constructs successfully prevent the formation of RCL (Escarpe, Zayek et al. 2003; Sastry, Xu et al. 2003; Miskin, Chipchase et al. 2005). Hence a safe use of LVs, as a gene delivery system in therapeutic applications, should be limited to a single transduction event without perturbation of the target

cellular genome. In this context, chromatin insulators were investigated as means to protect gene expression from neighboring enhancers or silencers and to reduce the risk related to viral integration (West, Gaszner et al. 2002; Sinn, Sauter et al. 2005; Hanawa, Yamamoto et al. 2009; Nielsen, Jakobsson et al. 2009).

1.2.3.1.3 LV Expression Constructs

LV elements are encoded by several plasmid constructs or expression cassettes (Fig 1.1). Vector particles are assembled in *trans* from constructs without most viral *cis*-acting sequences. The packaging construct codes for the core and enzymatic components of the usually HIV-1 derived virion. A second construct is responsible for expression of the viral envelope which is most commonly VSV-G (envelope construct). The viral *cis*-acting sequences are delivered by an expression cassette which encodes for the transgene of interest (transfer vector construct). These constructs need to be simultaneously expressed in a respective producer cell. We briefly summarize the most important properties and functions of these constructs and refer to extensive and more detailed reviews wherever possible.

Most recent LV production systems use 3rd generation plasmid constructs (Dull, Zufferey et al. 1998; Vigna and Naldini 2000; Quinonez and Sutton 2002) (Fig. 1.1 A). Whenever *tat*-dependent systems may result in mobilization of vectors upon transfer of *gag-pol* and *env* or HIV-1 superinfection (Bukovsky, Song et al. 1999), *tat* is removed in these constructs and its function replaced by a heterologous strong promoter (e.g. CMV). The accessory gene *nef* can be removed from LV constructs pseudotyped with the most commonly used pH-independent envelopes (Aiken 1997; Luo, Douglas et al. 1998; Chazal, Singer et al. 2001).

VSV-G is by far the most often used LV envelope (Fig. 1.1 B). This envelope protein has been already used in RV constructs to broaden vector tropism (McClure, Marsh et al. 1988; Akkina, Walton et al. 1996). Using an unknown, but ubiquitously found receptor on animal cells, the uptake of VSV-G pseudotyped LV then takes place by a receptor-mediated endocytic pathway (Coil and Miller 2004). VSV-G increases particle resistance to shear forces allowing vector particle processing by ultracentrifugation and its presence increases the thermostability of LVs produced by transient transfection (Aiken 1997; Carmo, Dias et al. 2009). However, LV pseudotyped with VSV-G produced in human cells are inactivated by human serum complement

(DePolo, Joyce D. Reed et al. 2000), and alternative envelopes for clinical *in vivo* applications of LVs have been suggested. Pseudotyping with other envelopes has been extensively reviewed elsewhere (Verhoeyen and Cosset 2004; Cronin, Zhang et al. 2005). To address the limitation of the widely used VSV-G pseudotyped LVs to transduce quiescent cells in the G0 phase of the cell cycle, recent findings indicate that LVs pseudotyped with measles virus glycoproteins and CXCR4-tropic HIV envelope are able to transduce quiescent T cells (Ramezani and Hawley 2002; Frecha, Costa et al. 2008). Other pseudotypes allow for the targeted transduction of certain organs, subsets of cells in tissue, or impart the vector with neural retrograde transport properties (Sena-Esteves, Tebbets et al. 2004; Verhoeyen and Cosset 2004; Wong, Azzouz et al. 2004).

The third construct (transfer vector) contains the required *cis*-acting sequences (3' and 5' LTR, packaging signal (Ψ), *rev* responsive element (RRE)) and typically a reporter gene such as GFP that can be replaced by the therapeutic transgene of interest (Fig. 1.1 C). *Tat*-independent 3rd generation packaging plasmids are most often employed in combination with self inactivating (SIN) vectors (Miyoshi, Blomer et al. 1998; Zufferey, Dull et al. 1998; Iwakuma, Cui et al. 1999). These constructs render transcription of full length RNA unlikely even in the presence of viral proteins. The desired transgene is expressed of an internal, heterologous promoter. These vectors reduce the risk of RCL formation (Miyoshi, Blomer et al. 1998; Iwakuma, Cui et al. 1999). Without titer loss, it is possible to insert a transgene with a size of ~8 kb in current 3rd generation transfer vectors. Several conserved regions are included in current constructs to improve LV functionality. The woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) significantly improves transgene expression when situated in the 3' untranslated region of the coding sequence (Zufferey, Donello et al. 1999; Cockrell and Kafri 2007). The central polypurine tract (cPPT), the central termination sequence (CTS) and the polypurine tract (PPT) incorporated in central or 5' vector location and adjacent to the 3'LTR, respectively, prime and terminate plus strand proviral DNA synthesis following reverse transcription of viral RNA, resulting in elevated transduction efficiencies (Follenzi, Ailles et al. 2000; Sirven, Pflumio et al. 2000; Zennou, Petit et al. 2000; Zennou, Serguera et al. 2001; Cockrell and Kafri 2007).

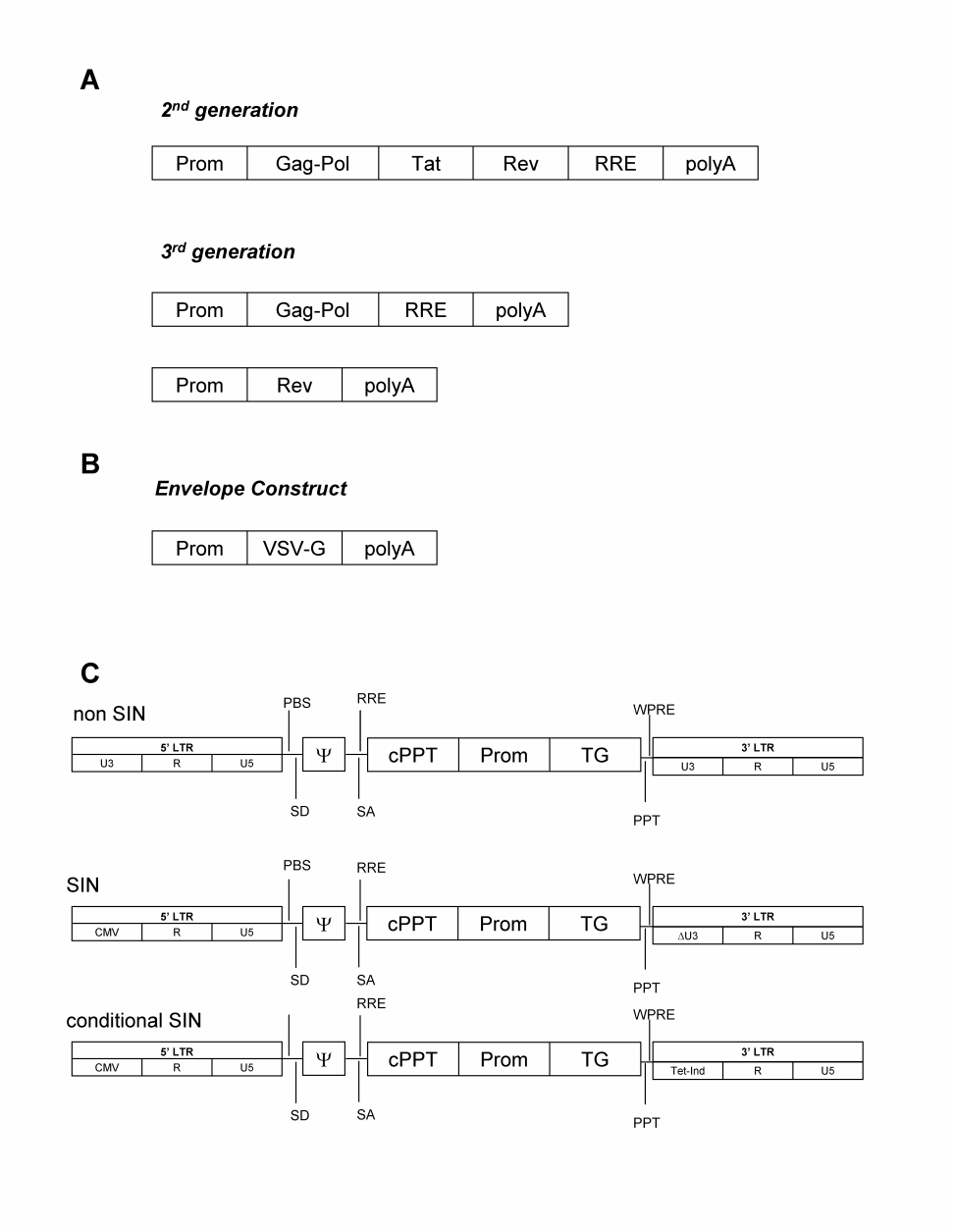

Figure 1.1 : Expression constructs for lentiviral vector production.

To generate LVs, three to four constructs need to be expressed simultaneously in producer cells. Only relevant parts of the constructs are shown.

(A): Lentiviral vector packaging constructs. Only constructs with advanced biosafety features are represented here, i.e. 1st generation constructs including the accessory genes *vif*, *vpr* and *vpu* are omitted. Whereas 2nd generation constructs are *tat*-dependent and in most cases only comprise a

single packaging plasmid, *rev* is placed on a second packaging plasmid in *tat*-independent 3rd generation constructs

(B): Envelope constructs. In most LV systems, VSV-G is used as envelope protein, see text for details on alternative pseudotypes

(C) Lentiviral transfer vector constructs. Early LV systems employed non-SIN vectors . In SIN vector systems, a deletion in the U3 region of the 3'LTR is transferred to the 5'LTR upon reverse transcription. Conditional SIN vectors provide additional safety and allow tissue specific transgene regulation (for review: [2]) or the inducible vector transcription in stable LV producer cell lines.

CTS: central termination sequence; LTR: long terminal repeat; PBS: primer binding site; polyA: Polyadenylation site; Prom: Promoters such as CMV, EFIa, RSV or others; ψ: cis packaging signal; SA/SD: splice donor and splice acceptor site

1.2.4 LV Particle Stability

The stability of LVs is generally low, with a half-life of only 3-18 h at 37 °C (Higashikawa and Chang 2001; Watson, Kobinger et al. 2002; Croyle, Callahan et al. 2004). Nevertheless, they show a better stability than mouse RV (Mühlebach, Schmitt et al. 2003). The viral decay depends on the LV pseudotype and also the presence or absence of serum and is mainly driven by a loss of the capacity to perform reverse transcription (Watson, Kobinger et al. 2002; Croyle, Callahan et al. 2004; Carmo, Panet et al. 2008; Carmo, Alves et al. 2009; Carmo, Dias et al. 2009). LV stability is significantly increased at 4 °C. Only few studies investigated the effect of different parameters on the stability of VSV-G pseudotyped LV. Among the parameters tested were the influence of temperature, pH, freeze-thaw and different serum treatments (Higashikawa and Chang 2001; Croyle, Callahan et al. 2004). Lentiviral vectors were found to have a half-life of ~10 h at 37 °C and were showing biphasic decay at 4 °C. After freeze-thaw, a half life of 3.8 cycles was found for the first 5 rounds of treatment. LVs were stable at pH 7 and showed a marked decrease in stability at pH 6 or 8. The biphasic decay during multiple freeze-thaw cycles and temperature incubation is explained by the occurrence of two viral vector populations with different properties within the same vector preparation (Higashikawa and Chang 2001; Croyle, Callahan et al. 2004). LVs pseudotyped with amphotropic envelopes rapidly lose their infectivity

in non-optimized storage buffer formulations. Their stability can be increased significantly by the addition of serum, proteins such as albumin, lipoproteins and lipids (Carmo, Alves et al. 2009).

1.2.5 Quantification of LVs

Since this review focuses on production of LVs, it is essential for the understanding of the challenges and improvements in LV production processes and for a critical assessment of the yields reported in the literature, to provide an overview of quantification methods for LVs. These methods can be broadly divided in two categories: First, direct methods detect viral particles or major structural components in culture supernatants. Second, indirect methods or functional assays are most often cell-based biological assays that assess viral vector function after transduction of suitable target cells.

1.2.5.1 Quantification of LVs in culture supernatants (direct methods)

To assess the number of LV particles after production, methods for the direct quantification of LV components in supernatants are frequently employed. These assays comprise the quantification of viral RNA content (as RNA molecules or viral genomes (vg) per mL) by PCR (RNA titer), reverse transcriptase (RT) activity measurements (mU RT/mL) or quantification of the capsid protein CAp24 by ELISA (Eberle and Seibl 1992; Sastry, Johnson et al. 2002; Tonini, Claudio et al. 2004; Geraerts, Willems et al. 2006). The most common of these assays is probably the CAp24 ELISA although it is the least reliable for evaluation of functional vector particles and subject to high variability (Geraerts, Michiels et al. 2005; Ricks, Kutner et al. 2008). Results are typically reported as the mass amount of CAp24 protein per volume of supernatant (pg/mL) or as the calculated number of total/physical particles (TP/PP) (Naldini, Blomer et al. 1996; Farson, Witt et al. 2001; Beyer, Westphal et al.). Generally, only a small amount of the total produced viral particles is functional (Higashikawa and Chang 2001; Mitta, Rimann et al. 2005). All detection methods for total LV particles therefore largely overestimate functional titers. Various sources can additionally give false positive results in these assays, e.g. carryover of DNA from vector production (when estimating RNA titer) or unincorporated CAp24 that is not related to viral particles (ELISA), in particular when crude vector preparations are titered (Ricks, Kutner et al. 2008). Direct methods are consequently mainly used for normalization of vector preparations prior to transduction, for screening of high producer clones during the development of stable LV

packaging and producer cell lines, for vector optimization (Geraerts, Michiels et al. 2005) and to evaluate and validate the quality of vector preparations (via the ratio of functional to total LV particles).

1.2.5.2 Functional assays (indirect methods)

To assess the number of infectious particles, assays need to be performed after transduction of target cell lines. Typically, the cell line and exact protocol employed depend on the final application. For example, transduction-enhancing additives such as Polybrene® (PB) or cell cycle arrested (e.g. mitomycin C treated) cells (Chang, Urlacher et al. 1999) might be used in these assays. Transduction efficiencies are generally cell-line dependent. Functional titrations of LV preparations are consequently most often tested on two cell lines, using the cell line of interest for the final application (e.g. hematopoetic stem or other primary cells) and a universally used cell line with a well established protocol to allow for titer comparisons. Among the universally used cell lines which have been tested for transduction, HEK293 cell variants show a higher efficiency than HeLa and Mus dunni cells (Sastry, Johnson et al. 2002). Transduction efficiency is generally cell-line dependent with long term transgene expression depending on the characteristics of the transduced cell (Chang, Urlacher et al. 1999). Other candidates include the HT1080 (Böcker, Rossmann et al. 2007), NIH 3T3 (murine embryonic fibroblast) (Cronin, Zhang et al. 2005) and other cell lines that were tested for LV production such as TE671 human rhabdomyosarcoma cells (Chang, Urlacher et al. 1999)

In general, transduction protocols need to be stringently standardized as minor modifications can result in significant titer differences. Vector titer can vary by up to 50-fold when modifying conditions of the transduction protocol such as inoculum volume (non-proportional effect on titer), type and number of target cells, vector stability and length of vector exposure to target cells (Zhang, Metharom et al. 2004). Transduction titers need to be estimated after dilution to ensure that the final MOI is <<1, resulting in a low percentage of transduced cells. This procedure minimizes the risk of multiple integrations in target cells and ensures assay linearity (Geraerts, Willems et al. 2006; Kutner, Zhang et al. 2009). Transduction assays also have inherent limitations because not each particle that transduces a target cell causes transgene expression. Expression from non-integrated vector forms can occur (Wu 2004; Nightingale, Hollis et al. 2006), but only 10-18 % of the transduced RNA genomes integrate in host-cell DNA, with the

large majority being degraded after reverse transcription (Butler, Hansen et al. 2001; Van Maele, De Rijck et al. 2003). Transgene expression might also not be detectable if insertion takes place in regions of reduced transcriptional activity. Furthermore, transduction inhibitors like proteoglycans in non-purified material can negatively impact transduction efficiency (Le Doux, Morgan et al. 1996).

Several days after transduction, the target cells are tested for LV gene transfer. The detection can be performed at several levels, quantifying cellular genomic DNA (proviral DNA), cellular mRNA or the target protein expression level. PCR methods offer the flexibility to detect LVs at all levels of their life cycle if suitable targets are selected. Common target sequences include well conserved regions in current HIV-1 vector constructs such as WPRE and LTR (Geraerts, Willems et al. 2006; Böcker, Rossmann et al. 2007). For a useful overview on PCR-based assays and other methods for LV quantification, extensive work is available (Delenda and Gaillard 2005; Sastry, Johnson et al. 2002; Geraerts, Willems et al. 2006).

If a reporter gene such as GFP or β-galactosidase is present in the LV construct, a fast quantification by FACS analysis or fluorescence microscopy as GFP transducing units (gtu) or transducing units (tu) per mL is preferable (Kuate, Wagner et al. 2002; Geraerts, Willems et al. 2006). Due to its simplicity, this method is the most popular mean to quantify functional LVs. When GFP is used, the method assumes that all integrated vectors create an expression that is above the detection threshold of assay. If only the percentage of GFP-positive cells is quantified, these assays are not sensitive for multiple transgene copies in target cells. The mean fluorescence intensity of transduced cells does, however, correlate with the GFP copy number per genome (Zhang, La Russa et al. 2004). Control transductions including LV inhibitors such as AZT should be used to rule out pseudo-transduction that is defined as false GFP-positive cells detected because of diffusion of GFP or transfer of GFP-encoding DNA (Liu, Winther et al. 1996; Nègre, Mangeot et al. 2000; Nash and Lever 2004). Other, less frequently used, methods include drug resistance assays in which results are reported as colony forming units (CFU)/mL) (Metharom, Takyar et al. 2000; Tonini, Claudio et al. 2004).

1.2.6 Production of LVs

Production protocols for LVs were first described by transfecting adherent 293T cells with three different plasmids (Naldini, Blomer et al. 1996; Naldini, Blomer et al. 1996). Although the interest in these vectors in research and development steadily increased over the years, protocols for the production have undergone only slight modifications. Comparatively small quantities of vectors are required for standard research purposes. Conventional protocols can generate supernatants that lead after concentration to small volumes of LV preparations with titers of ~10^9 tu/mL. This is sufficient for most *in vitro* experiments and for *in vivo* testing in small animal models (Klages, Zufferey et al. 2000). Protocols for the production of LVs are therefore still rudimentary, largely empirical and poorly standardized with the large majority based on production in adherent 293T cells, rendering a transfer to industrial mass production impractical. Although LVs mostly are and, in the near future, will be used for *ex vivo* transduction, the total vector amount for early phase clinical trials is in the range of at least 10^{11}-10^{12} functional particles. Production in large batch preparations will also clearly be advantageous, minimizing the number of tedious quantification, RCL assays and other contaminants. With several candidates moving towards advanced clinical trials, there is consequently an increasing need of scalable production strategies. In the following sections, current protocols and strategies for the production of LVs will be reviewed and compared.

Two production strategies for LVs are currently used: 1) transient transfection with several plasmid constructs and 2) expression of required vector components in stable, inducible packaging cell lines. The development of a stable packaging cell line is time-consuming but is generally expected to lead to higher yields. A significantly faster and cost-effective approach is transient gene expression/transfection. It offers advantages in terms of flexibility and overall process time. Improved scalable transfection protocols should allow producing enough material for phase I clinical trials (Derouazi, Girard et al. 2004; Ansorge, Lanthier et al. 2009). Both strategies will be discussed and compared in the following, in particular with respect to their large-scale applicability for the production of LVs.

1.2.6.1 Transient Transfection for the Production of LVs

Transient transfection is defined as the unstable insertion of DNA into host cells. For in-depth reviews on transient transfection in mammalian cells, readers are referred to excellent work by others (Wurm and Bernard 1999; Baldi, Muller et al. 2005; Pham, Kamen et al. 2006). Commercially available cationic lipids (LipofectAMINE, FuGENE, 293fectin, etc.) are classically used in small-scale transfection experiments and result in high expression levels (Pham, Kamen et al. 2006). However, their elevated costs preclude their use for large-scale applications. Large-scale production is until now only economically feasible when using either calcium phosphate ($CaPO_4$-) precipitation or polyethylenimine (PEI) mediated transfection.

In the past, transfection was commonly regarded as a non-scalable technology. Its use in combination with suspension-grown cells has now been established for the large-scale production of biopharmaceuticals and viral vectors (Durocher, Perret et al. 2002; Geisse and Henke 2005; Park, Lim et al. 2006; Pham, Kamen et al. 2006; Durocher, Pham et al. 2007; Hildinger, Baldi et al. 2007). It is therefore an attractive alternative for the rapid production of recombinant proteins and viral vectors within 24-72 h (Naldini, Blomer et al. 1996; Wurm and Bernard 1999; Blesch 2004). As medium exchange is necessarily required for $CaPO_4$-precipitation, PEI is becoming the agent of choice for large-scale transfection campaigns (Pham, Kamen et al. 2006). Although it shows cytotoxic effects (Godbey and Mikos 2001; Kunath, von Harpe et al. 2003; Sun, Goh et al. 2006), it offers advantages in terms of simplicity of use and compatibility with currently used media and is most suitable for large-scale applications as an exchange of medium is often not necessary (Pham, Kamen et al. 2006; Toledo, Prieto et al. 2009).

Efforts are still needed to optimize the volumetric productivities of transient transfection protocols. One critical limitation is that current transfection protocols are confined to rather low cell densities of up to 2×10^6 c/mL (Derouazi, Girard et al. 2004) with most optimized protocols being in the range of only $0.5-1\times10^6$ c/mL at the time of transfection (Durocher, Pham et al. 2007). In order to increase process yields, strategies that allow for operation at high cell density and/or an increase in cell specific productivity are needed (Ansorge, Lanthier et al. 2009).

An advantage of transfection protocols for LV production is the possibility of using cytotoxic/cytostatic transgenes and/or vector components. This holds for many HIV-1 derived

proteins and for the commonly used VSV-G envelope glycoprotein (references in section 1.4.2 and (Bartz, Rogel et al. 1996)). Various transgenes of interest and envelope glycoproteins with alternative cell tropisms can be tested within a short period of time.
Table 1.1 compares published transfection protocols for LV production. The methods are divided into conventional and improved methods. Conventional methods include calcium phosphate-based or cationic lipid-mediated transfection in adherent HEK293T cells cultured in serum containing standard medium (Follenzi and Naldini 2002). Improved methods encompass all the protocols which reported significant improvements, resulting in higher yields, better cost-effectiveness or standardization.

Several mammalian cell lines have been successfully used for LV production. Among those are several variants of HEK293 cells (293T and subvariants like FT and T/17, 293E6E, 293SF) but also COS-1, COS-7, CV-1, HeLa, HT1080 TE671 ((Chang, Urlacher et al. 1999; Ikeda, Takeuchi et al. 2003) and see Table 1.1). Compared to 293T, COS-7 and CV-1 were found to result in less efficient LV production (Reiser, Harmison et al. 1996; Smith and Shioda 2009). HeLa cells secrete less CAp24 for a given level of RNA expression than 293T and HT1080 (Ikeda, Takeuchi et al. 2003). In contrast, COS-1 have been described as an advantageous packaging host system for small scale production of high quality LVs. They adhere strongly to culture surfaces, producing packaging cell contamination-free supernatant after transfection with a higher amount of functional relative to total particles at similar titers (Smith and Shioda 2009). This system is therefore an interesting alternative for automated high throughput functional screens based on lentiviral vectors for which additional purification steps are impractical.

HEK293 variants remain the most widely used host system for LV production. They are advantageous because of their human origin and provide a safe track record in the production of RV. It is possible to adapt HEK293 for growth in suspension and they show only a moderate dependence on accessory genes such as *vif*, *vpu*, *nef* to generate infectious virus (Garnier, Côté et al. 1994; Côté, Garnier et al. 1998; Farson, Witt et al. 2001). The cell density reported in the literature for LV production varies and depends on the employed production medium. In adherent cell cultures, optimal densities were found to be 2.1×10^7 cells/15 cm plate (18-24 h prior to transfection) (Sena-Esteves, Tebbets et al. 2004), whereas transfection of suspension cultures is typically performed at $\sim1\times10^6$ c/mL. Recently, LV production after transfection at high cell

density (5×10^6 c/mL) was described (Ansorge, Lanthier et al. 2009). Although these formulations will be an absolute necessity for LV mass production, serum and animal component-free media were only evaluated in a handful of publications (Reiser 2000; Sena-Esteves, Tebbets et al. 2004; Segura, Garnier et al. 2007; Ansorge, Lanthier et al. 2009; Kuroda, Kutner et al. 2009).

Other transfection agents than $CaPO_4$ and PEI have been tested to generate LVs but either gave lower yields for multi-component lipid systems (Karolewski, Watson et al. 2003; Mitta, Rimann et al. 2005) or are not widely used because the associated costs are much higher than for protocols based on $CaPO_4$ or PEI (Coleman, Huentelman et al. 2003). These methods are not suitable for mass production of LVs and are consequently not mentioned in Table 1.1. PEI has been only employed in recent work but was already demonstrated to give more reliable results (Segura, Garnier et al. 2007; Ansorge, Lanthier et al. 2009; Kuroda, Kutner et al. 2009; Toledo, Prieto et al. 2009). $CaPO_4$ protocols can result in highly efficient transfection but results may be inconsistent when critical parameters are not stringently monitored. For example, slight shifts in pH, precipitation kinetics and impurities in starting material were shown to reduce transfection efficiency dramatically (Kuroda, Kutner et al. 2009; Toledo, Prieto et al. 2009). For PEI-based transfection, process conditions such as the PEI:DNA ratio and the amount of polyplexes per cell differ in the literature and seem to depend on the employed system (media, cell line, plasmid constructs, product) (Durocher, Perret et al. 2002; Derouazi, Girard et al. 2004; Backliwal, Hildinger et al. 2007; Durocher, Pham et al. 2007). Optimization of these parameters results in several-fold productivity improvements (Durocher, Perret et al. 2002; Ansorge, Lanthier et al. 2009).

Another important cost-driving factor for large-scale transfection protocols is the amount of DNA required to achieve efficient transfer of the genetic information to producer cells. Highly pure DNA preparations need to be employed, corresponding to spectrometric ratios of (260/280) nm superior to 1.8 (Tom, Bisson et al. 2007). Commercial kits are now widely accepted for plasmid purification and were demonstrated to result in similar LV titers compared to cesium chloride and ethidium bromide based protocols (Karolewski, Watson et al.). The amount of recombinant DNA used for the production of LVs is generally high for $CaPO_4$-based methods, with a wide range from ~1-15 $\mu g/1\times10^6$ cells. As the DNA mass to transfected cell ratio can be significantly

reduced using PEI, it appears also from that perspective to be the most cost-effective transfection agent, with only 0.4-3.5 μg of DNA/1×10^6 cells. Accordingly, recent protocols based on transfection in suspension cultures employ DNA amounts which are 20-30 fold lower than in conventional protocols (Segura, Garnier et al. 2007; Ansorge, Lanthier et al. 2009), thereby reducing costs and also the likelihood for RCL formation by recombination.

The optimal mass ratio for multi-plasmid transfection with the LV constructs has been evaluated in a number of studies. It is a consistent finding that a higher mass ratio of transfer vector plasmid DNA compared to the other packaging components leads to highest titers (Mitta, Rimann et al. 2005; Segura, Garnier et al. 2007). These ratios are, however, dependent on the respective vector constructs and vary in the literature (Table 1.1).

LV titers generally decrease with increasing vector insert size and improved biosafety, with 3rd generation systems showing lower yields than 1st and 2nd generation constructs (Al Yacoub, Romanowska et al. 2007). Differences of several orders of magnitude have been reported for other envelopes than VSV-G that are possibly less cytotoxic (Sena-Esteves, Tebbets et al. 2004). Similar findings are reported for cases in which the transgene of interest shows cytotoxicity because high levels of transgene expression in LV producer cells are typical for VSV-G pseudotyped systems (Segura, Garnier et al. 2007).

Assessing the cell-specific productivity is often difficult for LV production protocols. For production in adherent cells, the exact cell density at the time of transfection is rarely determined or productivity is reported based on the size of the culture dish only (Kuroda, Kutner et al. 2009). The cell density was therefore in several cases extrapolated and sometimes the volume of the harvested supernatants estimated to compare the yields of the different methods in Table 1.1.

It was previously reported that the cell specific productivity after transfection is approximately 50-500 fg CAp24/cell×d, resulting in average cell culture yields of 100-1000 ng CAp24/mL×d for 2-3 days, corresponding to 10^6-10^7 tu/mL after titration on HeLa cells (Farson, Witt et al. 2001). These values are well in the range of the yields found for all studies that form a part of the present review, with average cell specific titers of 1-50 tu/cell×d. Only one study reported titers that are several orders of magnitude higher than these expected values, possibly due to the use of CHO K1 cells for titration or the concentration of LVs before titration (Mitta, Rimann et al. 2005).

Table 1.1: Comparison of transient transfection protocols for LV production

System (Cell Line for Production, Culture Medium[1])	LV system (generation, vector type, transgene)[2]	Transfection (method, details[3])	Plasmid ratio[4]	Cell Number/ Density at Transfection ($x10^6$ cells, $x10^6$ cells/mL)[5]	DNA amount ($\mu g/10^6$ cells)	Remarks[6] additive, concentration	Titration (cell line, polybrene addition, titration method)	Harvest Number/ Time (#: hpt)	Validation of Scalability, Scale[7]	Yield (titer (tu/mL), spec. prod. (tu/c), envelope)[8]	Reference
Conventional methods											
HEK293T, DMEM-10	1st, non-SIN, lacZ, Lucif	$CaPO_4$, BBS	1:3:4	~3	~13	-, -	208 F (rat fibroblasts), + 8 µg/mL PB, β-gal positive foci or luminescence	I: 62	-, 10 cm plate	~$4x10^5$, ~1, VSV-G	Naldini, 1996
HEK293T, DMEM-10	3rd, SIN, lacZ or GFP	$CaPO_4$, HBS	5:12:3:20	~4	~10	-, -	HeLa, -, HEK 293 T	I: 28	-, 10 cm plate	$1x10^6$, ~2.5, VSV-G	Zufferey, 2000
HEK293T, DMEM-10	3rd, SIN, GFP	$CaPO_4$, HBS/BBS	7:13:5:20	~20	~2.5	-, -	HEK293T, + 8 µg/mL PB, microscope counting or flow-cytometry for GFP+	I: 48 II: 72	-, 15 cm plate	~$6x10^5$-6 $x10^6$, ~2, VSV-G	Blesch, 2004
Improved methods											
HEK293T, UC	1st, non-SIN, G418	$CaPO_4$, HBS	1:1:1	~0.8	19	chloroquine, 25 µM	HOS, + 8 µg/mL PB, G418 selection	I : 24 II : 48 III : 60 IV :83	-, 6-well plate	$1x10^7$ cfu/mL, ~65 cfu/c, VSV-G	Reiser, 2000
HEK293T, DMEM-10	2nd, SIN, nlacZ	$CaPO_4$, BBS	1:3:4	~0.5	~3	butyrate, 10 mM	HEK293T, -, microscope counting β-gal positive foci	I: 24 II: 48	+, cell factory (1 L supernatant)	$1.2x10^6$ at 2 harvests, ~3 tu/cell, VSV-G	Karolewski, 2003
HEK293T, DMEM-10, OptiMEM-I, UC [9]	2nd, SIN, GFP	$CaPO_4$, HBS	2:3:3	40	~1	butyrate, 10 mM (Mokola)	HEK293T, + 8 µg/mL PB, microscope counting GFP+	I : 36	-, 15 cm plate	$1.5x10^8$, ~40, VSV-G; ~1 $x10^7$, ~3, Mokola[10]	Sena-Esteves, 2004
HEK293T, ADMEM [11]	2nd, non-SIN, EYFP	$CaPO_4$, HBS	1:1:2	~1.7	~3.5	chloroquine, 25 µM	CHO-K1, -, microscope counting YFP+	I : 48	-, 6-well plate	~ $2.9x10^9$, ~1700[11], VSV-G	Mitta, 2005
HEK293T/17, ADMEM, ADMEM + supplements (Gibco)	3rd, SIN, cond. SIN, GFP	$CaPO_4$, HBS	1:1:2	~1.5	12.4-16.4	chloroquine, 25 µM	HeLa, + 8 µg/mL PB, flow cytometry GFP+	I :30 II :54	-, 10 cm plate	$1x10^5$-1 $x10^7$ [13], ~0.1-10 [14], VSV-G	Al Yacoub, 2007
HEK293T, DMEM-0[14] (D), UC	2nd, SIN, lacZco	PEI, linear (40 kD)	1:2:3	0.5	0.7 -1 (D) 0.5-0.7 (UC)	chloroquine, 25 µM, (for 48 hpt (D) and 16 hpt (UC)	HOS, microscope counting (X-gal staining)	I: 48 (+16)	-, 15 cm plate	1-1.25$x10^7$ (D), ~ $3.5x10^7$ (UC), ~10, VSV-G	Kuroda, 2009
HEK293FT, DMEM-10	3rd, SIN, GFP	PEI, branched (25 kD)	1:2:2:3	~3	2.66	-, -	HEK293FT, flow-cytometry GFP+	I :48	-, T 75 flaks	$1x10^7$, ~30	Toledo, 2009
HEK293E, (clone 6E), Freestyle	3rd, SIN, GFP	PEI, linear (25 kD)	1:1:1:2	20, 1	1	-, -	HEK293E, + 8 µg/mL PB, flow-cytometry GFP+	I: 72 II: 96 III: 120 [15] (small scale) discontinuous harvest (1V/d) from 24-144 hpt in BR	++, 3 L batch reactor	$2x10^6$, 2, VSV-G	Segura, 2007
HEK293SF-3F6, HyQ	3rd, SIN, GFP	PEI, linear (25 kD)	1:1:1:2	100, 5	0.4	butyrate, 5 mM	HEK293E, + 8 µg/mL PB, flow-cytometry GFP+	I: 24 II: 48 III: 72 IV: 96 (small scale) continuous harvest at 1V/d in BR	+++, 3 L perfusion reactor	~$1x10^8$, ~70	Ansorge, 2009

Remarks for Table 1.1

[1]: if several culture media were tested, only best formulations are mentioned

[2]: vector types: non-SN, SIN, conditional (cond.) SIN

[3]: details: buffer system ($CaPO_4$) or polymer type (PEI)

[4]: Mass ratio of plasmid constructs for transfection: Envelope: Packaging 1: (Packaging 2): LV transfer vector

[5]: if cell density at transfection is not determined, predicted cell density is given (from cell density before transfection); cell concentration only for protocols in suspension, if several cell densities were tested, only optimal density is mentioned

[6]: if several additives, best conditions are mentioned only, largest reported scale mentioned

[7] : positive signs for fully validated scale-up (maximum 3 degrees, negative sign for no validation of scale-up; - : no scale up validated (small-scale (culture dish or T-flask) only), +: scale up by increase in culture volume without increase in volumetric productivity, ++ : scaled up for large-scale production, scalability limited, +++ readily scalable, bioreactor scale validated

[8]: values were extrapolated or estimated (critical parameters like cell density at transfection or harvested LV-containing supernatant were not given);

titer as virus concentration in non-concentrated culture supernatants; titers in tu/mL if not further specified,

[9]: best three media formulation are shown

[10]: more envelope constructs were tested, two constructs with highest titers are shown

[11]: Advanced D-MEM medium: +2% fetal calf serum, (vii) and a culture additive containing 0.01 mM cholesterol, 0.01 mM egg's lecithin and 1× chemically defined lipid concentrate

[12] : not clear if reported LV titers relate to non-concentrated culture supernatant or concentrated preparations after ultracentrifugation

[13]: insert sizes from 4-7.5 kb were tested; titers decreased with vector construct insert size

[14]: DMEM-0: DMEM without serum with high glucose concentration was used

[15]: harvest continued until 168 hpt but low yields after 120 hpt

1.2.6.2 Production of LVs with Packaging and Stable Producer Cell Lines

Although transfection as a platform for LV production offers advantages in terms of flexibility and a short time to product, several intrinsic disadvantages hamper its use for LV mass production. These include variation in transfection efficiency when protocols are not optimized, costs associated to plasmid DNA production and additional quality control for the transfection

agents. The required transfection steps complicate the process compared to simple routine production processes of other biologics. Significant efforts are therefore undertaken to generate stable packaging and producer cell lines. Packaging cell lines express all LV components except the vector constructs and still require transfection for vector production. Stable producer cells express all necessary vector components and are consequently generated after introduction of the vector construct. The ideal LV producer cell line should be stable over extended culture periods, produce large quantities of LVs and preferably grow in suspension under serum-free conditions for easy scale-up (Kuroda, Kutner et al. 2009). It is a general expectation that, once established, stable producers will outperform transfection methods and deliver standardized production batches for optimized downstream processing of the produced material. Significant efforts have therefore been undertaken to establish stable packaging and producer systems. Table 1.2 summarizes recent publications on stable LV packaging and producer cell lines. For this comparison, reports on systems lacking important biosafety features or exhibiting low yields and poor stability were not considered.

Due to the cytotoxic and cytostatic effects of several LV vector components, in particular *gag*, *rev* and VSV-G (if selected as envelope protein), these components need to be tightly regulated when stably expressed in a cell line (Kaplan and Swanstrom 1991; Miyazaki, Takamatsu et al. 1995; Sparacio, Pfeiffer et al. 2001; Quinonez and Sutton 2002). The basal expression of LV components in the 'off-state' hence needs to be below the cytotoxicity threshold (Sparacio, Pfeiffer et al. 2001).

Most systems employ the tetracycline (Tet)-inducible system to achieve the required regulation (Kafri, van Praag et al. 1999; Farson, Witt et al. 2001; Ni, Sun et al. 2005). The classical systems are in off-state in the presence of doxycycline (dox), a tetracycline analog. The subsequent removal of dox induces expression of the LV components. The complete removal of dox is only easily feasible with adherent cells and induction takes typically several days until high expression levels are reached. These systems are hence not amenable to large-scale production. The counterpart to Tet-off systems, Tet-on, was consequently used to ease the scalability and practicability of induction (Broussau, Jabbour et al. 2008). Early classical Tet constructs were genetically (gene loss) or transcriptionally instable (gene silencing) after prolonged cultivation periods. The use of additives that are known to promote transcription such as sodium butyrate

have been used to circumvent this problem (Xu, Ma et al. 2001; Cockrell, Ma et al. 2006; Stewart, Leroux-Carlucci et al. 2009). Other approaches to achieve improved stability include multi-level regulation of the cytotoxic LV components to account for the commonly observed leakiness of the Tet system (Farson, Witt et al. 2001; Ni, Sun et al. 2005; Broussau, Jabbour et al. 2008; Throm, Ouma et al. 2009). Alternatively to the Tet-inducible system, the insect derived ecdysone (Ecd) is used in several studies (Pacchia, Adelson et al. 2001; Sparacio, Pfeiffer et al. 2001; Kuate, Wagner et al. 2002; Seraphin, Ralf et al. 2002). It is attributed with a lower basal expression, a higher level of induction and a faster peak expression after ponasterone A (an Ecd analog) addition but still needs to demonstrate its potential for high-yield LV production and requires further evaluation.

Stable packaging cells are typically produced by transient cotransfection and subsequent selection of highly productive clones (Pacchia, Adelson et al. 2001; Cockrell, Ma et al. 2006; Stewart, Leroux-Carlucci et al. 2009). Alternatively, transduction with RVs or LVs can be used (Klages, Zufferey et al. 2000; Farson, Witt et al. 2001; Xu, Ma et al. 2001; Kuate, Wagner et al. 2002; Ikeda, Takeuchi et al. 2003; Broussau, Jabbour et al. 2008) as an efficient way to insert the vector construct in stable packaging cells. This approach results in more efficient expression of LV related proteins compared to transfection (Ikeda, Takeuchi et al. 2003). Transfected or transduced cells are then screened for *gag*, CAp24 expression or RT activity to select for the best producing clones, to generate LVs or to establish stable producers. With expression of vector RNA in producer cells being one of the major rate limiting steps for efficient LV production, serial transductions of packaging cell lines result in higher titers (Kafri, van Praag et al. 1999; Farson, Witt et al. 2001). SIN vectors are, however, difficult to insert stably into packaging cells with traditional, high-yield methods. Therefore, a third approach for the generation of stable producers, concatemeric array transfection, has been recently described. This method includes the generation of DNA structures (arrays) by *in vitro* ligation, containing multiple copies of SIN vector and drug resistance expression cassettes which are then transfected in stable LV packaging cells. This approach allows for the efficient generation of cell lines that stably express SIN LVs (Throm, Ouma et al. 2009).

Challenges for the generation of stable LV producing cell lines include gene silencing, in particular for long term genetic stability of the constructs. Sufficient expression of *rev* is also

known to be critical for high-yield production (Ikeda, Takeuchi et al. ; Broussau, Jabbour et al. 2008).

Although not routinely reported, cell specific productivities for stable packaging cell lines and producers are more often found in the literature (Klages, Zufferey et al. 2000; Ikeda, Takeuchi et al. 2003). If not directly reported, it is in contrast almost impossible (because of the expansion of the producer cell population), to infer specific productivities for stable producers. The current stable systems give yields of 10^5-10^8 tu/mL with similar specific productivities compared to transient transfection. They also suffer from small production windows of a few days due to the cytotoxicity of the produced vector components. With the majority of current systems being VSV-G pseudotyped LV, in which self-infection of producer cells is a common observation, long production windows could increase the risk of recombination, might compromise genetic stability and are consequently not desired (Farson, Witt et al. 2001).

Whereas cellular growth after transfection dilutes the transiently transferred genetic information over time, a stable cell line often leads to superior total yields at equal specific productivity when the cell population is expanded during production.

Table 1.2 : Comparison of stable packaging and producer system

System (name[1], LV-type (env), basal cell line, medium)	LV system (generation of packaging cassette, vector type prod./pack, transgene)[2]	Generation of stable producer[3], method	Induction (system, additives (concentration)	Titration (cell line, polybrene addition, titration method)	Validation of Scalability, scale[4]	Yield (Titers, spec. prod., Production Window[5] (titer packaging cells (pack), stable producer (SP))	Reference
HIV-1 based systems without induction							
293TGPRT1+R1, HT-STAR/STAR, HIV-1 (Ampho and GALV), HEK293T, HT1080, DMEM-10	2nd, codon optimized GagPol, γ-retrovirus envelopes, GFP	+, stable transduction with MLV vectors	-	293T, + 8 µg/mL PB, flow-cytometry GFP+	-	$1x10^7$ tu/mL, 20 tu/cell, 3 months[6] (SP)	Ikeda, 2003
Tet-inducible HIV-1 based systems							
LV^G, HIV-1 (VSV-G), HEK293T, DMEM-10	3rd, non-SIN, GFP	+, transduction with non-SIN LV	Tet-off	HeLa, -, flow-cytometry GFP+	-	$5 x10^6$ tu/mL, 1-20 tu/c*d, 7 d (SP)	Klages, 2000
Lentikat, (HIV-1) VSV-G), HEK293G, IMDM-10	2nd, non-SIN, GFP	+, serial transduction with non-SIN LV	Tet-off	HeLa, + 8 µg/mL PB, flow-cytometry GFP+	-	$3.5 x10^6$ tu/mL, 1-10 tu/cell , 8 d (SP)	Farson, 2001
SODk1, HIV-1 (VSV-G), HEK293, DMEM-10	2nd, cond. SIN	+, transduction with cond. SIN LV	Tet-off, butyrate, 0.5/5 mM	293T, -, flow-cytometry GFP+	-	$2 x10^6$ tu/mL, n.a., 6 d[7] (SP)	Kafri, 1999, Xu, 2001
17B-5, HIV-1, (VSV_G), HEK293 (Invitrogen), DMEM-10	2nd codon-optimized, non-SIN, GFP	-	Tet-off, 3-level cascade	HeLa, -, flow-cytometry GFP+	+, cell factory	$3.5 x10^7$ tu/mL, 15-25 tu/cell , 11 d (pack)	Ni, 2005
SODk3, HIV-1 (VSV-G), HEK293/ SODk0, DMEM-10	2nd with gag/pro, vpr/pol split, Cond. SIN	+. stable cotransfection and clonal selection	Tet-off, butyrate, 5 mM	293T, -, fluorescence microscopy GFP+	-	$2 x10^6$ tu/mL (pack), n.a.[8], $1x10^7$ tu/mL (SP), 3 d	Broussau, 2008
293SF-PacLV, HIV-1 (VSV-G), 293SF-3F6, LC-SFM +GPN3,	3rd, cond SIN, GFP	+, transduction with cond. SIN	Tet-on and cumate inducible double switch	293A, + 8 µg/mL PB, flow-cytometry GFP+	+++, 3.5L BR	$3-8 x10^6$ tu/mL, ~10 tu/cell (pack), $1-3x10^7$ tu/mL, ~100 tu/cell (SP), 5 d	Cockrell, 2006
GPRG, HIV-1 (VSV-G), HEK293/T17, DMEM-10	2nd, SIN, GFP and IL2RG	+, concatemeric array transfection	Tet-off, two-level	HeLa, + 8 µg/mL PB, flow-cytometry GFP+	++, WAVE (1-10 L),	$5 x10^7$ tu/mL (pack), 1-10 tu/cell, $5 x10^7$-$2 x10^8$ tu/mL (SP)[9], 1-10 tu/cell (BR), 5 d	Throm, 2009
Other systems							
REr1.35, HIV-1 (VSV-G), HEK293T, MEM-10	3rd, non-SIN	+, transfection and clonal selection	Ecd-on	HeLa, + 50 µg/mL PB, puromycin resistance and flow-cytometry GFP+	-	$1 x10^5$ tu/mL, <1 tu/cell, 3-5 d (SP)	Pacchia, 2001
293-Rev/Gag/Pol_i, HIV-1 (VSV-G), HEK293, DMEM-10	1st-3rd, non-SIN	-	Ecd-on, butyrate, 5 mM	HeLa, + 8 µg/mL PB, flow-cytometry GFP+	-	$1-3 x10^5$ tu/mL, n.a., 2 d (pack)	Sparacia, 2001
PC48.2, EIAV (VSV-G), HEK293T, DMEM-10	-	+, stable cotransfection	Tet-on, butyrate, 10 mM	COS (D17), + 8µg/mL PB, microscope counting of β-gal positive foci	-	10^5-10^6 tu/mL, ~1 tu/cell, 2 d (SP)	Stewart, 2009
Sgp-G, SIV (VSV-G),EcR-293, DMEM-10	-	+, serial transduction with SIV and clonal selection	Ecd-on	HEK293, -, fluorescence microscope GFP+	-	10^5 tu/mL (pack and SP), n.a., 2-4 d	Kuate, 2002

Remarks for Table 1.1

[1]: name of packaging cell line

[2]: vector types of producer cell line or packaging cell line (after tranfection/transduction) : non-SIN, SIN, conditional (cond.) SIN

[3]: +: stable producer generated, - no stable producer described

[4]: positive signs for fully validated scale-up (maximum 3 degrees, negative sign for no validation of scale-up); - : no scale up validated (small-scale only (culture dish or T-flask)), +: scale up by increase in culture volume without increase in volumetric productivity, ++ : scaled up for large-scale production, scalability limited, +++ readily scalable, bioreactor scale validated

[5]: maximum titers, production window = length of production time with consecutive high titers

[6]: clone-dependent decrease over prolonged cultivation observed

[7]: no kinetics, one point titers only

[8]: cell density neither reported nor possible to estimate

[9]: cell specific productivity could not be calculated (neither cell densities at induction nor harvested volume reported

1.2.6.3 Improvement of LV Production by Medium Additives

Several process parameters govern the efficient generation of LVs. Despite the differences in the production methods, several compounds that are critical to achieve a high-yield LV production have been already identified and are used in several of the reviewed protocols (Tables 1.1 and 1.2).

1.2.6.3.1 Sodium Butyrate

Gene silencing of at least some of the LV expression cassettes is a common observation after transient transfection and also in stable packaging and producer cell lines. The loss in expression can be dramatic with a decreased CAp24 production of 10-30 fold. As gene loss has been excluded as a possible reason, gene silencing is the most likely underlying mechanism (Kafri, van

Praag et al. 1999; Sparacio, Pfeiffer et al. 2001). Sodium butyrate in a concentration range of 2-10 mM is well described as a powerful inducer of LV production. It causes hyperacetylation of histones, leading to higher transcription and increased expression of transfected DNA (Altenburg, Via et al. 1976; Kruh 1981; Gloger, Arad et al. 1985). It also stimulates the activity of the HIV-1 LTR and CMV promoters (Gasmi, Glynn et al. 1999). When two strong promoters are close together, as in most 3rd generation vector constructs, sodium butyrate significantly reduces effects caused by promoter interference (Jaalouk, Crosato et al. 2006). It can consequently be used to boost LV production at least temporarily, leading to peak titers over a few consecutive days (Kafri, van Praag et al. 1999; Sparacio, Pfeiffer et al. 2001; Ansorge, Lanthier et al. 2009). Sodium butyrate was elsewhere reported to have a mixed effect on LV titers (Sena-Esteves, Tebbets et al. 2004), with no increase in titers of VSV-G pseudotyped particles but a marked increase for other pseudotypes. In conclusion, sodium butyrate only seems to have a major effect if the expression efficiency of the affected construct is non-optimal (Gasmi, Glynn et al. 1999). It acts on several levels, since increases in VSV-G mRNA, CAp24 expression and vector RNA levels are reported (Kafri, van Praag et al. 1999; Jaalouk, Crosato et al. 2006; Al Yacoub, Romanowska et al. 2007). Whereas no significant effects were found for any envelope construct at low concentrations (0.1 mM) (Al Yacoub, Romanowska et al. 2007; Ansorge, Lanthier et al. 2009), it resulted in productivity improvements of 4-15-fold at higher concentrations of 5-20 mM (Karolewski, Watson et al. 2003; Ansorge, Lanthier et al. 2009).

1.2.6.3.2 Chloroquine

Chloroquine is an amine that raises the pH of endosomes and lysosomes. This increase in lysosomal pH may prevent the degradation of transfected DNA (Karolewski, Watson et al. 2003). Chloroquine is commonly used for LV and RV production (Park, Ohashi et al. 2000; Reiser 2000; Mitta, Rimann et al. 2005; Al Yacoub, Romanowska et al. 2007; Kuroda, Kutner et al. 2009). However, its effect depends on the transfection agent used, improving yields after calcium phosphate precipitation but resulting in reduced titers when PEI is used for transfection (Kuroda, Kutner et al. 2009).

1.2.6.3.3 Cholesterol and Lipids

Enveloped viruses such as LVs, RVs, Influenza and Ebola bud from the membrane of an infected cell and thereby obtain a host-cell derived lipid bilayer. As various lipids show a heterogeneous distribution within cell membranes, it was hypothesized that sphingolipids and cholesterol segregate in cell membrane microdomains called membrane or lipid rafts. According to this theory, cholesterol stabilizes membrane rafts from which virus budding and also entry probably takes place (Chazal and Gerlier 2003). The lipid composition of different viral envelopes varies and suggests that some viruses bud from membrane rafts. These membrane rafts are highly dependent on cholesterol (Chazal and Gerlier 2003). Indeed, a disruption of lipid rafts by cholesterol depletion hinders HIV-1 particle production from cells and cholesterol depletion from viral particles reversibly impairs HIV-1 infection (Ono and Freed 2001; Popik, Alce et al. 2002). Significant lipid alterations are observed in infected cells compared to uninfected cells and the life cycle of HIV has also shown to be cholesterol-dependent. It was hypothesized that cellular cholesterol is an important requirement for the infectious form of the virus (Maziere, Landureau et al. 1994). Excess cholesterol is thus expected to increase the budding of enveloped viruses (Chazal and Gerlier 2003; Holm, Weclewicz et al. 2003; Mitta, Rimann et al. 2005).

The addition of lipid cocktails is therefore a common strategy to improve the production yield of viral vectors based on budding virus forms. Cholesterol, lipid cocktail and also lipoprotein addition before transfection (>24 h) favors the budding of enveloped viruses and increases LV infectivity (Mitta, Rimann et al. 2005; Chen, Ott et al. 2009).

Findings for RV suggest that the cellular lipid metabolism and content plays a critical role in vector production (Merten 2004; Coroadinha, Alves et al. 2006; Coroadinha, Ribeiro et al. 2006; Coroadinha, Silva et al. 2006). On the other hand, retroviral particle lability has been shown to parallel with viral membrane cholesterol content (Beer, Meyer et al. 2003; Carmo, Faria et al. 2006; Coroadinha, Alves et al. 2006). To further optimize yields, studies are needed for LVs that decorrelate the cellular capacity of virus release and virus degradation or stability.

1.2.7 Scalable Production Strategies and Process Operation

For the development of a cost-effective LV production process, a scale-up should generally imply a larger culture volume and enhanced volumetric productivity. This is not the case when only the

number of production vessels or the surface area of adherent cultures (from T-flasks to roller bottles and cell factories) is increased (Merten 2004). Nevertheless, cell factories have been used to generate LV material for first clinical trials. The production of multi-liter volumes of LV supernatant is feasible with this approach but it is labor intensive and not cost-effective (Schonely, Afable et al. 2003). Similar efforts include the use of aerated high-performance flasks that suffer from similar limitations when compared to readily scalable suspension-based methods (Kutner, Puthli et al. 2009).

Although many of the reports on LV production claim a large-scale applicability, most rely on adherent cell lines cultivated in standard serum-containing medium. These characteristics render the scalability of these processes challenging and cumbersome. Surprisingly, this problem is only rarely addressed in the literature. It can be expected that only bioreactor-based systems in which cells are grown on microcarriers (microspheric supports for adherently growing cell lines) or suspension-grown cell lines can satisfy future demands for LV mass production. Problems typically occurring with microcarrier cultures in large-scale cultures include the absolute need for bubble-free aeration and minimization of mechanical shear stress to allow for ideal growth on the surfaces (Merten 2004). The adaptation of stable packaging and producer cell lines to suspension and even microcarrier-based culture is additionally difficult because changes in cellular morphology and membrane properties during adaptation and/or serum reduction can lead to loss in viral productivity. Significant increases in LV yield will only be achieved by higher volumetric productivities. The yield of adherent cell cultures is by definition limited by the surface area of the culture dish or carrier. One study describes the scale-up of adherent cell cultures for LV production using fibrous discs (Throm, Ouma et al. 2009). This process, however, still requires discontinuous medium exchanges for induction and/or LV harvest.

Suspension-grown cell lines should be preferable as they show the best and most straightforward scalability and allow the high-yield production of LVs by transfection at high cell density in shake flask and continuous bioreactor perfusion cultures (Segura, Garnier et al. 2007; Ansorge, Lanthier et al. 2009). When employing stable LV cell lines, it should be noted that the classical Tet-off system requires medium exchange and complete inhibitor removal, and is consequently difficult to scale-up. To avoid this problem, the generation of a stable packaging cell line in

serum-free suspension culture using the corresponding Tet-on regulation system has recently been described (Broussau, Jabbour et al. 2008).

Only a handful of studies have addressed the scalability of LV production (Segura, Garnier et al. 2007; Broussau, Jabbour et al. 2008; Ansorge, Lanthier et al. 2009; Throm, Ouma et al. 2009). Further efforts are required to improve these processes and ensure their effectiveness and robustness.

Low final yields in non-optimized production systems and low LV stability complicate the establishment of viable industrial processes (Cruz, Almeida et al. 2000; Merten 2004). Perfusion processes with rapid and regular exchange of medium are consequently highly recommended for LV production. For adherent cell lines, microcarrier cultures are preferable whereas conventional stirred tank bioreactors operated in perfusion mode should be chosen for suspension-grown cell lines (Merten 2004). All systems need to ensure tight control of process parameters such as pH and osmolality to mainain LV infectivity (Higashikawa and Chang 2001; Torashima, Yamada et al. 2006). For RVs and LVs, processes in which the cell culture is maintained at 37°C and the harvested supernatant cooled down are advantageous (Cruz, Almeida et al. 2000; McTaggart and Al-Rubeai 2000; Ghani, Garnier et al. 2006; Ansorge, Lanthier et al. 2009). Process optimization at the bioprocess engineering level has been performed for RV (Cruz, Almeida et al. 2000) and will also be required for LV processes.

1.2.8 LV Purification and Final Product Characterization

In conventional protocols for LV production ultracentrifugation remains the concentration and purification method of choice (Naldini, Blomer et al. 1996; Reiser 2000; Follenzi and Naldini 2002). This method is easily accessible and convenient for small-scale applications, providing high-titer preparations after one or several rounds of concentration. It is, however, greatly limited by the volume of the preparation and consequently not scalable. Scalable purification strategies are required to process supernatants from multi-liter production runs. As LVs share very similar structural, physical and biochemical properties with RV, the field can benefit from extensive reviews covering the topic (Braas, Searle et al. 1996; Lyddiatt and O'Sullivan 1998; Segura, Kamen et al. 2006; Rodrigues, Carrondo et al. 2007). In summary, suitable purification methods should remove contaminants, such as transduction inhibitors, host cell derived proteins, endotoxins and free DNA remnants while preserving vector functionality. Chromatography-based

methods such as anion exchange chromatography appear to be the most viable options for scalable purification (Scherr, Battmer et al. 2002; Slepushkin, Chang et al. 2003; Yamada, McCarty et al. 2003). In particular, Mustang Q anion exchange membrane chromatography was described as a promising technology in several studies (Marino, Kutner et al. 2003; Slepushkin, Chang et al. 2003; Ricks, Kutner et al. 2008; Kutner, Puthli et al. 2009). The downstream processing strategy to purify LV preparations for the first clinical trial has also been published, reporting an overall recovery of 30 % and demonstrating the feasibility to generate material for *ex vivo* use. RV and LV preparations can also be polished using size exclusion chromatography (Slepushkin, Chang et al. 2003; Transfiguracion, Jaalouk et al. 2003). Particular challenges for purification include the low stability of LV after multiple freeze-thaw cycles, at increased temperature and the narrow pH and osmolarity range for processing of the vector. The process steps and materials and methods for scalable LV mass production by current state of the art methods are presented in Figure 1.2.

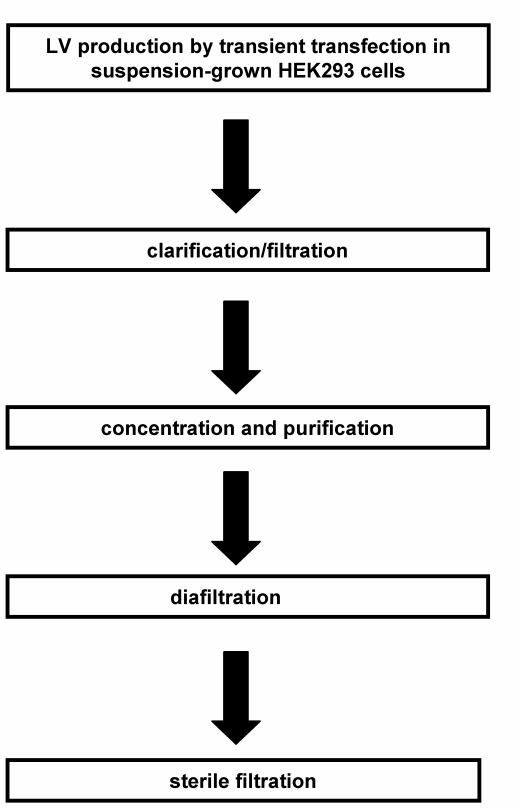

Figure 1.2 : Flowchart of a scalable process for LV mass production.

Materials and methods that are used comprise PEI-based transfection in bioreactors using 3rd generation packaging (Gag-Pol, Rev) and envelope (VSV-G) constructs and a self-inactivating (SIN) lentivector in serum- and animal-component-free medium. LV-containing supernatant is then continuously harvested from 1-4 dpt from the perfusion culture. Further processing of the supernatant includes sequential microfiltration with decreasing pore size (0.8/0.45 μm), followed by (membrane) anion-exchange chromatography and/or size exclusion for polishing. Final steps are a benzonase treatment to remove free DNA, buffer exchange for storage/final formulation and sterile filtration. Quality control assays as described in section 1.2.5 and 1.2.8

Before LV preparations can be used in clinical applications, a variety of regulatory aspects need to be considered. Detailed descriptions of the regulatory requirements are well beyond the scope of this review but would be found in the specialized reviews cited below. The first clinical trial using lentiviral vectors in humans gave important insights into the concerns of the regulatory agencies and how these can be addressed from a manufacturing and quality control perspectives (Slepushkin, Chang et al. 2003; Manilla, Rebello et al. 2005; Levine, Humeau et al. 2006). Extensive reviews dealing with the manufacturing of viral vectors might also provide helpful guidance on these topics (Lusky 2005; Mandel, Burger et al. 2008; Thorne, Takeya et al. 2009; Wright 2009). In general, current good manufacturing practice (cGMP) guidelines for the production of biologicals in the United States and Europe are defined by the respective regulatory agencies Food and Drug Administration (FDA/CBER) and European Medicines Agency (EMEA). Other sources include international regulations of the International Conference on Harmonisation (ICH) and the World Health Organization (WHO) or other country-specific codes. Particular concerns related to the use of lentiviral vectors in humans include vector mobilization, insertional oncogenesis and generation of RCL. A variety of respective assays has consequently been developed for quality control release testing of LV lots and transduced cellular products (Schonely, Afable et al. 2003; Manilla, Rebello et al. 2005). These assays comply with the standard approach for release testing of biopharmaceutical products by testing for safety, identity, strength, potency, purity and biological properties of the final product.

To prevent vector mobilization after infection and rescue by wild-type HIV-1 helper virus, it was suggested that the clinical implementation of LVs should be mainly based on SIN vector constructs (Miyoshi, Blomer et al. 1998; Zufferey, Dull et al. 1998; Bukovsky, Song et al. 1999; Evans and Garcia 2000). Concerning insertional mutagenesis, recent data analyzing the integration patterns in the first LV-based clinical trial is now available, suggesting that LVs, in contrast to RVs, integrate into the entire intragenic region and do not favor integration near proto-oncogenes and that no selective expansion of the transduced population occurred over time (Mitchell, Beitzel et al. 2004; Ciuffi, Mitchell et al. 2006; Wang, Levine et al. 2009).

This LV construct will be further evaluated during a long term follow up of all patients involved in this trial (Manilla, Rebello et al. 2005). However, ongoing clinical trials might reveal if other modified lentiviral constructs, containing strong promoters, are intrinsically less likely to

transactivate cellular genes than RV or have other adverse effects (Montini, Cesana et al. 2006; Kohn 2007; Rossi 2009; Wang, Levine et al. 2009).

Although the generation of RCL is unlikely, their occurrence would be fatal as a novel human pathogen would be generated. It is therefore required to perform rigorous testing for the emergence of RCL. The assay design is generally difficult, as a putative RCL is of unknown structure. A number of different assays to detect RCL has been proposed (Sastry and Cornetta 2008). Some methods are most likely sensitive to RCL, such as assays targeting the VSV-G envelope or its DNA/RNA, CAp24 protein and HIV-1 *gag* DNA/RNA. Typical procedures of RCL assays therefore comprise the co-cultivation of HIV-1 permissive (indicator) cell lines, such as C8166-45 and MT4, with production cells and the final LV preparation for a period of several weeks to amplify possible RCL (Chang, Urlacher et al. 1999; Farson, Witt et al. 2001; Escarpe, Zayek et al. 2003). In parallel, a positive control is used in form of an attenuated HIV-1 and the supernatants tested for the presence of RCL. To date, no RCL emergence has been reported during production when advanced LV constructs were used (Escarpe, Zayek et al. 2003; Sastry, Xu et al. 2003; Schonely, Afable et al. 2003; Miskin, Chipchase et al. 2005). Only large production batches of ongoing and future clinical trials can however give insights on the RCL formation during large scale LV production.

1.2.9 Remaining Challenges for Efficient LV Mass Production

In the field of LV production, only limited basic bioprocess engineering principle-guided approaches have been reported. For example, optimal harvest times or kinetics of virus production are rarely reported and for current standard protocols the time of LV peak production is only rarely determined. Authors use different harvest points after transfection or induction with or without culture media exchange during production. Most studies therefore report results in which both, viral productivity and degradation or decay, are confounded (Beer, Meyer et al. 2003). A deconvolution of these parameters needs to be achieved to allow for a rational optimization, ideally based on mathematical models.

The assessment of cellular productivities (infectious or total viral particles per cell) is often difficult because protocol details such as cellular density or harvest volume are not reported. Whereas titers of up to 10-150 pg/cell have been reported for transient protein production, the specific productivity for high-yield LV systems is low, similar to other viral vectors that are

produced transiently (Wright 2009). For total LV particles, cell specific mass amounts of only 0.8-8 pg/cell×day are typically reported, with only ~0.1-1 % of total produced LVs being functional (i.e. ~1-50 tu/cell) (Higashikawa and Chang 2001; Mitta, Rimann et al. 2005). In contrast, cell-specific yields for other viral constructs such as adenoviral and adeno-associated viral vectors are in the range of 10^3-10^4 IVP/cell (Kamen and Henry 2004; Aucoin, Perrier et al. 2008). The production of other vectors is mostly based on systems that include several 'rounds' of infection. Transient transfection and the expression of transfected DNA constructs for LV production is probably less efficient compared to virus-based gene transfer (Elouahabi and Ruysschaert 2005; Collins 2006; Vaughan and Dean 2006). Stable systems suffer so far from similar productivity limitations and equally only allow for transient LV production with short production windows. The impact of medium additives and for example the importance of the lipid metabolism suggests that media optimization strategies are needed to further improve LV yields. Therefore more work integrating recent progress in metabolic and process engineering is required to significantly increase the LV production yields.

The many different protocols and methods for LV quantification are another challenge that complicate advances in process development. Whereas it is suggested that both functional and total vector particles are quantified, most methods are time-consuming and standardization is urgently needed. The field would highly benefit from the development and adaptation of fast quantification methods targeting the total and intact viral particles that have been described for other viral vectors (Transfiguracion, Bernier et al. 2001; Shen, Meghrous et al. 2002; Transfiguracion, Coelho et al. 2004; Transfiguracion, Bernier et al. 2008).

A clinical use of LVs will require optimized formulations to prevent a rapid loss in viral activity. Additional studies in different buffer compositions on the stability of purified preparation of pseudotyped LVs are urgently needed.

1.2.10 Summary and Conclusion

In this review, we point out recent advances in the generation of LV. A comparison of existing protocols for LV production is generally difficult because of the difference in production systems and operating conditions. Nevertheless, significant improvements of conventional transfection protocols, increasing functional titers by several orders of magnitude, have now been reported (Karolewski, Watson et al. 2003; Mitta, Rimann et al. 2005). Optimization of production

parameters and the use of scalable suspension-based strategies can lead to improved volumetric yields of more than 100-fold and allow for cost-effective LV production (Mitta, Rimann et al. 2005; Ansorge, Lanthier et al. 2009).

The LV system remains a field that is in development and improvements in its molecular architecture will depend on the acceptance and success of ongoing clinical trials. The optimal LV system is not yet found and depends on the respective therapeutic application. The rising interest in non-integrating LV forms can serve as one example for the ongoing development in the field (Nightingale, Hollis et al. 2006; Wanisch and Yanez-Munoz 2009). Methods offering a high degree of flexibility such as transient transfection are consequently instrumental to respond to the demand in LVs.

Stable systems will be of highest importance for LV production with regards to routine mass manufacturing of final commercial material. However, these systems are not yet widely used, probably because the overall benefit compared to transient transfection remains relatively modest. It is questionable if the efforts to produce a stable packaging cell line are beneficial in the early stages of an LV-based evaluation of a therapeutic strategy. Using different or modified vector components in early stages of LV vector development is often required and transient expression offers the appropriate flexibility, whereas a stable cell line strategy would require generation of a new cell line for each desired vector pseudotype (Ni, Sun et al. 2005). Both production strategies, transient transfection and stable packaging and producer cell lines, will therefore be used in parallel in the foreseeable future.

For research applications, protocols using adherent cultures in T-flasks or culture dishes are sufficient, whereas readily scalable suspension-based approaches should be developed for pilot and large-scale productions. It would be preferable to have a scalable production system in place when preclinical studies and phase 1 clinical trial are being contemplated to avoid serious production constraints that might turn into a bottleneck in the latest clinical evaluation phases.

There is still a possibility to further improve current LV production processes by several orders of magnitude. The yields of current LV systems could first of all be increased rationally by optimization of the harvest rate as function of vector stability in culture supernatants. Production at higher cell densities and optimized media formulations should further increase productivity of current processes. Finally, improvements in the molecular design of LV systems, targeting an

improved stability of the viral particles should allow for more efficient production. Although many aspects require further research and development efforts, recent advances in LV production should enable the cost-effective and simple mass production of clinical LV material under GMP conditions in the near future.

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CHAPTER 2 METHODOLOGY FOR SCALABLE LV PRODUCTION AND PROCESS CHARACTERIZATION

As indicated in the introduction, an optimization strategy was developed in this thesis to produce LV in a scalable manner. Here we describe in detail the methodologies that were used during this work.

First, the selected approach for LV production is described. This includes production of LV in perfusion after transient transfection. Operation in perfusion mode was combined with the transient transfection technology previously developed within our group. Extensive experience with the transfection technology (Durocher, Perret et al. 2002; Durocher, Pham et al. 2007; Tom, Bisson et al. 2007) and earlier work (Segura, Garnier et al. 2007) demonstrating the feasibility of LV production in suspension culture formed the basis of this work.

The analytical methods used to assess LV quantity and quality are then described in detail and compared. We then give details on the other analytical and monitoring tools that were employed in this work and provide a brief theoretical background when needed. These tools comprise both offline and online methods for measurement of cell counts, permittivity, biovolume, cell size and metabolite concentrations.

2.1 Production of Lentiviral Vectors

2.1.1 Cell Culture

The HEK293SF-3F6 cell line, originally developed for the production of adenoviral vectors, was used in this study (Côté, Garnier et al. 1998). This cell line is highly transfectable and grows in suspension under serum-free conditions. In most parts of this work, we refer to this cell line simply as HEK293.

Several culture media were tested for their ability to sustain production of LV in suspension: Two formulations of LC-SFM (Côté, Garnier et al. 1998) were used in small-scale experiments: LC-SFM L (LC-SFM + 0.1 % (v/v) lipid mixture (SIGMA, L-5146) (Pham, Perret et al. 2003) and LC-SFM GL (LC-SFM L supplemented with 0.5 % (v/v) gelatin peptone GPN3 (OrganoTechnie S.A. (La Courneuve, France)), (Pham, Perret et al. 2005)). For bioreactor experiments, LC-SFM L was supplemented with 0.1 % BSA (Celliance, Kankakee, IL); HyQSFM4TransFx293 (HyQ) (Hyclone, Logan, UT), a commercial medium specifically developed for transfection of HEK293 cells was evaluated as a second media formulation (Tom, Bisson et al. 2007). Whenever indicated HyQ was supplemented with 5 % (v/v) of stock solution of the regular formulation of cell boost 5 (CB5 reg) (Hyclone, Logan, UT) or a customized formulation of CB5 (CB5 mod) to obtain a fortified HyQ medium formulation (HyQ+). In contrast to CB5 reg, the latter formulation CB5 mod was not found to be inhibiting the transfection. The CB5 stock solutions were prepared at 35 g/L according to manufacturer's instructions. In general, precultures were passaged using standard media (i.e. LC-SFM L or HyQ) every 2-3 days to keep the cells in their exponential growth phase. After a maximum of three months, new cells were thawed from the working cell bank.

2.1.2 Transient Transfection

The transfection protocol has been published in detail elsewhere (Tom, Bisson et al. 2007). A PEI:DNA mass ratio of 2:1 was found to be optimal for transient transfection of HEK293SF-3F6 (Durocher, Perret et al. 2002; Pham, Perret et al. 2003). We used a GFP-encoding SIN transfer vector (pCSII-CMV5-GFPq), third generation packaging plasmids (pMDLg/pRRE#54 and pRSV-Rev) and a vector encoding the VSV-G envelope (pSVCMV-IN-VSVg) (Dull, Zufferey et

al. 1998; Broussau, Jabbour et al. 2008) for LV production in HEK293 cells (fig. 2.1). A plasmid mass ratio of 1:1:1:2 (VSV-G: Gag-Pol: Rev: Lentiviral Transfer Vector) was used for transfection according to previous results from our group (Segura, Garnier et al. 2007). Initial conditions involved transfection according to routine procedures using PEI-DNA complexes (polyplexes) with a total DNA amount of 1 $\mu g/10^6$ cells. Improved production conditions were then identified (chapter 3) with transfection at a cell density of $5x10^6$ c/mL in HyQ medium, a total DNA amount of 0.4 $\mu g/10^6$ cells and the addition of sodium butyrate (5 mM) at 16 hpt. If not mentioned otherwise, improved production conditions comprised a medium exchange rate of 1 "sequential discontinuous medium exchanges per day (DMD)" or 1 volume(s) of medium per reactor volume per day (VVD) in small scale and bioreactor cultures, respectively.

Initial small scale experiments were performed using a constant concentration of PEI-DNA complexes with varying polyplex volumes, i.e. for transfection at $5x10^6$ c/mL, 40 μg of DNA were used for transfection per shake flask (working volume of 20 mL), corresponding to a total DNA concentration of 20 μg/mL in the polyplex mixture.

For some of the experiments presented in appendix II, the polyplex volume was maintained constant at 10 % of the final culture volume. Thus DNA and PEI concentrations were a function of the cell density at transfection and up to 4 times higher for transfection at $2x10^7$ c/mL.

Precultures for LV production experiments in HyQ+ were diluted in medium including 5 % (v/v) of CB5 mod (HyQ+) 2-3 d before transfection (see appendix II). Polyplexes were in all cases prepared in medium without supplement to avoid interference of medium components in HyQ+ and ensure comparability. After transfection, regular cell boost (CB5 reg) was used as feed (in bioreactor cultures) or for discontinuous medium replacement (in small-scale) starting at 24 hpt.

For bioreactor cultures, polyplexes were prepared by manual shaking of the DNA-PEI mixture that corresponded to ~7-9 % of the final culture volume (i.e. 200-250 mL).

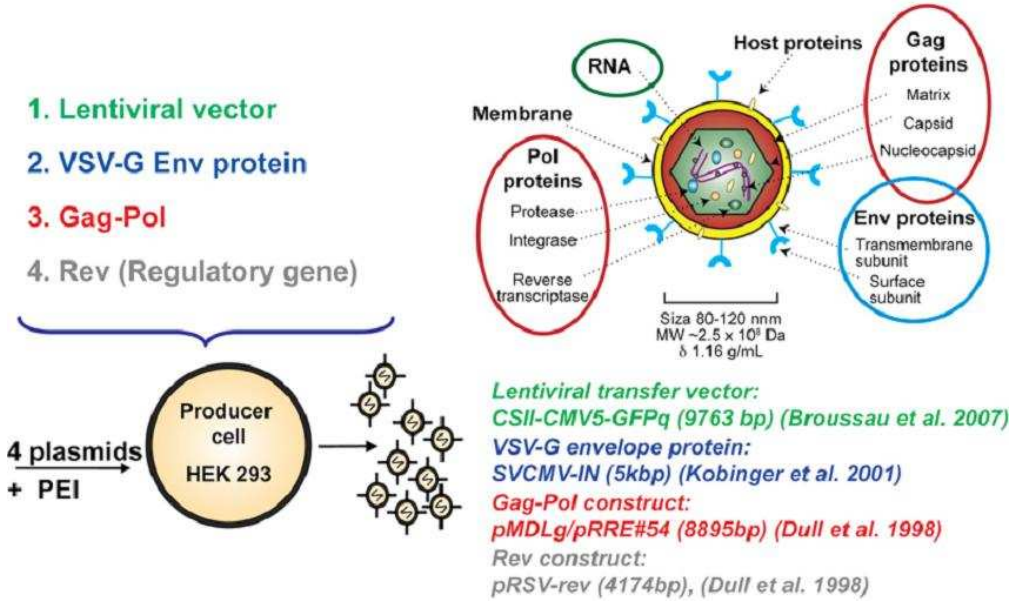

Figure 2.1 : LV production by PEI-based transient transfection.

Simultaneous transfection of four different plasmids encoding for the essential LV components into HEK293 cells leads to generation of LV particles

2.1.3 Stability and Harvest of LVs

Preliminary results of experiments on LV stability in culture supernatants confirmed literature reports showing that LVs rapidly loose infectivity at standard production temperature. In these preliminary experiments, incubation at 37°C for ~12 h resulted in loss of ~50 % of functional titer whereas the viral infectivity decay was greatly reduced if the produced vectors were maintained at 4°C (figure 2.2). Further results on LV stability in culture supernatants are presented in appendix I.

The production of HIV-forming proteins is initiated as early as 5-6 h after transfection (Jouvenet, Bieniasz et al. 2008). To maximize the yield in functional LVs and to address the low vector stability, a daily harvest of LV was thus performed starting 1 day post-transfection (dpt) in the starting set of operating conditions.

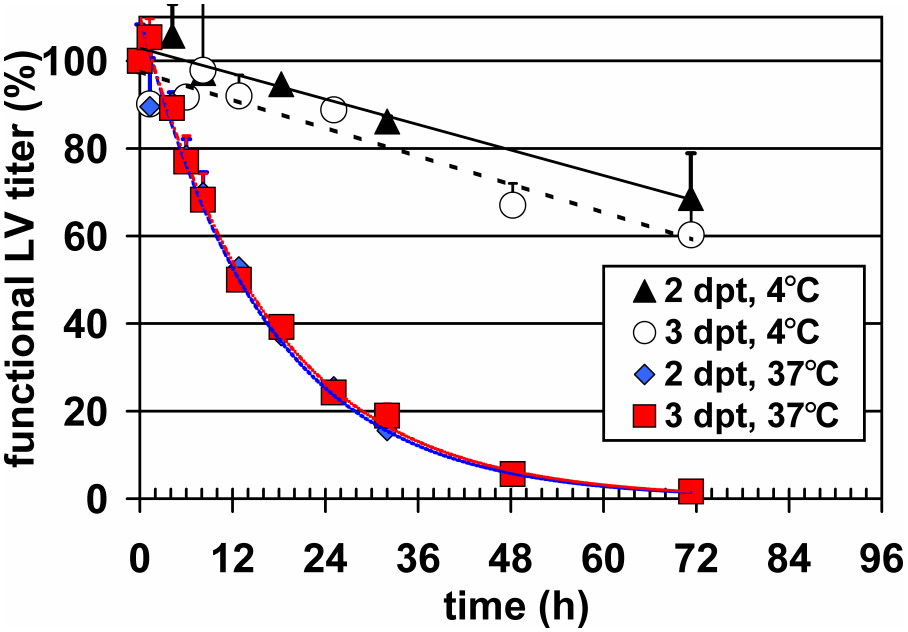

Figure 2.2: LV stability in cell free bioreactor supernatants.

Supernatants were harvested from continuous bioreactor cultures at 2 and 3 days post-transfection (dpt) and incubated at 4 and 37°C prior to GTA analysis

2.1.4 LV Production in Small Scale Cultures

To mimic perfusion conditions and continuous LV production in bioreactor scale at high cell density, an experimental approach using sequential medium replacement was used in small scale cultures. Few hours before transfection, the cell suspension was centrifuged (300×*g*, 5 min) and resuspended in fresh medium at the targeted cell density for transfection. This simplified protocol was used to facilitate the execution of experiments and increase the experimental throughput. LV harvest was then conducted using complete daily medium exchanges by centrifugation (300×*g*, 5 min) starting 1 dpt (i.e. at 24, 48, 72 and 96 hours post-transfection (hpt)). With the exception of data presented in chapter 3, we refer to this approach as “sequential discontinuous medium exchanges per day (DMD)”. Initial conditions, as presented in chapter 3, include transfection at $1x10^6$ c/mL and 1 DMD. In improved production conditions, cell density at transfection was increased to $5x10^6$ c/mL and medium was exchanged at 1 or 2 DMD. At 2 DMD, media was exchanged at 16, 24, 40, 48, 64, 72, 88 and 96 hpt. When LVs were produced at higher cell densities than $5x10^6$ c/mL, centrifugation time and relative centrifugal force were increased from 5 to 10 minutes and from 300 to 350×*g*, respectively.

Each medium exchange decreased the LV titers in supernatants to very low and in comparison insignificant values (fig. AIV.2). Each harvest only represents the production of LV which occurred from one harvest to the subsequent one, thereby greatly facilitating the analysis of production kinetics.

After centrifugation, the LV-containing supernatants that had been harvested were filtered through 0.45 µm HT Tuffryn membranes (diameter of 25 mm) (Pall, Ann Arbor, MI) to remove residual cells and cellular debris and stored at -80°C until further analyses. In general, not more than 3 mL of supernatant were subjected to filtration. At cell densities >5x10^6 c/mL, increased backpressure was observed during filtration. In these cases, membranes with larger filtration area (diameter of 32 mm) or sequential filtration through 0.8 µm Supor membranes (diameter 32 mm) followed by 0.45 µm filtration (25 or 32 mm) was performed. None of these methods decreased functional LV titer compared to non-filtered supernatants (results not shown). Stocks of sodium butyrate were prepared at a concentration of 1 M from n-butyric acid (SIGMA, B-2503) and neutralized with 10 M NaOH. This stock solution was added directly to the cell culture to reach a concentration of 5 mM whenever indicated. A basic description of the LV production protocol can, in a slightly modified form, also be found in the literature (Segura, Garnier et al. 2010).

2.1.5 LV Production in Bioreactor Cultures

A similar bioreactor setup to the one used in this study has been described previously (Henry, Kamen et al. 2007). A schematic representation of the bioreactor setup is presented in fig. 2.3. Minor modifications compared to this setup were the following: agitation rate was set to 85 rpm, pH was controlled in the range of 7.1-7.2 by addition of CO_2 via the surface or sodium bicarbonate solution (7.5 % (w/v)). The vessel (working volume 2.7 L) was equipped with probes to measure and control pH, DO and temperature. A Biomass System® (Fogale nanotech, Nîmes, France) as well as a Biomass Monitor 220 (Aber Instruments, Aberystwyth, UK) (standard *in situ* probes for 25 mm ports) were employed for the measurement of permittivity across the β-dispersion frequency spectrum of ~0.1-10 MHz. In perfusion mode, cells were retained in the bioreactor using a 10 L acoustic filter (AppliSens, Schiedam, Netherlands) operated in backflush mode (full recycling of cell suspension into bioreactor at each backflush) with an interval of 30 min and a run/stop ratio of 55/5 s. The use of acoustic filter technology for perfusion cultures of

mammalian cells has been extensively described in the literature (Trampler, Sonderhoff et al. 1994; Henry, Dormond et al. 2004; Shirgaonkar, Lanthier et al. 2004).

Bioreactor cultures were inoculated at cell densities around 5×10^5 cells/mL (viable cell count (*vcc*)). Perfusion was started after initial growth in batch mode at cell densities above 1×10^6 cells/mL. Perfusion was initiated at 1 or 2 volume(s) of medium per reactor volume per day (VVD). After reaching the targeted cell density (1×10^6 *vcc* (starting conditions) or $5-8\times10^6$ *vcc* (improved production conditions) in perfusion mode, the culture was transfected by the addition of polyplexes and perfusion was stopped for 3 h. After transfection, the LV-containing supernatant was kept at 4ºC until clarification and sodium butyrate was added following the same procedure as in small scale. The harvested supernatant was filtered through a Supor double membrane with pore sizes of 0.8 and 0.45 μm (Pall, Ann Arbor, MI). Bioreactor samples were filtered according to the procedure described above (section 2.1.4). Clarified supernatants and samples were subsequently stored at –80ºC until further analyses.

The DO was controlled at 40 % of air saturation by sparging pure oxygen in pulse mode into the culture at increasing flow rates (depending on the biomass content in the reactor). From the total oxygen volume sparged into the bioreactor, the oxygen sparging rate (OSR in mL/min) was calculated as an indicator of the volumetric oxygen consumption.

A summary of the bioreactor cultures performed in this work can be found in table AII.2.

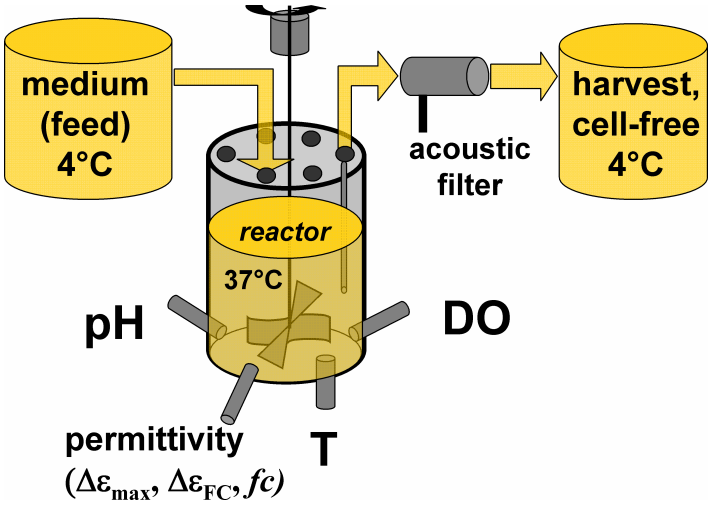

Figure 2.3 : Schematic representation of bioreactor setup and continuous operation for LV production.

For all cultures, bioreactors were equipped with *in situ* sterilizable probes to measure pH, temperature (T), dissolved oxygen (DO) and permittivity (see section 2.5 and chapter 5).

2.2 Analytical Methods for Assessment of LV Quantity and Functional Titers: Variability Assessment and Comparison of Quantification Methods

To appreciate the challenges in LV production process development, it is fundamental to assess the intrinsic variability of LV quantification methods (see also section 1.2.5). This section first provides a short theoretical introduction on the quantification methods used in this work. We focus on practical constraints of the different methods and assess inter- and intra-assay variations. It is critical to determine the accuracy of each quantification method to decide whether or not differences in titers observed using modified production conditions are significant.

Quantification methods can be generally divided in two categories: The techniques assess either 1) LV quantity, i.e. total/physical particle titers or 2) functional LV titers. The ratio of functional vectors to total particles in a given preparation is an indicator of LV quality.

Methods targeting total/physical particle titers are directly assessing the LV concentration in cell culture supernatants. The results largely depend on how a total/physical particle is defined by the

experimental approach, i.e. which part of the viral particle is quantified. The most widely used direct LV quantification methods, the CAp24 ELISA and quantitative RT-PCR, assess the concentration of major structural components such as the most abundant LV protein, the capsid CAp24, and the viral genetic information in culture supernatants, respectively. From these two methods, RT-PCR is best suited to measure the amount of total particles (VG titer) (Gerearts, Willems et al. 2006). It is known that after transfection without the Gag-Pol plasmid which is one of the main drivers of LV particle formation, viral RNA secretion is significantly reduced, corresponding to one thousandth of values from control transfections (Ikeda, Takeuchi et al. 2003). Nevertheless, viral RNA can even be detected if no functional LV particles are present when the VSV-G construct is omitted during transfection (Geraerts, Willems et al. 2006).

In contrast, the CAp24 ELISA (VP titer) can be affected by false positive results from free non-viral related CAp24, is less reliable for evaluation of functional vector particles and also subject to high variability (Ansorge, Henry et al. 2010; Geraerts, Michiels et al. 2005; Ricks, Kutner et al. 2008). Nevertheless, the CAp24 ELISA remains to date the most widely used assay.

Generally, all direct methods overestimate functional titers and indirect methods must therefore be used to quantify the number of functional LVs. Functional assays (indirect methods) assays determine viral vector function or transgene transfer after transduction of suitable target cells. Functional titers are a function of the respective transduction protocol. More specifically, functional LV titers are dependent on (see also section 1.2.5 and tables 1.1-2):

- the target cell line and the culture conditions including the medium,
- the addition of transduction-enhancing additives (e.g. Polybrene),
- the cell cycle phase/arrest of target cells,
- the transduction conditions such as inoculum volume and number of target cells,
- the vector stability, length of exposure,
- the transgene, its promoter, vector backbone, i.e. transgene expression efficiency,
- the transduction detection method used in the assay.

The assays are generally dependent on the final application in which LVs are intended to be used. In most cases, functional assays are consequently conducted on two cell lines: First, LV

preparations are titered on a universally used cell line using a standard protocol to allow for comparison of production yields. Second, a protocol including the cell line of interest for the final application is performed to predict gene transfer efficiency and the target dose.

In the following, we will summarize the experimental approach of the three main quantification methods used in this work.

2.2.1 Assays for the Determination of Total/Physical Particles

2.2.1.1 LV Quantification by Quantitative RT-PCR

Real-time quantitative PCR is widely used to quantify LVs. An excellent review on the topic (Delenda and Gaillard 2005) provides a comparison of existing methods, their constraints and pitfalls. Thus herein we give only a short summary on the topic and focus on the approach that was developed in the present work. In general, RT-PCR offers the flexibility to detect LVs at several levels, i.e. either by quantifying viral particles in supernatants (VG titer), the cellular genomic DNA after transduction (proviral DNA) or transgene mRNA levels after transduction to estimate the transgene expression level.

The two most common approaches comprise VG titer and proviral DNA quantification (fig. 2.4).

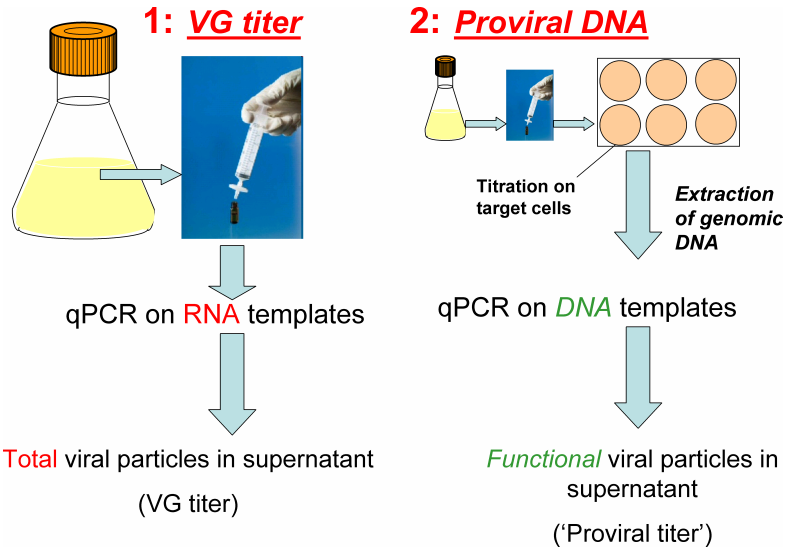

Figure 2.4 : Flow diagram comparing the two most common methods for LV particle quantification by RT-PCR, VG titer (1) and proviral DNA (2) quantification.

In this work, RT-PCR was exclusively used to assess VG titer as a measure of the total viral particle numbers. Several protocols employing different purification methods and primer targets have been published for that purpose. Table 2.1 summarizes recently published methods for VG titer determination.

Based on this comparison, a quantification method was developed using commercially available kits for purification, DNAse treatment and RT-PCR. WPRE (woodchuck hepatitis virus posttranscriptional regulatory element) was chosen as primer target as it is present in most of the recent LV constructs and can be detected at all levels of the LV life cycle. In particular when estimating VG titer after transient transfection, carryover of DNA from vector production can lead to false positive results in RT-PCR assays. A DNAse treatment was therefore included into the sample preparation procedure prior to PCR analysis. Only the most recent protocols describe the use of an RNA standard to calculate the number of viral genomes per PCR reaction. Routinely used DNA standards do, however, neglect differences in the reverse transcription process. A RNA standard based on the LV transfer vector pCSII-CMV5-GFPq (section 2.1.2) was thus developed.

Table 2.1 : Comparison of RT-PCR based methods for VG titer quantification (direct method)

Reference	LV system, production mode	PCR method	Purification	Primer Target	Linearity, Standard	Ratio functional : total (%)	Remarks
Geraerts et al., 2006	2nd generation, triple transfection	TaqMan principle, one-step RT-PCR	RNAqeuous – Micro Kit (Ambion) + DNAse	WPRE, GFP, LTR-gag	10^3-10^8 RNA copies/rxn, RNA standard	0.1-10 % (GTA: PCR) (vector-dependent)	RNA standard, rT-control
Lizee et al., 2003	2nd generation SIN, triple transfection	TaqMan principle, 2-step (rT before PCR)	RNA: Rneasy columns (Qiagen + DNAse. DNA: Easy-DNA kit	WPRE	10^3-10^8 copies/rxn, DNA standard	0.5 % (ratio proviral DNA : RNA) (GFP-GTA 6-fold lower than proviral DNA)	DNA standard, internal controls for normalization albumin (TVGs), β-actin (TEU)
Sastry et al., 2002	3rd generation SIN, Cell Genesys, quadruple transfection	TaqMan, two-step (rT before PCR)	RNA: Rneasy columns (Qiagen + DNAse before purification)	primer binding site of HIV-1	~$5x10^3$-$5x10^6$ copies/rxn, DNA standard	0.05 % (proviral DNA: RNA) GFP 10-fold lower than proviral DNA	DNA standard, rT-control
Ikeda et al., 2002	2nd generation, triple transfection; EIAV was also tested	TaqMan, one-step RT-PCR	QIAamp viral RNA mini kit + DNAse treatment	eGFP	n.a., n.a.	0.2 %, control transfections with empty Gag-Pol plasmid gave 1000-fold lower VG titers	rT-control
Scherr et al., 2001	2nd generation SIN, triple transfection	TaqMan, two-step (rT before PCR or no rT for cDNA target)	Sanburn and Cornetta (1999)	U5/R region	~10^1-10^6, DNA standard	< 1%	minus strong-stop codon cDNA as primer target
Martin-Rendon, 2002	EIAV, triple transfection	TaqMan, one-step RT-PCR	Viral RNA extraction kit (Qiagen) + DNAse treatment	CMV	4 orders of magnitude, no standard	n.d.	Only Ct values are reported, rT- control

RT-PCR method used in this work (VG titer)

The VG titer, i.e. the amount of total viral particles expressed as viral genomes (vg)/ml), was determined using a SYBR-Green® I (SGI) quantitative RNA RT-PCR assay (Roche Applied Science, Laval, Qc). An in-house RNA standard was used to quantify LV in supernatants from production runs. The RNA standard was produced using the MEGASCRIPT kit (Applied Biosystems, Foster City, CA). For this purpose, WPRE from the LV transfer vector (pCSII-CMV-GFPq) was cloned into pUC19 T7. The resulting plasmid pUC19 T7-LVWPRE was then linearized and *in vitro* transcribed. The transcript (RNA standard) with a length of 260 bases was purified (MEGACLEAR kit, Applied Biosystems, Foster City, CA), quantified by spectrophotometry, aliquoted at the final standard concentrations ($1x10^9$-$1x10^4$ copies/reaction) in DEPC-treated water and finally characterized (fig. 2.5). Specificity of the PCR reactions was confirmed by melting curve and agarose gel analysis. The lower detection limit of this method is in the range of $5x10^5$ vg/mL.

After RNA purification from LV-containing supernatants using the High Pure Viral RNA kit (Roche Applied Science, Laval, Qc), samples were treated with DNAse (DNA-free Kit, Applied Biosystems, Foster City, CA). RT-PCR was then performed in a Carousel-based LightCycler (Roche Applied Science, Laval, Qc) or a Mastercycler ep realplex system (Eppendorff, Hamburg, Germany). Primers (P1, P2) targeting a sequence in the WPRE sequence were selected using clone manager (Sci-Ed Software, Cary, NC). Each reaction had a volume of 20 µL with a concentration for each primer of 0.5 µM (P1: LVWPREF: AGT-TGT-GGC-CCG-TTG-TCA-GG, P2: LVWPRER: AGG-CGA-GCA-GCC-ATG-GAA-AG), amplifying a sequence in the WPRE element of 249 bp.

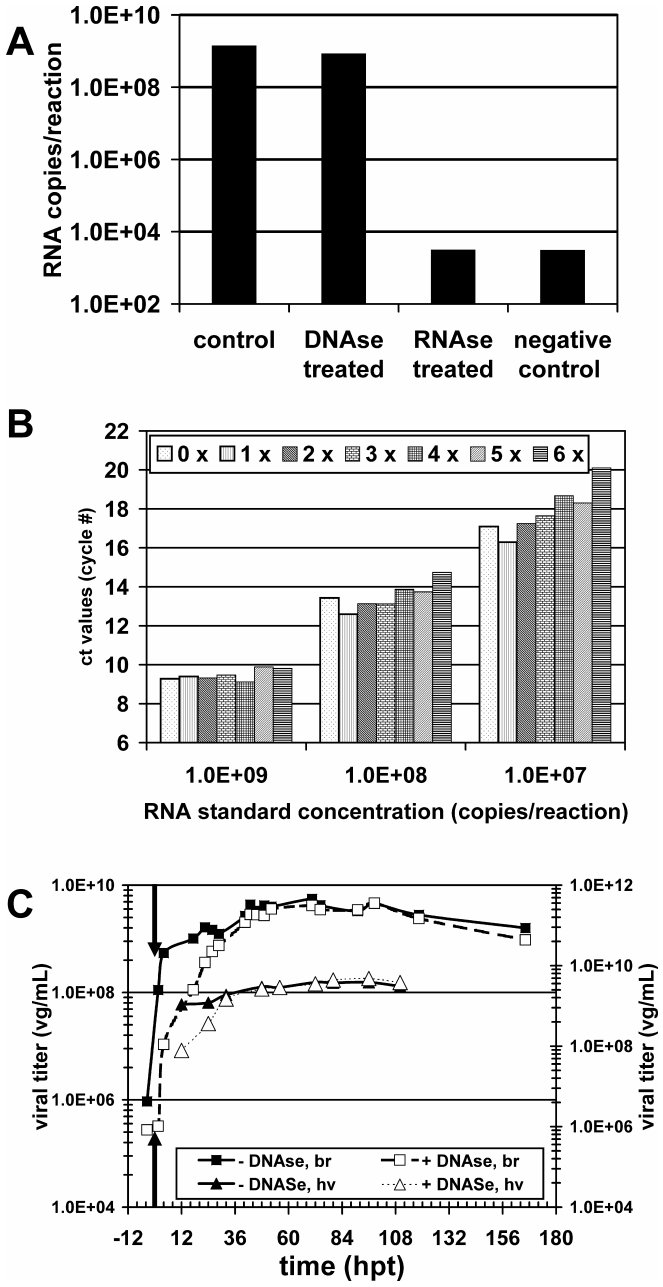

Figure 2.5 : Characterization of in-house RNA standard and qualification of PCR method with process samples.

(A) DNAse and RNAse treatment of RNA standard; (B) evolution of RNA standard PCR results after sequential freeze-thaw cycles, legend indicates number of cycles; (C) PCR analysis of bioreactor (run #5) samples without (- DNAse) and with (+ DNAse) DNAse treatment, arrows mark time of transfection; br: samples taken from bioreactor; hv: sample from the harvested cell-free supernatant

Figure 2.5 presents the characterization of the RNA standard and qualification of the PCR method during a bioreactor production run. We observed that residual DNA in the preparation was insignficant as copy numbers were not affected by additional DNAse treatment. Additionally, RNAse treatment could bring the signal back to the baseline value of the negative control (fig. 2.5 A). The freeze-thaw stability of the RNA standard was evaluated after sequential freeze-thaw cycles (fig. 2.5 B). No significant loss in RNA copy number was found up to three cycles and we thus defined this number as a maximum after which the RNA standard aliquots were discarded.

When comparing samples from bioreactor production with and without DNAse treatment prior to PCR, we found that it was in particular decreased during the first 24 hours post-transfection. In later process stages, the difference in VG titer of treated and non-treated samples was lower. This indicates that residual plasmid DNA was most likely the main reason for increased PCR signals and that the DNAse treatment successfully addressed the carryover of plasmid DNA after transfection (fig. 2.5 C).

The inter-assay variability of the developed PCR method was very low and the results between different assays, based on the RNA standard curve, were highly reproducible (fig. 2.6, table 2.2).

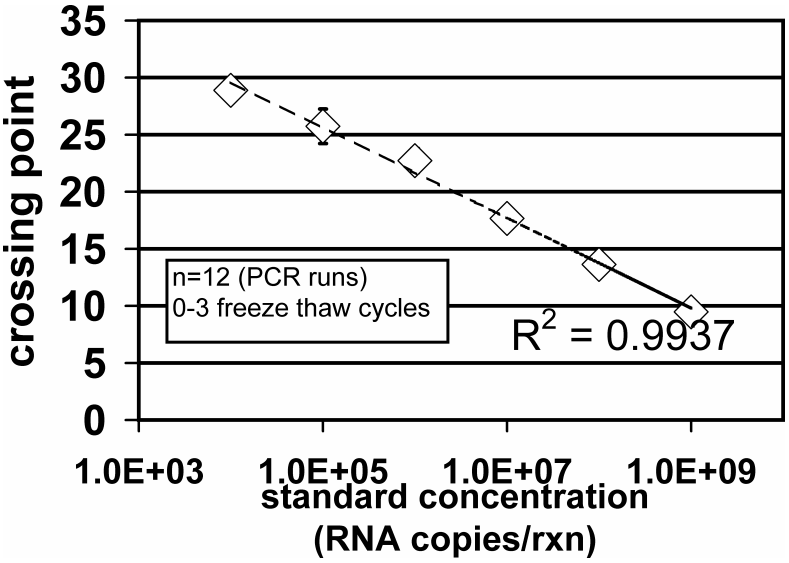

Figure 2.6 : Average standard curve from independent PCR analyses (n = 12); crossing point is identical to ct values shown in fig. 2.5 B

2.2.1.2 LV Quantification by CAp24 ELISA (VP titer)

As briefly mentioned earlier, various sources such as unincorporated CAp24 that is not related to viral particles can lead to false positive results in CAp24 ELISA assays (ELISA), in particular when crude vector preparations are titered (Ricks, Kutner et al. 2008). In addition, CAp24 is released from cells after transfection even though no functional LV is present (Geraerts, Willems et al. 2006).

In the present work, a commercial CAp24 ELISA kit was used according to the manufacturer's instructions (QuickTiter Lentivirus Titer Kit (LV-associated HIV p24); Cell Biolabs Inc, San Diego, CA) to determine VP titer. The manufacturer of this kit claims that the sample preparation method allows quantifying of the LV Cap24 core protein only, i.e. free capsid protein is excluded from the measurement.

Processing of samples according to this protocol was particularly tedious and time consuming. Only a limited number of process samples were therefore analyzed for comparison with the other LV quantification methods. A further disadvantage of the method is its restricted linear range (>1.5–50 ng/ml, average standard curve shown in fig. 2.7). The resulting extensive dilution of LV samples will likely affect reproducibility.

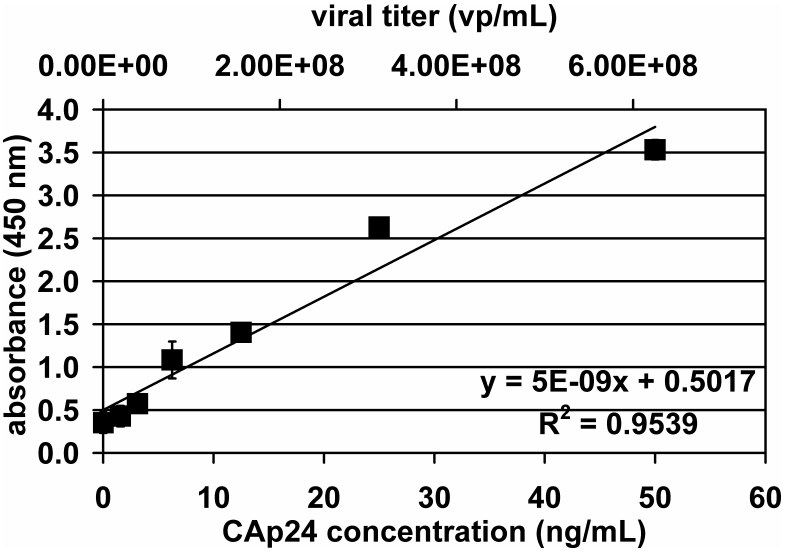

Figure 2.7 : Average standard curve from independent CAp24 ELISA assays (n = 3).

Remark: the regression coefficient was slightly higher (R2 = 0.99) when values until 25 ng/mL only were considered for the regression

2.2.2 Functional LV Titer Quantification by Gene Transfer Assay (GTA)

For the assessement of functional LV titer, it is necessary to conduct indirect methods that allow for the quantification of the LV ability to transfer its genetic information to suitable target cells (section 1.2.5.2, 2.2). In this work, a gene transfer assay (GTA) using suspension-grown cells was used to quantify functional LVs. The method is simple, allowing for a high throughput while minimizing assay variability. For this purpose, HEK293E cells (clone 6E) were cultured in Freestyle^{TM} 17 medium (Invitrogen). Transduction and titer determination was performed as previously described (Segura, Garnier et al. 2007). In brief, the target cells were diluted at the day of transduction to a cell density of $5.5x10^5$ c/mL. Polybrene was directly added to the cell suspension at 8 μg/mL. After 15-30 min of incubation at 37°C, 0.9 mL of the cell suspension was loaded into each well of a 12 well plate and 0.1 mL of diluted LV containing samples were added. Two days later, the cells were harvested by centrifugation, resuspended in PBS and fixed by formaldehyde addition (final concentration of 2 %). The samples were then scored for GFP expression by FACS analysis. Functional LV titer was calculated using the formula:

tu/mL = % GFP positive cells * dilution factor * cell count at time of transduction (c/mL) *assay volume (0.9 mL)/100

The limit of detection of this assay is $\sim 1\times10^5$ tu/mL. We used an in-house LV standard in all GTA experiments to assess inter-assay variability. All final titers were calculated relative to this LV standard that was found to be stable over a period of more than one year after storage at -80°C in culture medium. The average standard concentration was calculated from independent GTA experiments (n = 40) and gave a standard deviation of 27.7 % (fig. 2.8).

To confirm that GFP-expression was not the result of pseudotransduction, which is defined as detection false GFP-positive cells caused by diffusion of GFP or transfer of GFP-encoding DNA, control experiments with selected samples were performed in the presence of azidothymidine (AZT) (SIGMA, 100 μM). In all performed control experiments, the number of GFP-positive cells was at least reduced by 90 % compared to titration without AZT (figure AIV.3). In contrast, the addition of butyrate had no direct effect on LV titer quantification during the assay (fig. AIV.1).

The only disadvantage of the described method is the rather high detection limit of $1x10^5$ tu/mL and the need for large volumes of LV containing samples. The approach is probably amenable to smaller volumes but this was not tested in this work as routine production conditions already resulted in sufficient volumes of detectable LV amounts.

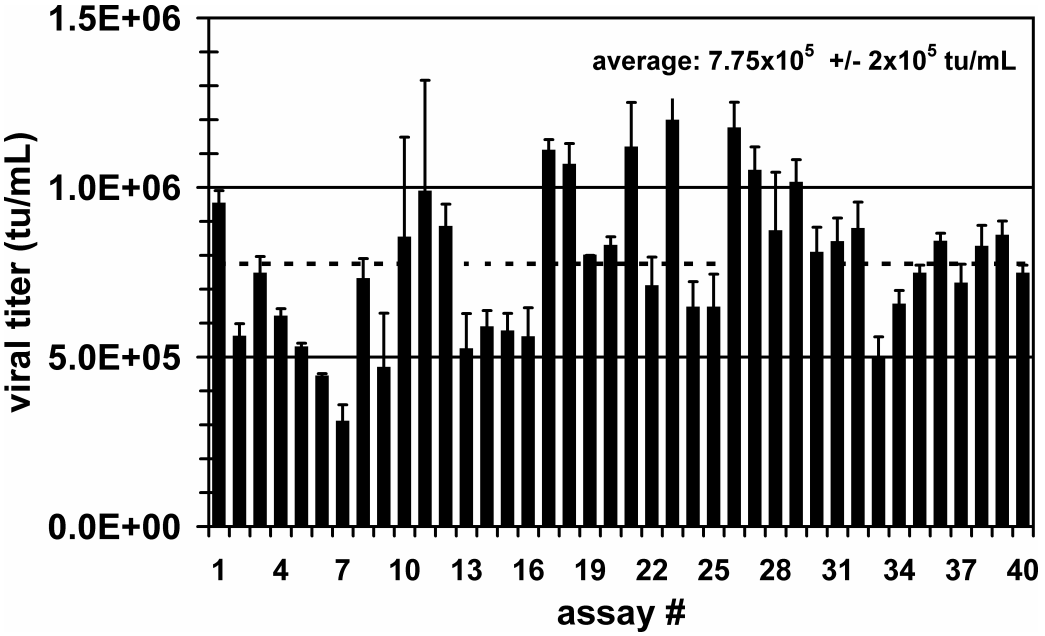

Figure 2.8 : Variation in functional LV titer of in-house GTA standard.

Each bar represents the average value of one GTA assay; dashed line represents the average values from n = 40 independent assays; cell density at the time of transduction was on average: 5.19 +/- 0.935 $x10^5$ c/mL

2.2.3 Summary and Comparison of LV Quantification Methods

In summary, available LV quantification methods are a considerable constraint for process development (see table 2.2 for a comparison of the different methods). All approaches are of high work intensity, limiting the number of samples that can be analyzed by a single operator per day. In-house standards were instrumental to evaluate variability of all non-commercial methods and allowed comparing the results of samples that were processed in independent assays. In general, the time to result using the different protocols is long, in particular for functional titer determination for which results are not available earlier than 2 days after viral transduction of the target cells.

As the FACS-based GTA assay is dependent on the expression of GFP in transduced cells, the low flexibility with respect to other LV constructs is the main limitation of the present approach. Although strictly controlled conditions for target cell transduction were used here, the observed inter-assay variability was nevertheless quite high. In this context, it was put forward by others that small variations in cell density and random integration are responsible for variations in LV titration assays (Geraerts, Willems et al. 2006; Zhang, Metharom et al. 2004). In this work, a reduced intra-assay variability compared to the inter-assay variability was observed. It is thus believed that the state of the target cell line, its passage number and history could have impacted the GTA results. This suggests that a stringent control of target cell line history could be evaluated to reduce assay variability.

RT-PCR based methods are particularly promising for LV quantification as the time to result for VG titer determination is short and inter-assay variability is low. The results suggest the evaluation of RT-PCR for functional LV determination after target cell transduction (approach 2 in fig. 2.4) as this might reduce the time to result of the GTA assay significantly and additionally provide the flexibility to use the same test for several LV constructs.

In contrast, LV quantification by CAp24 ELISA was the least advantageous method suffering from a high variability, a long time to result and the highest labor intensity. This method remains nevertheless essential due to its specificity for the LV capsid protein, for comparison of results of different LV-based constructs and when PCR-based methods are not available.

The detection limits of the quantification methods were not directly compromising the comparison of different production conditions for the model system. Routine starting conditions (chapter 3) already resulted in significant amounts of LVs that were detectable by GTA and PCR. However, it remains to be evaluated if this will hold for other LV constructs which are produced at lower yields.

Table 2.2: Comparison of LV quantification methods used in this work

LV Quantification Assay/ *assay parameter*	**GTA**	**PCR**	**ELISA**
Method	in-house, similar to previously described approach (Segura et al., 2007)	in-house/kit-based, see section 2.2.1.1	kit-based
Assay standard	in-house, aliquot from bioreactor run #2	in-house, in vitro transcript of WPRE sequence	kit-based
Inter-assay variability[I]	**27.7 %,** (n = 40)	**4 +/-1 %** (n = 12)	**15 +/- 12.5 %**[X], (n = 3)
Intra-assay variability[II]	8 +/- 16 %, (n = 250)	9 +/- 8 % (n = 9)[VII]; 22 +/- 15 % (n = 17[VIII])	**21 +/- 20 %**, (n = 34) [XI]
Time to result/ throughput per day[III]	**2 d[VI], ~60**	**3-6 h[IX], 40-50**	**2 d, 40-50**
Labor intensity[IV]	+	+	-
Flexibility[V]	+	+++	++
Detection limit (DL)	0.5-1x10^5 tu/mL	5x10^5 vg/mL	1.95x10^7 vp/mL
Remarks	highest number of samples processed with this method	most advantageous LV quantification method	low number of assays was available for analysis

Remarks Table 2.2 :

+++: recommended/advantageous

++: recommended but intrinsic constraints

+: significant intrinsic constraints

-: not recommended

I: standard deviation based on assay standards; n corresponds to the number of assays

II: average standard deviation from duplicate analyses of identical samples (n = # of duplicates)

III: maximum number of samples that can be analyzed by one trained operator per working day

IV: results from duplicate analysis of identical samples using independent purification and DNAse treatment steps

V: method flexibility concerning the analysis of varying LV constructs/particles

VI: time to result could possibly be reduced by using advanced detection methods (PCR)

VII: results from duplicate analysis by RT-PCR in a single assay

VIII: results from duplicate analysis by RT-PCR (two assays) after purification with High Pure Viral RNA kit and DNAse treatment

IX: depends on the number of samples (purification step is time-consuming if many samples need to be analyzed)

X: standards at low concentrations with higher variations than at high concentrations

XI: duplicate analysis at two different dilutions for each sample

2.3 Sample Analyses

Cell Counting and Cell Size Measurements

Hemacytometer counts using erythrosine B dye exclusion were used to assess cellular density and viability. The volume weighted arithmetic mean cell diameter (*d*) was determined using a Z2™ Coulter Counter® (Beckman Coulter, Mississauga, ON) with an aperture diameter of 100 µm, followed by analysis of the size distributions with the Accucomp® software package (Beckman Coulter). The upper and lower analysis limits were 7.31 and 23.8 µm, respectively. To calculate *d*, the distributions were plotted as volume (%) against cell diameter (µm). The lower analysis limit was then manually increased to select only that part of the distribution corresponding to viable cells for the calculation.

Each value of *d* represents the average of at least two distributions. Using *d* and the viable cell count per mL (*vcc*), the biovolume (*bv)* was then calculated (chapter 5.2.3.4.1 and appendix III).

GFP measurement

In the present work, the total expression of GFP in samples was quantified offline in 96 well plates using a SpectraMax Gemini EM plate reader (Molecular Devices, Sunnyvale, CA). $1x10^5$ cells were distributed in each well and fluorescence was measured at wavelengths of 485/538/495 nm (excitation/emission/cutoff) after fixation with 4 % formaldehyde. One of the limitations of the method was that the total GFP expression signal is a function of the number of producer cells distributed in the well prior to measurement. Any variation in cell density (obtained from manual counting) is thus reflected in the GFP signal (unpublished observation).

Metabolite Concentrations

Virus-containing samples were filtered by centrifugation (13000 rpm for 5 min, Biofuge Hereaeus, pico) through Microcon Centrifugal Filter Devices (Ultracel YM-30) with a nominal molecular weight limit (NWL) of 30,000 prior to amino acid analysis by HPLC and/or metabolite analysis.

Glucose, lactate, and ammonia assays were performed using the Biolyzer (Kodak, New Haven, CT). Amino acid concentrations in fresh media and supernatants were quantified by HPLC (Waters Alliance System, Waters Corp., Milford, MA) using a modification of the Waters AccQ.Tag method as describedin the literature (Cohen 2000; Pham, Perret et al. 2003; Kamen, Tom et al. 1991).

2.4 Data Treatment and Calculation of Growth and Metabolic Rates and Productivity

Growth and metabolic rates were calculated using standard equations (Henry, Dormond et al. 2004) after filtering of the raw data (online and offline measurements). The calculation of specific rates was based either on the viable cell count (*vcc*) or the online dual-frequency permittivity signal ($\Delta\varepsilon_{FC}$). Offline raw data were fitted using the smoothing spline regression function of the kyplot software. Online data were treated with Matlab (The MathWorks, Natick, MA).

The cell-specific viral productivity for our experiments (tu/cell; vp/cell; vg/cell) was calculated by dividing the total produced LV particles after 4 days post-transection by the number of cells at the time of transfection.

2.5 Permittivity Measurements

The underlying theory on the dielectric properties of biological cells has been described in detail elsewhere (Kell et al. 1985; Harris and Harris 1987; Pethig and Kell 1987; Markx and Davey 1999). In brief, when placed in an alternating electric field, polarization of the cells occurs due to their insulating plasma membrane (fig. 2.9).

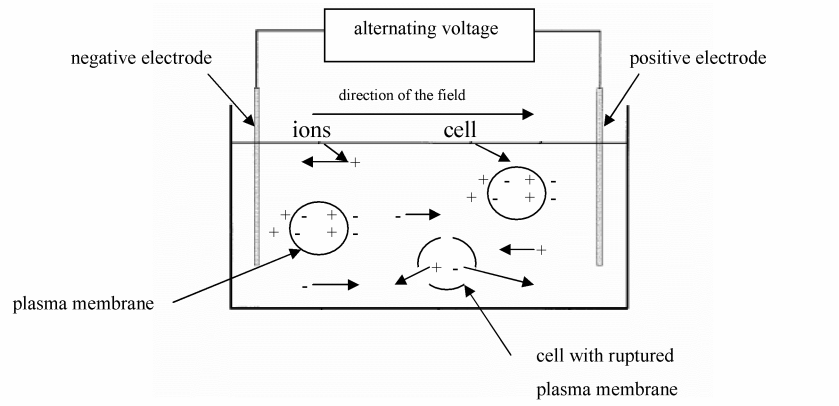

Figure 2.9 : Biological cells act like tiny capacitors in an electrical field

This polarization or charge separation can be measured in terms of the permittivity of the cell suspension. Hence, intact biological cells act as tiny capacitors in electrical fields whereas cells with ruptured membranes theoretically do not contribute to the permittivity. Any given cell suspension shows a characteristic decrease in permittivity when applying alternating currents at increasing frequencies in the range of 0.1-10 MHz. This decrease, the β-dispersion, is caused by the polarization of cell membranes and can be defined by several dielectric parameters (details on the parameters are described in chapter 5). The permittivity is decreasing in the range of the β-dispersion starting on a low frequency plateau that is produced by the complete polarization of the cell membrane whereas the high frequency plateau is due to the polarization of suspending medium or liquid where frequency is too high to cause polarization of the cell membrane.

Additional dispersions can be observed at lower and higher frequencies caused by different effects (Markx and Davey, 1999; Foster and Schwan 1989).

However, only the β-dispersion is of relevance for biomass estimation as the permittivity signals observed in the low-frequency range of the β-dispersion can be used for biomass estimation. Generally, it is the biovolume or membrane enclosed volume fraction that is estimated by permittivity measurements. Several commercial systems are now available. All of these comprise *in situ* sterilizable probes that, in most cases, use a four-electrode probe design to reduce electrode polarization effects (Figure 2.10). The system then computes permittivity, conductivity and the β-dispersion parameters in real-time.

Details on the measurement of permittivity and its related parameters and practical applications of the technology are further described in chapter 5 and appendix III.

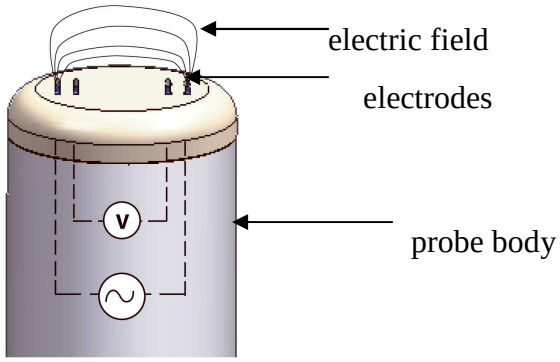

Figure 2.10 : Probe tip of a typical commercial permittivity-based biomass probe (shown here: Fogale Biomass System®)

CHAPTER 3 DEVELOPMENT OF A SCALABLE PROCESS FOR HIGH-YIELD LENTIVIRAL VECTOR PRODUCTION BY TRANSIENT TRANSFECTION OF HEK293 SUSPENSION CULTURES

3.1 Article Presentation

This chapter presents the journal article **"Development of a scalable process for high-yield lentiviral vector production by transient transfection of HEK293 suspension cultures"**. This article was published in the ***Journal of Gene Medicine***. This thesis aims to develop a production strategy suitable for the mass production of lentiviral vectors. To date, two complementary LV production methods exist: 1) the use of stable packaging or producer cell lines and 2) transient transfection. Transient transfection offers advantages in terms of flexibility and a short time to product. This strategy should therefore be ideal for the rapid production of clinical trial lots and is the focus of this work.

The present paper describes the development of a scalable process for the high-yield LV production by transient transfection in perfusion cultures. The main hypothesis was that operation at high cell density would increase LV titers while production in perfusion mode could be used to recover functional LV and thus address the issue of low LV stability. Several strategies to increase specific and volumetric productity were employed in this work. Results suggest that, using this high-yield production process, LV could be generated in an efficient manner for phase I clinical trials in a single pilot scale bioreactor.

In this work, we refer to results from two bioreactor runs that are presented in this paper as run #2 (routine starting conditions) and run # 3 (improved production conditions).

3.2 Development of a Scalable Process For High-Yield Lentiviral Vector Production by Transient Transfection of HEK293 Suspension Cultures

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3.2.1 Abstract

Background

Lentiviral vectors (LVs) offer several advantages over other gene delivery vectors. Their potential for integration and long term expression of therapeutic genes render them an interesting tool for gene and cell therapy interventions. However, large scale LV production remains an important challenge for the translation of LV-based therapeutic strategies to the clinic. The development of robust processes for mass production of LV is needed.

Methods

A suspension-grown HEK293 cell line was exploited for the production of GFP-expressing LVs by transient Polyethylenimine (PEI)-based transfection with LV-encoding plasmid constructs. Using third-generation packaging plasmids (Gag-Pol, Rev), a VSV-G envelope and a self-inactivating (SIN) transfer vector, we employed strategies to increase volumetric and specific productivity. Functional LV titers were determined using a flow cytometry-based gene transfer assay.

Results and Discussion

A combination of the most promising conditions (increase in cell density, medium selection, reduction of PEI-DNA complexes per cell, addition of sodium butyrate) resulted in significantly increased LV titers of more than 150-fold compared to non-optimized small-scale conditions,

reaching infectious titers of ~10^8 transducing units (tu)/mL. These conditions are readily scalable and were validated in 3 L-scale perfusion cultures.

Conclusions

Our process produces LV in suspension cultures and is consequently easily scalable, industrially viable and generated more than 10^{11} total functional LV particles in a single bioreactor run. This process will allow the production of LV by transient transfection in sufficiently large quantities for phase I clinical trials in 10-20 L bioreactor scale.

3.2.2 Introduction

Lentiviral vectors (LVs) are an increasingly important tool for gene and cell therapy interventions. Each virus type offers an inherent set of properties for specific gene delivery applications (Kay, Glorioso et al. 2001). LVs offer advantages over other vectors such as their ability to transduce quiescent and non-dividing cells, the irreversible integration in the genome and a large packaging capacity (Quinonez and Sutton 2002). Compared to γ-retroviral vectors, LV do not show a preference for integration near transcription start sites (Montini, Cesana et al. 2006). LVs have already been used in phase I clinical trials in which efficient and safe *ex vivo* gene delivery to T cells with good persistence *in vivo* was demonstrated (Levine, Humeau et al. 2006; Kohn 2007).

Readily scalable and cost-effective LV production is of primary importance to successfully implement LV-based therapy approaches in the clinic. As an example, for a phase I clinical trial, at least 5×10^{11} functional LV particles would be required (MacGregor 2001). Although the interest in lentiviral vectors in research and development steadily increased over the years, protocols for their production have been given only minor attention. The processes still rely on the classical approach of transfecting adherent cell cultures (Naldini, Blomer et al. 1996; Coleman, Huentelman et al. 2003; Schonely, Afable et al. 2003; Slepushkin, Chang et al. 2003; Sena-Esteves, Tebbets et al. 2004; 2005; Warnock, Merten et al. 2006; Lejeune, Truran et al. 2007; Bellintani, Piacenza et al. 2008; Kuroda, Kutner et al. 2009).

To ease scale-up, attempts have been made to develop stable, inducible packaging or producer cell lines that stably express LV components. However, their generation, in particular for self-

inactivating (SIN) vectors, resulted only in low yields (Kafri, van Praag et al. 1999; Klages, Zufferey et al. 2000; Sparacio, Pfeiffer et al. 2001; Ikeda, Takeuchi et al. 2003; Cronin, Zhang et al. 2005; Ni, Sun et al. 2005; Cockrell, Ma et al. 2006; Warnock, Merten et al. 2006; Broussau, Jabbour et al. 2008; Throm, Ouma et al. 2009).

Currently, transient transfection remains the only viable alternative to generate LVs, in particular for early phases of production campaigns. Transient transfection is a fast method for the production of sufficient amounts of viral vectors (Naldini, Blomer et al. 1996; Blesch 2004). The production of viral vectors by transient transfection in large-scale suspension cultures has recently been described by several independent studies (Durocher, Pham et al. 2007; Hildinger, Baldi et al. 2007; Segura, Garnier et al. 2007). Transfection protocols also allow for the flexibility to use cytotoxic/cytostatic transgenes and/or vector components (Bartz, Rogel et al. 1996). Recently, it was demonstrated that PEI-based transfection leads to better reproducibility of LV production compared to common calcium phosphate-based protocols (Kuroda, Kutner et al. 2009). PEI also allows the production of LV in suspension culture by transient transfection without the need of medium exchange prior or after transfection, making the process scalable (Segura, Garnier et al. 2007).

The efficiency of transient transfection is highly culture medium dependent and seems to be a function of additives (e.g. anti-clumping agents) and nutrient availability before, during, and after transfection (Pham, Perret et al. 2003; Pham, Perret et al. 2005; Pham, Kamen et al. 2006). Enrichment of medium with peptones (or their addition after transfection) results in superior productivity following PEI-mediated transfection (Pham, Perret et al. 2003; Pham, Perret et al. 2005). The presence and concentration of charged entities and polymers that interfere with transfection varies from one medium to another. In practice, the choice of media candidates for transfection is therefore limited. In many processes, the optimization of transfection protocols consequently results in significant productivity improvements (Durocher, Perret et al. 2002; Karolewski, Watson et al. 2003; Dormond, Meneses-Acosta et al. 2009). Transfection protocols for suspension cultures are typically performed in batch mode. A limitation of this approach is that common protocols are confined to rather low cell densities. Current studies report cell densities of up to 2×10^6 cells/mL (Derouazi, Girard et al. 2004) with most optimized protocols being in the range of $0.5\text{-}1\times10^6$ cells/mL at the time of transfection (Durocher, Pham et al.

2007). As a result, efforts are still needed to optimize volumetric productivities of transfection protocols.

A low half-life of 4-16 h is reported for LVs and retroviral (RV) vectors (Higashikawa and Chang 2001; Watson, Kobinger et al. 2002). This presents a challenge for the production of LV and RV vectors, rendering processes based on regular batch or fed-batch mode unattractive (Cruz, Almeida et al. 2000; Cruz, Goncalves et al. 2000; McTaggart and Al-Rubeai 2000). In a previous study, the issue of a low LV stability was addressed by producing LVs in batch replacement mode (Segura, Garnier et al. 2007). However, a transfer of this process to industrial operations is difficult (Geisse 2009). Perfusion mode using acoustic cell filter devices for cell retention has already been validated as an advantageous strategy for adenoviral and retroviral vector production (Ghani, Garnier et al. 2006; Henry, Kamen et al. 2007). It allows a rapid harvest of produced viral particles (Merten 2004) and also alleviates nutrient limitations, leading to higher specific productivity (Henry, Kamen et al. 2007).

One important limiting step in LV production appears to be the expression of LV-related genes, possibly due to transcriptional silencing of the transfected plasmid DNA as observed for LV and RV packaging cell lines (Kafri, van Praag et al. 1999; Jaalouk, Eliopoulos et al. 2000; Ni, Sun et al. 2005). Therefore, the addition of n-butyric acid or its salt, sodium butyrate, in a concentration range of 5-20 mM is often described in LV and RV production protocols (Soneoka, Cannon et al. 1995; Sakoda, Kasahara et al. 1999; Karolewski, Watson et al. 2003; Merten 2004; Sena-Esteves, Tebbets et al. 2004). Butyrate leads to higher transcription and increased expression of transfected DNA (Altenburg, Via et al. 1976; Kruh 1981; Gloger, Arad et al. 1985; Gasmi, Glynn et al. 1999; Davie 2003).

In this study, we present a scalable process for the production of LV by transient transfection of suspension-grown HEK293 cells in perfusion cultures. The goal of this work was the development of a process that could be easily transferred to industrial bioreactor scale. We conducted a complete daily medium exchange after transfection starting 1 day post-transfection in small-scale experiments. This allowed harvesting of LV particles, removal of remaining polyplexes and also provided additional nutrients for the transfected cells. Overall, we demonstrate how the combination of several strategies to increase the productivity of the process (increase in cell density, medium selection, optimization of transfection conditions and addition

of the expression-enhancing additive sodium butyrate) led to an improvement in LV yield of more than 100-fold compared to non-optimized production conditions. Bioreactor perfusion cultures were then performed for validation of the conditions with a continuous medium exchange rate of one reactor volume per day. With these improved yields, the process can allow the production of LVs by transient transfection in sufficiently large quantities for phase I clinical trials in a single run using a pilot scale bioreactor.

3.2.3 Materials and Methods

3.2.3.1 Cell Culture

The HEK293SF-3F6 cell line, originally developed for the production of adenoviral vectors, was used in this study (Côté, Garnier et al. 1998). This cell line is highly transfectable and grows in suspension under serum-free conditions.

Several culture media were tested for their ability to sustain production of LV in suspension: Two formulations of LC-SFM (Côté, Garnier et al. 1998) were used in small-scale experiments: LC-SFM L (LC-SFM + 0.1 % (v/v) lipid mixture (SIGMA, L-5146) (Pham, Perret et al. 2003) and LC-SFM GL (LC-SFM L supplemented with 0.5 % (v/v) gelatine peptone GPN3 (OrganoTechnie S.A. (La Courneuve, France)), (Pham, Perret et al. 2005)). For bioreactor experiments, LC-SFM L was supplemented with 0.1 % BSA (Celliance, Kankakee, IL); HyQSFM4TransFx293 (HyQ) (Hyclone, Logan, UT), a commercial medium specifically developed for transfection of HEK293 cells was evaluated as a second media formulation (Tom, Bisson et al. 2007).

Hemacytometer counts using erythrosine B dye exclusion were used to assess cellular density and viability.

Stocks of sodium butyrate were prepared at a concentration of 1 M from n-butyric acid (SIGMA, B-2503) and neutralized with 10 M NaOH.

3.2.3.2 Transient Transfection

The transfection protocol has been published in detail elsewhere (Tom, Bisson et al. 2007). A PEI:DNA mass ratio of 2:1 was found to be optimal for transient transfection of HEK293SF-3F6 (Durocher, Perret et al. 2002; Pham, Perret et al. 2003). We used a GFP-encoding SIN transfer

vector (pCSII-CMV5-GFPq), third generation packaging plasmids for Gag-Pol and Rev (pMDLg/pRRE#54 and pRSV-Rev) and a vector encoding the VSV-G enevelope (pSVCMV-IN-VSVg) (Dull, Zufferey et al. 1998; Broussau, Jabbour et al. 2008). A plasmid mass ratio of 1:1:1:2 (VSV-G: Gag-Pol: Rev: Lentiviral Transfer Vector) was used for transfection according to previous results from our group (Segura, Garnier et al. 2007). Experiments were conducted according to routine procedures using PEI-DNA complexes (polyplexes) with a total DNA amount of 1 $\mu g/10^6$ cells. Precultures were passaged every 2-3 days to keep the cells in their exponential growth phase. A few hours before transfection, the cell suspension was centrifuged ($300\times g$, 5 min) and resuspended in fresh medium at the targeted cell density for transfection. In bioreactor experiments, the culture was grown to the targeted cell density in perfusion mode and transfected thereafter.

3.2.3.3 Harvest of LVs, Plasmid Production

As the production of HIV-forming proteins is initiated as early as 5-6 h after transfection (Jouvenet, Bieniasz et al. 2008), we conducted daily harvest of produced LV and a complete medium exchange by centrifugation ($300\times g$, 5 min) starting 1 day post-transfection in small-scale experiments. LV-containing harvests were filtered through 0.45 μm HT Tuffryn membranes (Pall, Ann Arbor, MI) to remove cell debris and stored at -80°C until further analysis. During bioreactor cultivations, the harvested supernatant was filtered through a Supor double membrane with pore sizes of 0.8 and 0.45 μm (Pall, Ann Arbor, MI).

The four LV-encoding plasmid constructs (Segura, Garnier et al. 2007) were produced using an in-house anion-exchange purification method (St-Laurent et al., manuscript in preparation) or commercially available purification kits (QIAGEN Plasmid Giga Kit, Qiagen, Mississauga, ON).

3.2.3.4 Viral Quantitation and Productivity

Viral titer was determined using a flow cytometry-based gene transfer assay (GTA). For this purpose, HEK293E cells (clone 6E) were cultured in Freestyle™ 17 medium (Invitrogen). Transduction and titer determination was performed as previously described (Segura, Garnier et al. 2007). The limit of detection of this assay is $\sim 1\times10^5$ tu/mL. We used an in-house LV standard in all GTA experiments to minimize inter-assay variability. All final titers were calculated relative to this LV standard that was found to be stable over a period of more than one year after

storage at -80°C in culture medium. The average standard concentration was calculated from independent GTA experiments (n = 21). We observed a standard deviation of ~30 % in these experiments (# of total LV standard tests = 115). To confirm that GFP-expression was not the result of pseudotransduction, control experiments with selected samples were performed in the presence of AZT (SIGMA, 100 μM). In all performed control experiments, the number of GFP-positive cells was at least reduced by 90 % compared to titration without AZT (data not shown).

The cell-specific viral productivity for our experiments (tu/cell) was calculated by dividing the total functional produced LV particles after 4 days post-trasnfection by the number of cells at the time of transfection.

3.2.3.5 Bioreactor Cultures

A similar bioreactor setup to the one used in this study has been described previously (Henry, Kamen et al. 2007). Minor modifications compared to this setup were the following: agitation rate was set to 85 rpm, pH was controlled in the range of 7.1-7.2 by addition of CO_2 via the surface or sodium bicarbonate addition. The vessel (working volume 2.7 L) was equipped with probes to measure and control pH, DO and temperature and a Biomass Monitor 220 (Aber Instruments, Aberystwyth, UK) for the measurement of the culture capacitance. The DO was controlled by sparging pure oxygen in pulse mode. Cells were retained in the bioreactor using a 10 L acoustic filter (AppliSens, Schiedam, Netherlands) operated in backflush mode (full recycling of cell suspension into bioreactor at each backflush) with an interval of 30 min and a run/stop ratio of 55/5 s. Bioreactor cultures were inoculated at cell densities around 5×10^5 cells/mL. Perfusion was started after initial growth in batch mode at cell densities above 1×10^6 cells/mL. The medium exchange rate was set in all experiments to one volume per day.

3.2.4 Results

3.2.4.1 Small Scale Production of LV

3.2.4.1.1 Production of LV at high cell density (HCD)

We conducted small-scale shake flask experiments to identify improved LV production conditions. The goal was an increase in volumetric yield of the LV production process, targeting an operation at high cell density (HCD). In a first effort, we increased the cell density at the time of transfection from the routine cell density of 1×10^6 cells/mL to 5×10^6 cells/mL (fig. 3.1). The yields from transient transfection and the production of viral vectors are highly dependent on the physiological state of the cell culture (Pham, Perret et al. 2003; Pham, Perret et al. 2005; Pham, Kamen et al. 2006; Tom, Bisson et al. 2007). A cell density was hence chosen which could be supported by the selected medium and our chosen production strategy (daily medium replacement). When using the medium formulation LC-SFM L, we observed cell growth in batch mode up to a cell density of $\sim 2.5\times10^6$ cells/mL. In perfusion mode at one medium exchange per day, the culture grew exponentially up to 1×10^7 cells/mL (data not shown). A cell density of 5×10^6 cells/mL was consequently selected for the production of LV at HCD as it corresponded to early/middle exponential growth phase in perfusion mode under initial experimental conditions.

We observed that it was possible to keep a constant specific productivity of ~2 tu/cell at HCD, thereby achieving higher volumetric titers (fig. 3.1 A). When transfecting at a cell density of 1×10^6 cells/mL we reached maximum viral titers at 3 days post-transfection (dpt) of $<1\times10^6$ tu/mL. At HCD, the LV titer was in the range of $\sim 5\times10^6$ tu/mL. The cell density increased in both cases after transfection and viability remained high in all experiments until 4 dpt (>75 %) (fig. 3.1 B).

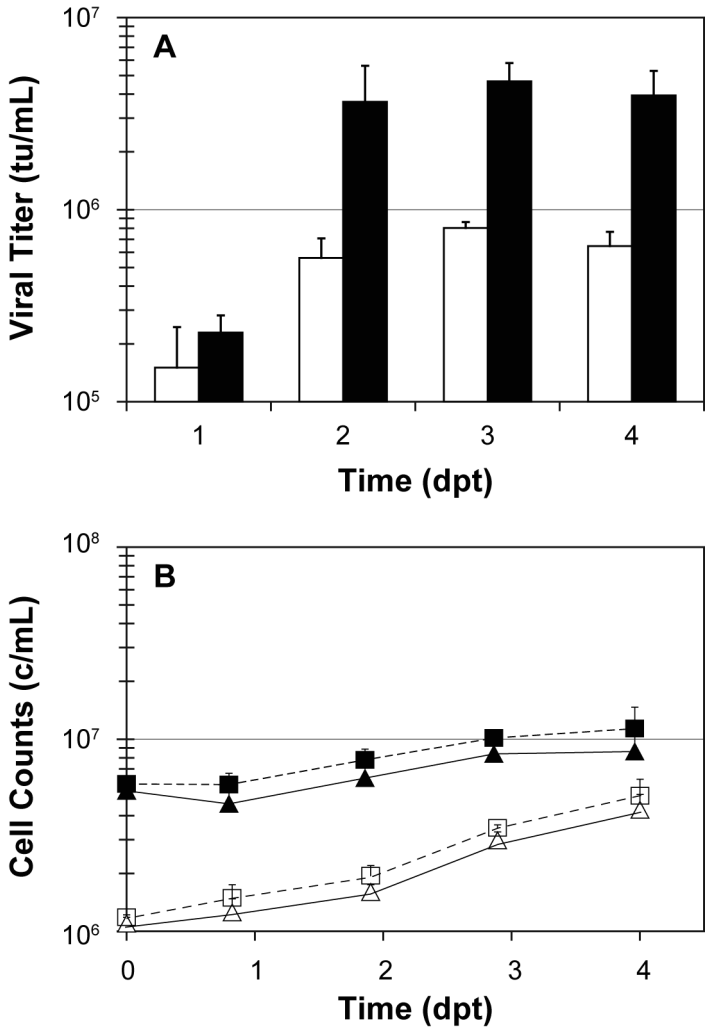

Figure 3.1 : Increase in cell density at transfection leads to higher viral titers at constant specific productivity in small scale experiments.

Experiments were conducted at low (LCD) and high cell density (HCD) in LC-SFM L. A: viral titer. LCD (open bars), HCD (solid bars); B: cell density. total cell count (squares), viable cell count (triangles), HCD (solid symbols), LCD (open symbols)

3.2.4.1.2 Medium Selection for LV Production and Optimization of Transfection Conditions

We consequently pursued this strategy and tested several media for the transfection at HCD. We sought to identify a medium formulation devoid of inhibiting components that required no

complete exchange of medium by centrifugation prior to or after transfection. Medium exchange should primarily be used to harvest LV and maintain high cell densities.

Although several commercial media formulations for transient transfection are available today, the choice of possible media candidates was limited, i.e. only a few media (LC-SFM L; LC-SFM GL; HyQ) showed the indispensable characteristics for this study: growth to high cell densities (with daily medium replacement) and 'suitability' for transfection (no interference with the transfection process itself). An additional preferable feature of possible media candidates was minimization/avoidance of animal-derived components. Preliminary growth tests revealed that cell growth was equivalent or superior in LC-SFM GL and HyQ (data not shown). Transfection was then performed at LCD and HCD in the three media candidates (fig. 3.2). LC-SFM GL was included to test if enriched medium formulations would be beneficial for LV production. GPN3 is, however, an animal-derived component and was consequently not of interest for further development of the production process. Indeed, LC-SFM GL gave highest titers after transfection at HCD (6-8$\times 10^6$ tu/mL) at 2 and 3 dpt. HyQ also showed high potential at LCD with a higher productivity of ~6 tu/cell. This high productivity could however not be transferred to HCD (~2 tu/cell). A possible reason for the lower cell-specific titers at HCD was increased aggregation and formation of a cell ring at the shake flask wall starting 2 dpt (not shown). PEI is known to have toxic effects (Godbey and Mikos 2001; Kunath, von Harpe et al. 2003; Sun, Hia et al. 2008) and might have therefore indirectly induced this phenomenon.

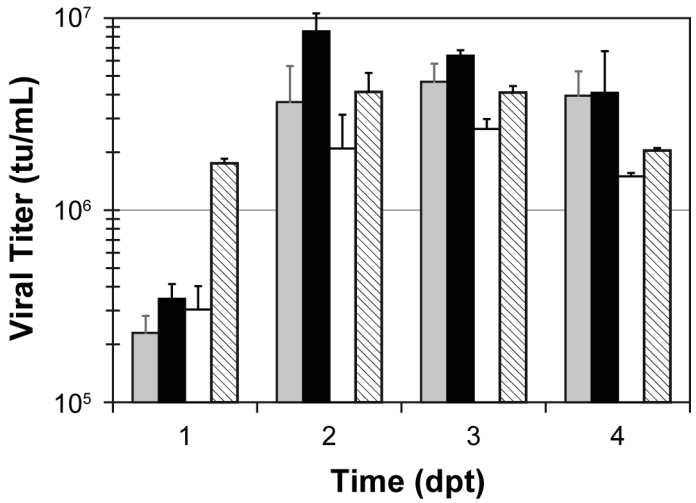

Figure 3.2 : The effect of different culture media on the production of LVs.

Experimental conditions were transfection at: HCD in LC-SFM L (solid grey bars), HCD in LC-SFM GL (solid black bars), LCD in HyQ (open bars) and HCD in HyQ (hatched bars)

Consequently, the impact of a reduction in polyplex amount was evaluated. Highest titers were observed when transfecting with 0.4-0.6 µg of total DNA/10^6 cells (data not shown). This modification of the transfection protocol allowed more than doubling maximum titers 3 dpt to ~9×10^6 tu/mL while using less than half of the total DNA amount for transfection (fig. 3.3). Because of the high potential of HyQ at LCD, the superior cell growth characteristics and medium properties (animal-derived component free medium), the optimization strategy was continued using HyQ.

3.2.4.1.3 Effect of sodium butyrate on LV production

In preliminary experiments, we evaluated the addition of sodium butyrate in batch cultures. We observed reduced cell growth of HEK293SF-3F6 at concentrations higher than 2 mM. At concentrations up to 5 mM, viability was however not significantly affected over a period of four days in preliminary batch cultures (data not shown). We therefore tested sodium butyrate addition after transfection at concentrations ranging from 0.1-5 mM (fig. 3.3). Sodium butyrate was added 16 hours post-transfection (hpt) (Karolewski, Watson et al. 2003) and the concentration was kept constant after each subsequent medium replacement. Using this strategy, maximum LV titers of

10^8 tu/mL 2 dpt could be reached when adding butyrate at a concentration of 5 mM. The amount of produced LV decreased thereafter exponentially. Higher concentrations of butyrate could not significantly increase the yield of LV (data not shown).

The kinetics of LV production under different experimental conditions was changed significantly when adding butyrate (compare fig. 3.2 and 3.3). In all experiments, LV could be detected starting 1 dpt. Without the addition of butyrate, maximum LV titers were observed 3 dpt. Before and after the maximum we found ~75 % (2 dpt) and up to 80 % (4 dpt) relative to the maximum titer for each experiment. When adding butyrate, the maximum in LV titer was found earlier (2 dpt), with an exponential decrease of LV titer on the subsequent days (~50% after 3 dpt, ~20% after 4 dpt).

The cell-specific viral productivity was in the range of 2-70 tu/cell in small scale experiments (table 3.1). Without the addition of butyrate, only ~2-6 tu/cell were produced. These numbers were increased up to 10-fold (~7.5-70 tu/cell) by the addition of butyrate at a concentration of up to 5 mM. At cell densities $>5\times10^6$ cells/mL, we observed a decrease in specific productivity (results not shown).

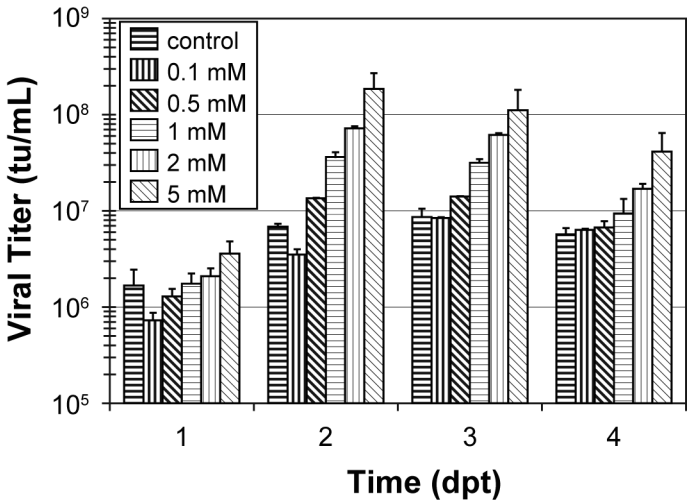

Figure 3.3 : Effect of sodium butyrate addition at different concentrations on the production of LVs.

All experiments shown were conducted in HyQ medium, at high cell density (HCD) and with 0.4 µg of total DNA/10^6 cells. Concentration of butyrate is shown in the figure legend

3.2.4.2 Bioreactor Scale LV Production in Perfusion Mode

In order to evaluate the scalability of the process, LV production was conducted in a lab scale bioreactor cultivation in perfusion mode (fig. 3.4). First, non-optimized production and transfection conditions were used. After transfection at a cell density of 1×10^6 cells/mL, perfusion was stopped for 3 h to allow for polyplex uptake. The harvested LV-containing supernatant was stored at 4°C and collected once per day for purification. After transfection at 1×10^6 cells/mL, cell growth continued until the end of the experiment. Viability remained higher than 80 % during the whole experiment. Detectable amounts of LV were found in the bioreactor from 1-6 dpt. Maximum viral titers were reached at 3 dpt with $\sim7\times10^5$ tu/mL. The titers measured in the harvested supernatant followed the same profile as in the reactor, indicating no significant loss during storage at 4°C for (a maximum of) 24 h.

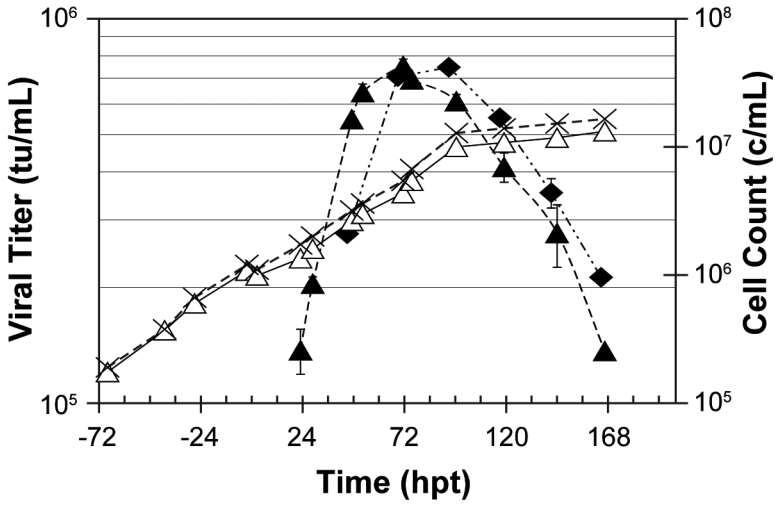

Figure 3.4 : Production of LV in lab scale bioreactor perfusion system.

Initial conditions were used (transfection at LCD in LC-SFM L). LV titer in harvest (solid diamonds), LV titer in reactor (solid triangles), total cell count (crosses), viable cell count (open triangles)

After demonstrating the feasibility of LV production in perfusion mode, we validated the improved experimental conditions from small scale experiments (fig. 3.5). Perfusion was started

at a cell density of $\sim 2\times10^{6}$ cells/mL and transfection was performed at $\sim 4.5\times10^{6}$ cells/mL. Sodium butyrate was added 16 hpt at a concentration of 5 mM. Cell growth slowed down after transfection, reaching a maximum viable cell density of $\sim 6\times10^{6}$ cells/mL 2 dpt. Viability remained high during the whole experiment (>80 %). Maximum LV titers of $\sim 8\times10^{7}$ tu/mL were found 2 dpt in the bioreactor supernatant. Compared to non-optimized production conditions, an increase in maximum LV titers of ~100-fold was achieved. First viral release was observed 1 dpt, starting a few hours after the addition of butyrate.

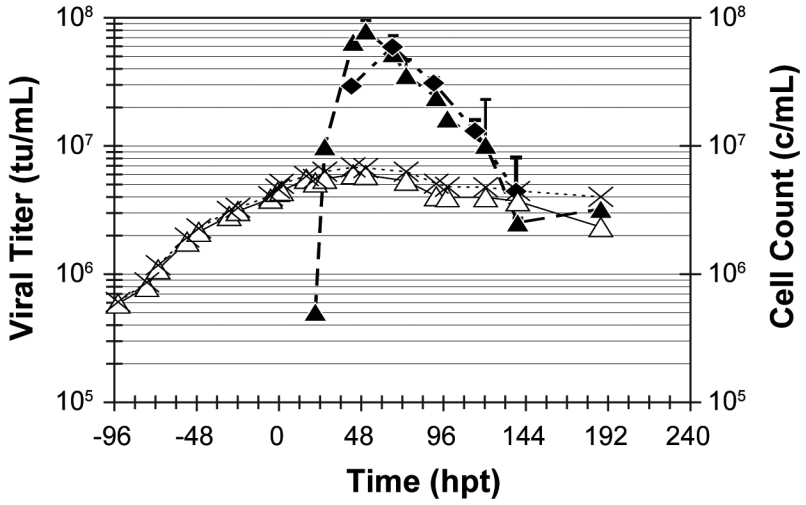

Figure 3.5 : Profile of LV production in perfusion culture under improved conditions.

Improved experimental conditions were used (transfection at HCD in HyQ with total DNA amount of 0.4 µg of total DNA/10^{6} cells), addition of butyrate 16 hpt. LV titer in harvest (solid diamonds), LV titer in reactor (solid triangles), total cell count (crosses), viable cell count (open triangles)

A total of $\sim 3.5\times10^{11}$ tu was produced in a single bioreactor run under improved conditions, compared to $\sim 6\times10^{9}$ tu when initial conditions were used (fig. 3.6). The improved production resulted in a ~75-fold increase in total functional particle yield after 4 dpt. The differences in production kinetics caused by butyrate addition were significant. An almost linear increase of the

total functional produced particles was found under initial experimental conditions from 1-3 dpt. In contrast, a steep increase was observed from 1-2 dpt when butyrate was added. The gain in produced LV particles was low in both experiments after 4 dpt.

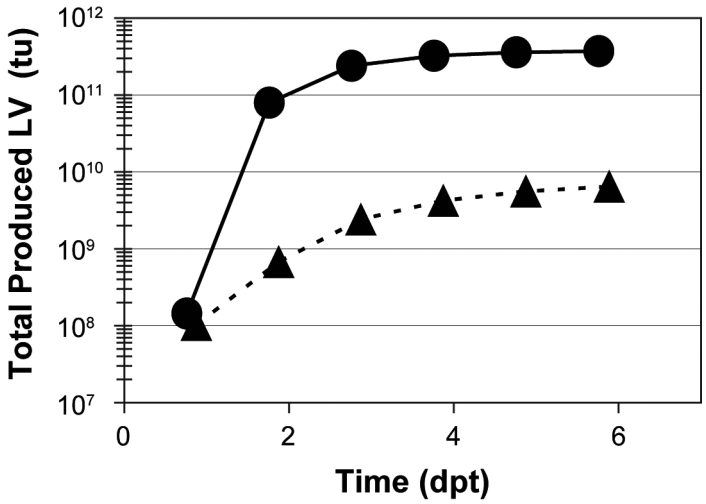

Figure 3.6 : Comparison of cumulative viral titers (total produced functional LV particles) of two bioreactor perfusion cultures.

Initial experimental conditions (triangles, dashed line), improved conditions (solid circles, solid line)

3.2.5 Discussion

We describe here transient transfection at high cell density (HCD) without the need of discontinuous medium exchange before or after transfection. We could circumvent the cell density limitation of routine transfection protocols by producing LV in perfusion mode at HCD. Previously, transfection protocols at HCD have been reported (Backliwal, Hildinger et al. 2008; Sun, Hia et al. 2008). These protocols require however discontinuous concentration or dilution of the cell suspension before or after transfection (Baldi, Hacker et al. 2007; Backliwal, Hildinger et al. 2008). In the present study, media is continuously exchanged to harvest produced LV and alleviate nutrient limitations. The process is consequently industrially viable and readily scalable.

The results from the comparison of different media suggest that the production of LV after transient transfection is dependent on nutrient availability. Enriched medium formulations (LC-

SFM GL, HyQ) outperformed our standard medium formulation (LC-SFM L). These results substantiate previous findings demonstrating the improvement in transient transfection yields by nutrient additions (Pham, Perret et al. 2003; Pham, Perret et al. 2005; Pham, Kamen et al. 2006; Tom, Bisson et al. 2007).

We observed that a significant decrease in polyplex amount leads to higher LV titers. Our results concur with findings from our (unpublished results) and other studies for calcium-phosphate and PEI-based transfection (Karolewski, Watson et al. 2003; Sena-Esteves, Tebbets et al. 2004; Sun, Hia et al. 2008; Kuroda, Kutner et al. 2009). Lower amounts of polyplexes probably minimize toxic effects of PEI, leading to higher productivity after transient transfection. Interestingly, other studies also found optimal LV and recombinant protein production at values of 0.4-0.6 μg of total DNA/10^6 cells for PEI-based transfection (Sun, Hia et al. 2008; Kuroda, Kutner et al. 2009). Whereas most transfection protocols do not take the number of transfected cells into account and report the quantity of transfection reagents on a volumetric basis (per volume of transfected cell suspension), the amount of polyplex used for transfection should therefore rather be calculated on a cell-specific basis. The DNA amounts used in the present study are significantly lower than what is routinely used in calcium-phosphate precipitation (Karolewski, Watson et al. 2003; Sena-Esteves, Tebbets et al. 2004). This further improves the cost-effectiveness of the method used here.

The significant impact of sodium butyrate on the production of LV underlines that this compound enhances the expression of limiting LV and retroviral (RV) gene products (VSV-G, viral packaging proteins and RNA) by increasing their transcription. Findings for RV indicate that butyrate in particular increases the production of packageable viral RNA (Olsen and Sechelski 1995; Jaalouk, Crosato et al. 2006). Without the addition of butyrate, LV titers were possibly also in our case significantly lower because of transcriptional suppression of the viral RNA (Jaalouk, Crosato et al. 2006). The maximum titer was reached earlier and the decline in titer relative to the maximum was accelerated when butyrate was added. It therefore seems to have an impact on the expression kinetics. Optimal expression of all four plasmid constructs for LV production took place 2 dpt. The exponential decrease of LV titer a few days after the addition of butyrate might be caused by a loss of transfected producer cells, in turn due to the expression of the toxic LV

proteins (in particular VSV-G and *gag*). Another explanation could be that butyrate has a different effect on the expression of the LV required proteins over time.

Comparing the results to literature findings, the LV titer and the cell specific productivity of functional LV after transient transfection were similar to what has been reported earlier by our group (Segura, Garnier et al. 2007). A comparison with results from other groups is generally difficult due to the variety of titration methods found in the literature. In addition, a comparison of cell-specific yields is not always possible since LV production protocols are often not providing all necessary values, e.g. the cell density at the time of transfection. Furthermore, differences in plasmid constructs might contribute to the variability in LV titers found in the literature. Using a 3-plasmid system, a yield of ~10 tu/cell was observed by others for LV production by transfection in adherent cells after 2-2.5 d (Kuroda, Kutner et al. 2009). This value is also well in the range of the present study for suspension-grown cells. In summary, the titers observed in the current study should be similar if not superior to what has been so far reported for protocols using adherent cells.

Table 3.1 summarizes the impact of changes in small-scale production conditions based on the total functional particle yield from each experiment after 4 dpt. The most significant increase in production of LV particles was reached by butyrate addition (~15 fold). A combination of the most promising experimental conditions led to an overall increase in total functional LV particles of ~150 fold in small-scale experiments.

Table 3.1: Impact of change in small-scale production condition based on the cumulative titers (total produced transducing particles 4 days after transfection)

Experimental condition	Medium	Cell density cells/ mL	Polyplex amount µg DNA/10^6 cells	Butyrate mM	Cumulative titer tu	Specific productivity tu/cell	Relative titer
Initial conditions, $1x10^6$ cells/mL	LC-SFM L	LCD	1	-	$4.3x10^7$	2	1.0
Cell density increase to $5x10^6$ cells/mL	LC-SFM L	HCD	1	-	$2.5x10^8$	2.3	5.8
Decrease of polyplexes	HyQ	HCD	0.4	-	$4.6x10^8$	4.4	10.6
Addition of butyrate	HyQ	HCD	0.4	5	$6.8x10^9$	70.5	158.3

The specific productivity was calculated based on the cell density at the time of transfection.

These conditions were then validated at the bioreactor scale, resulting in an increase of ~75-fold compared to a bioreactor production performed with non-optimized conditions. We observed similar LV titer trends in the bioreactor and harvested supernatant (stored at 4°C). These results are in-line with findings suggesting that LV have a much longer half-life at 4°C compared to 37°C (Higashikawa and Chang 2001) and indicate that the continuous harvest of product addressed the issue of the low LV stability.

The uptake of polyplexes was a fast process and resulted in production of LV one day after transfection. Transfection in perfusion mode is therefore feasible and an attractive alternative to alleviate the nutrient limitations in current protocols. Based on earlier results for the production of RV in bioreactor scale, we expected higher titers in bioreactor-scale compared to small-scale cultures due to constant and controlled culture conditions (Ghani, Garnier et al. 2006). We consequently attribute the lower LV yields in bioreactor scale to a less efficient transfection process. The use of acoustic cell filter should not directly influence LV titers but might cause an increased detrimental cellular aggregation before transfection, resulting in a less efficient plasmid transfer to cells. At this point, however, we can not exclude other possible explanations, in particular because lower yields for production of LV by transfection in bioreactor-scale (batch mode) compared to small-scale have also been reported earlier by our group (Segura, Garnier et al. 2007).

3.2.6 Conclusions

We describe here the development of a scalable process for the production of LV in bioreactor perfusion cultures by PEI-based transient transfection. Using a suspension-grown HEK293 cell line, a combination of several optimization strategies resulted in an improvement of LV yield of ~150-fold compared to non-optimized production conditions. We were able to generate maximum LV titers in non-concentrated culture supernatants of ~10^8 tu/mL. We observed that yields after transient transfection were dependent on the selected production medium and the amount of polyplexes used for transfection. The addition of sodium butyrate was shown to alter the production kinetics of LV and improved the production by ~15-fold. Our improved production conditions include transient transfection at high cell density in a medium that allows transfection without the need of discontinuous medium exchange. These results were successfully validated in bioreactor scale.

Future work will investigate an optimized harvest rate in the bioreactor based on LV stability, a further improvement of transfection parameters and understanding of the LV production kinetics.

To our knowledge this is the first study describing LV production in perfused suspension culture, resulting in an industrially viable process, i.e. without the need of discontinuous medium exchange (batch replacement) at any time of the process.

The production process is easily scalable, cost-effective and in particular less laborious than other current LV production methods. It should allow for the production of LV in sufficient quantities for phase 1 clinical trials in 10-20 L perfusion bioreactor scale.

3.2.7 Acknowledgements

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CHAPTER 4 CHARACTERIZATION OF LENTIVIRAL VECTOR PRODUCTION KINETICS USING OFFLINE QUANTIFICATION METHODS

4.1 Introduction

In routine production protocols, LV generation and degradation are confounded, rendering the analysis of production kinetics and evolution of LV quality (as the ratio of functional to total vector particles) difficult. These protocols involve one or two harvests at different time points after transfection from the supernatant of adherent cultures (see table 1.1; section 1.2.6). For a complete analysis of LV production kinetics generation and degradation processes would need to be deconvoluted. As described in chapter 3, LVs were harvested by sequential discontinuous medium replacement and continuous perfusion mode in small scale and bioreactor cultures, respectively. Using this production strategy, the released particles are either once or twice daily completely removed from the supernatant (small scale) or continuously harvested from the culture (bioreactor). This operation mode, in particular in bioreactor cultures, does not decouple the two processes (viral production and vector decay). It nevertheless facilitates the analysis of production kinetics in comparison to operation in batch mode.

Each LV quantification method targets a specific property or structural component of the vector. In general, direct methods that are used for LV quantification largely overestimate functional titers (see also section 1.2.5). These methods are nevertheless instrumental for process development as they provide the means to assess virus yield, recovery and product quality, i.e. process efficiency. For the development of the production process, the main interest in comparing the results from functional assays (indirect methods) and total particle quantification (VG/VP titer) is the estimation of the quality of the produced LV preparation via the ratio of functional to total particles. Only a handful of studies report on the analysis of LV quality in final preparations and during production (Higashikawa and Chang 2001; Mitta, Rimann et al. 2005; Geraerts, Willems et al. 2006). For example, it was found that LV quality is dependent on the vector backbone and might vary if transfection conditions are not stringently controlled (Geraerts, Willems et al. 2006).

The successful production of a functional particle is only possible if all of its components are expressed simultaneously in a producer cell. It was expected that the comparison of production kinetics and LV quality would therefore provide:

1) a better understanding of the transient nature of the production system, 2) important insights on why these kinetics were observed and 3) possible avenues for optimization of the LV production system.

In this chapter, the previously described LV quantification methods (described in chapter 2) were used with the goal to further characterize LV production kinetics under improved production conditions in small scale and bioreactor cultures. In contrast, chapter 3 only contains production kinetics for functional LV titers based on GTA assays.

First, this chapter presents the characterization of LV production kinetics after transient transfection in perfusion mode. A comparison of the results from several quantification methods is made to pull out similarities and differences in the evolution of vector titers after transfection. To that end, the experimental observations from small scale and bioreactor cultures under improved production conditions are analyzed. Second, the evolution of LV quality was investigated during production at different medium exchange rates. Its value was found to change significantly during the process and was a function of the production conditions such as medium exchange rate and sodium butyrate addition. Finally, the effect of butyrate on LV quality and production kinetics is evaluated and discussed.

4.2 Results

4.2.1 Comparison of LV Production Kinetics in Perfusion Mode

4.2.1.1 Functional LV Titer Production Kinetics

When producing LV using sequential medium replacement in small scale or continuous perfusion cultures, LV titers decreased exponentially after the maximum at 2 dpt. The LV production kinetics in perfusion mode was greatly affected by the addition of sodium butyrate and independent of cell density and medium composition with a maximum at 2 dpt (see figures 3.1-3 and section 3.2.4.1). Without the addition of butyrate similar kinetics were observed independent of the medium composition with maximum functional titers at 3 dpt but only slightly smaller

values for 2 and 4 dpt. In contrast, transfection in batch cultures resulted in almost constant functional LV titers from 2-4 dpt (fig. 4.1).

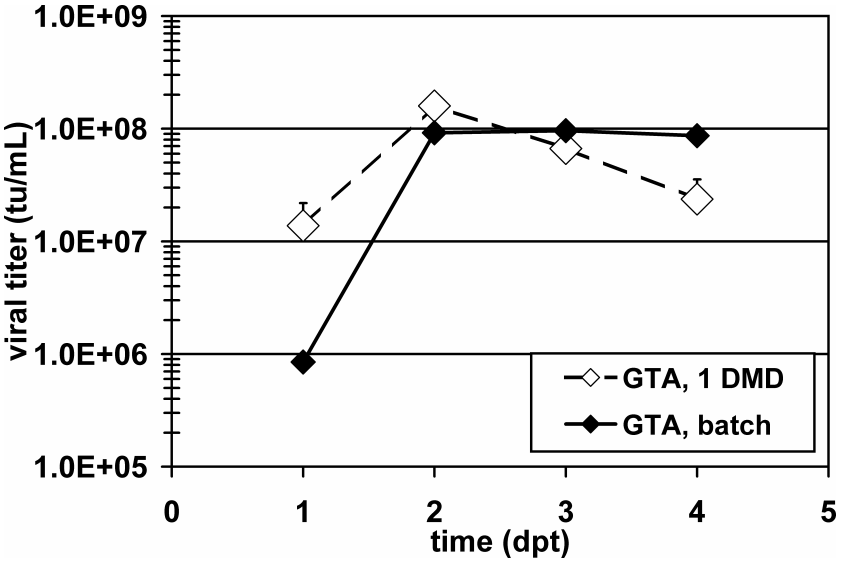

Figure 4.1: LV production in small scale at 1 “sequential discontinuous medium exchange per day (DMD) “ in comparison with production in batch mode.

LV production in batch mode was performed after transfection at the same cell density of a culture taken from bioreactor run #3.

Using improved LV production conditions (see section 2.1.4-5 for details) the kinetics for shake flask and bioreactor cultures was overall similar, with lower absolute values in bioreactor scale (fig 4.2). At 2 “sequential discontinuous medium exchanges per day (DMD)”, a faster decrease in transducing units was observed after the maximum in small scale cultures.

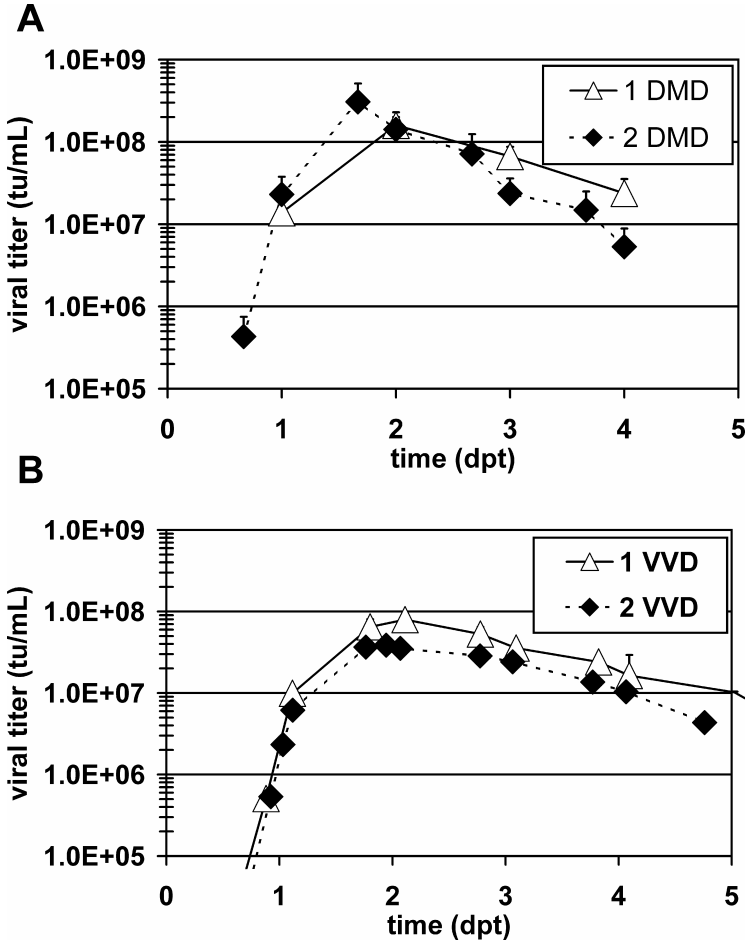

Figure 4.2 : Functional viral titer, quantified by gene transfer assay (GTA) in small scale and bioreactor experiments using improved production conditions

(A) small scale results; average kinetics for experiments at 1 discontinuous medium exchange per day (1 DMD; n = 3) and 2 DMD (n = 6) are presented

(B) bioreactor cultures, values correspond to a single culture performed at 1 and 2 VVD, respectively (run #3 and #4, see table 4.1 B)

4.2.1.2 Production Kinetics of Total Particles Quantified by ELISA (VP Titer)

Although only a limited number of CAp24 ELISA assays were performed for comparison of the different production conditions, the results gave consistent data (fig. 4.3). Maximum VP titers were observed at 3 dpt with $2x10^{10}$ vp/mL at 1 DMD (fig. 4.3 A). Slightly smaller values were found for 2 and 4 dpt. At a higher medium exchange rate, the titers were significantly reduced and a more distinct maximum was found at 3 dpt. In all conditions, the titers at 1 dpt were low or

even below the detection limit of the ELISA assay (for the chosen dilutions of the samples). Bioreactor cultures resulted in similar production kinetics (fig. 4.3 B). At 1 and 2 VVD, the maximum VP titer in those experiments was found at 3 dpt.

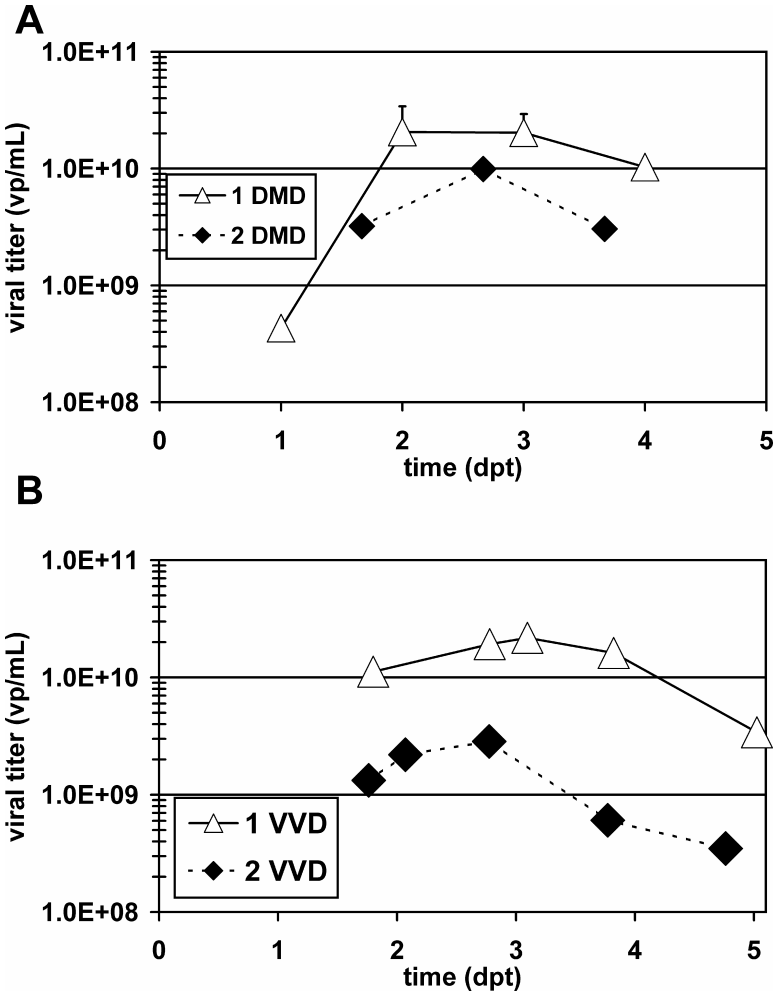

Figure 4.3 : LV production kinetics quantified by CAp24 ELISA assay (VP titer) in small scale and bioreactor experiments using improved production conditions

(A) small scale results, values correspond to single experiments at 1 and 2 DMD

(B) bioreactor cultures, values correspond to a single culture performed at 1 and 2 VVD, respectively (run #3 and #4)

4.2.1.3 Production Kinetics of Total Particles Quantified by RT-PCR (VG Titer)

In small scale experiments, the maximum in VG titer, i.e. the amount of lentiviral genomes quantified by RT-PCR, was found for medium exchange rates at 1 and 2 DMD at 2 dpt (fig. 4.4 A). However, the differences with the values observed for subsequent production days were small. In contrast, bioreactor cultures were showing the VG titer maximum at a later time point, while only minor differences for the values from 2-5 dpt were again observed (fig. 4.4 B). In summary, the VG titer remained almost constant from its maximum until 5 dpt, independently of production scale and medium exchange rate.

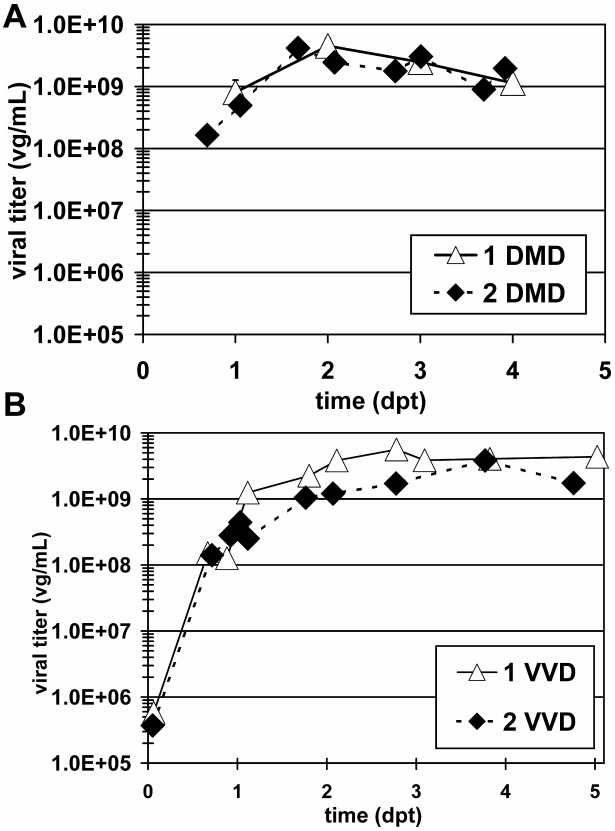

Figure 4.4 : LV production kinetics quantified by RT-PCR (VG titer)

(A) small scale results; average kinetics for experiments at 1 discontinuous medium exchange per day (1 DMD; n = 3) and 2 DMD (n = 2) are presented

(B) bioreactor cultures, values correspond to a single culture performed at 1 and 2 VVD, respectively (run #3 and #4)

4.2.1.4 Summary of LV Production Kinetics Results

Table 4.1: LV titer kinetics in small and bioreactor scale

(A) LV titer kinetics in shake flask cultures (small scale)

(B) LV titer kinetics in bioreactor scale

A	Functional titer (GTA)			VP titer (ELISA)[V]			VG titer (PCR)		
Operating condition/ *parameter*	**1 DMD** [II]	**2 DMD** [III]	**2 DMD, HyQ +** [IV]	**1 DMD**	**2 DMD**	**2 DMD, HyQ +**	**1 DMD**[V]	**2 DMD**[VI]	**2 DMD, HyQ +**
Time of maximum titer (dpt)	2	1.7	1.7	2=3	2.67	2.67	2	1.68	n.a.
LV titer at 4 dpt (in % of max)[I]	15	1.7	0.9	50	30	66	25	31	n.a.

B	Functional titer (GTA)			VP titer (ELISA)			VG titer (PCR)		
Operating condition/ *parameter*	**1 VVD, run #3**	**2 VVD, run #4**	**2 VVD, HyQ +, run #5**	**1 VVD, run #3**	**2 VVD, run #4**	**2 VVD, HyQ +, run #5**	**1 VVD, run #3**	**2 VVD, run #4**	**2 VVD, HyQ +, run #5**
Time of maximum titer (dpt)	2.1	1.67	1.67	3.1	2.78	n.a.	2.78	3.78[VII]	4.1[VII]
LV titer at 4 dpt (in % of max)[I]	25	29	14.5	73	21	n.a.	72	100	100

I: % of maximum titer is shown

II: values represent average results of n = 3 independent experiments

III: values represent average results of n = 6 independent experiments

IV: values represent average results of n = 3 independent experiments

V: values represent average results of n = 3 independent experiments

VI: values represent average results of n = 2 independent experiments

VII: almost constant values from 2-5 dpt all bioreactor cultures were performed only once at each operating condition

Table 4.1 compares LV production kinetics based on functional, VP and VG titer quantification in small and bioreactor scale. Functional vector titers found their maximum independent of operating condition and scale at around 2 dpt. Until 4 dpt, the functional titer then decreased dramatically to 1-15 % of its maximum value in small scale and 15-30 % in bioreactor cultures. In contrast, VP titer and VG titer were showing more uniform profiles from 2-4/5 dpt with almost constant values for that part of the production. Maxima in VP and VG titer were thus less clearly identifiable and in almost all cases found later than the highest concentration of functional LVs (also compare fig. 4.5 A).

Figure 4.5 compares LV production kinetics of two of the performed bioreactor perfusion cultures at 1 and 2 VVD (run #3 and 4). First, these two examples demonstrate the reproducible kinetics that was observed after quantification with different methods (fig. 4.5 A). Second, the differences in kinetics from the three quantification methods are also well represented. In both cultures, the viable cell count (*vcc*) increased after transfection and reached a maximum at ~2 dpt (fig. 4.5 B). This maximum was observed at the same time than the maximum in functional LV particles. Total GFP expression above background levels was measured starting at 1 dpt. From 1-3 dpt, the expression then increased linearly until 3 dpt after which it showed a smaller slope.

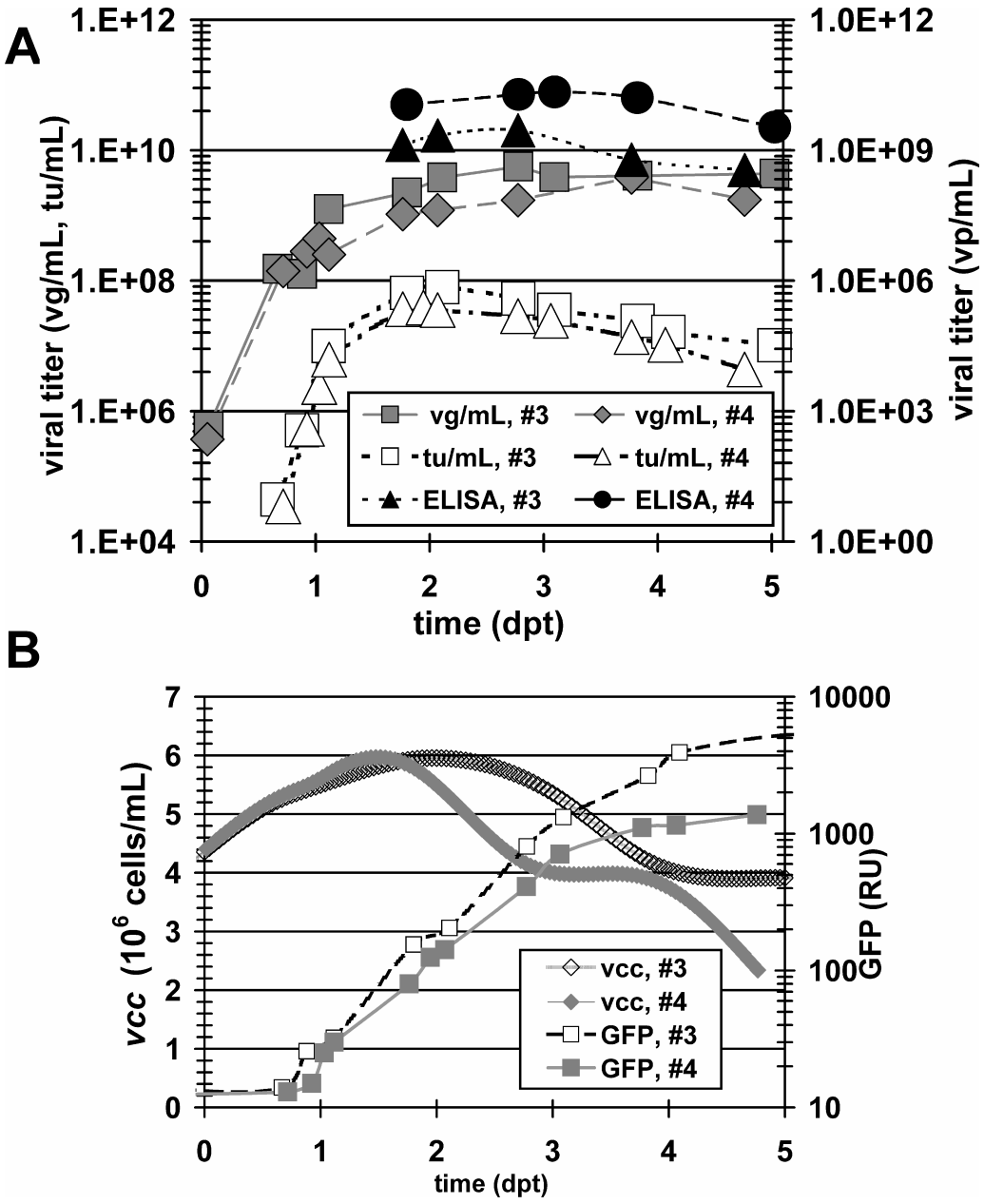

Figure 4.5 : Evolution of LV kinetics in bioreactor scale at 1 and 2 VVD

(A) functional, VG and VP titer

(B) *vcc* (the fit of offline cell counts taken 2-3 times daily is shown) and total GFP expression

4.2.2 Assessment of LV Quality Under Different Production Conditions

Most studies reported low values of 0.3-8 % for the ratio of transducing units to RNA titer. Mitta et al. (2005) proposed the term VP (lentiviral particle performance), as the ratio of functional to VP titer (CAp24 ELISA), and observed a wider range (<0.01-70 %). It has, however, been questioned if the VP titer is suitable for calculation of LV quality, as other groups found that CAp24 ELISA assays are the least reliable method for prediction of LV titers (Geraerts et al. 2006; Ricks et al. 2008). Routine protocols also measure free CAp24 in supernatants (Geraerts et al. 2006; Ricks et al. 2008). The results thus reflect CAp24 from viral particles alone only if purified samples are analyzed (Ricks et al. 2008); see section 1.2.5.1). Consequently, to calculate LV quality, we focused in the present work on the results from RT-PCR.

The evolution of LV quality in a typical experiment under improved production conditions is represented in figure 4.6. At 2 dpt, when the maximum functional titer was reached, LV quality based on the ratio of GTA to RT-PCR was maximal at ~4 %. Its value then decreased at 3 and 4 dpt (~ 3 and 2 %, respectively). For 2-4 dpt, a similar profile was observed for the LV quality based on the ratio of GTA to CAp24 ELISA. In contrast, the maximum was here already observed at 1 dpt. The ELISA results for samples at 1 dpt were, however, subject to high variability.

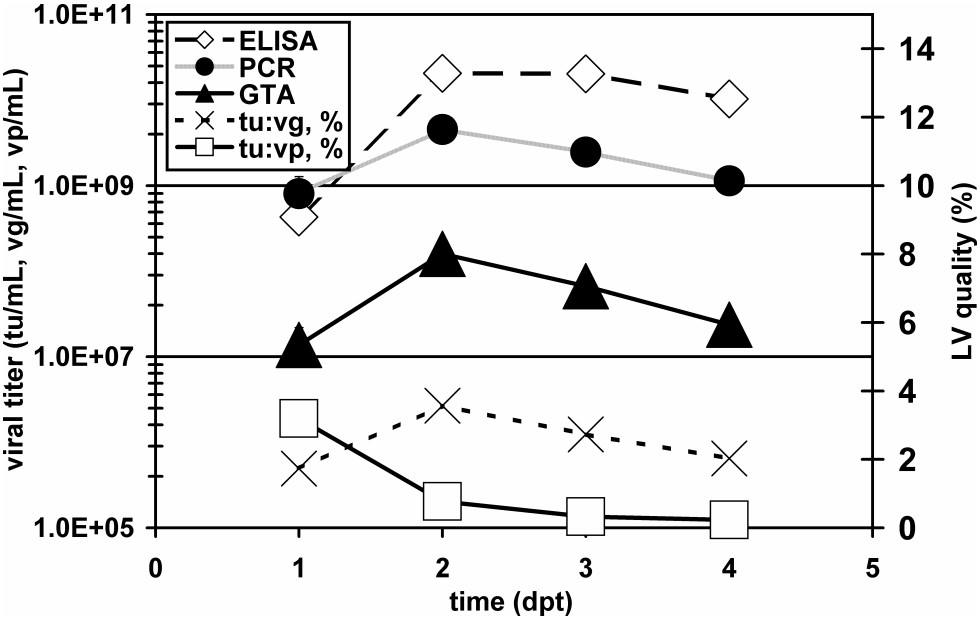

Figure 4.6 : Evolution of LV quality in typical small scale experiment using improved production conditions at 1 DMD.

Values represent the average of several experiments (functional titer: n = 3; VG titer: n = 3; VP titer: n = 1)

4.2.2.1 Increased Perfusion Rate Results in Higher LV Quality

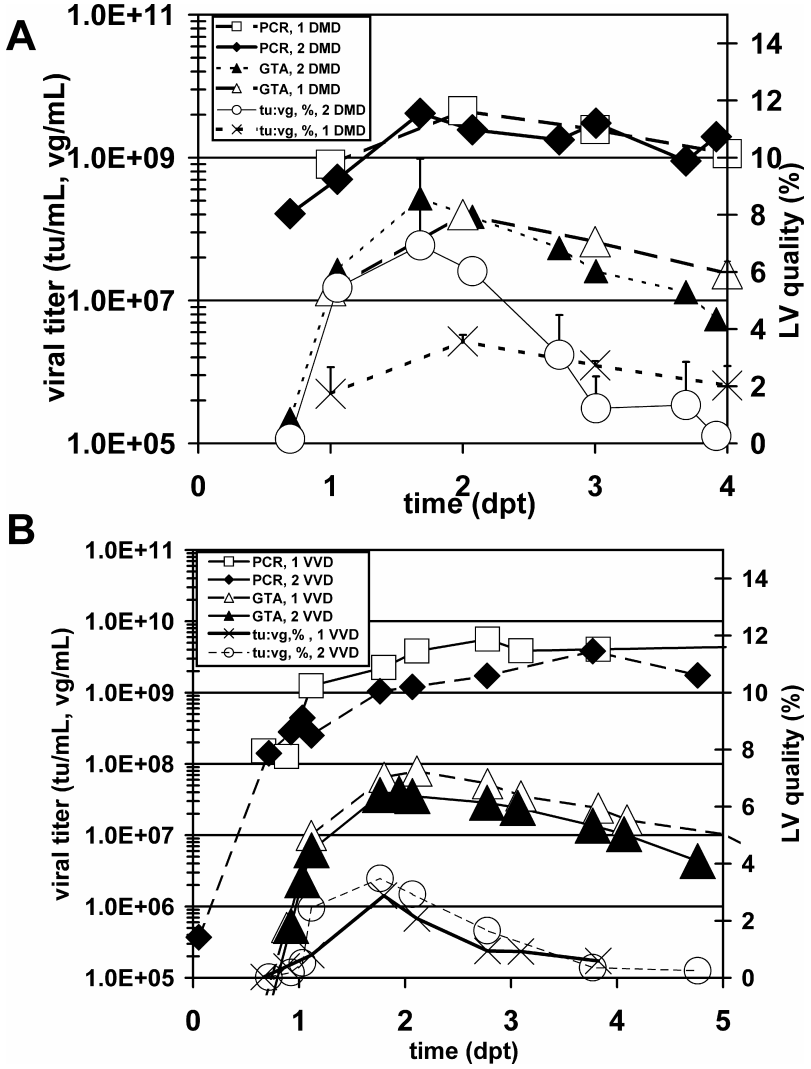

Figure 4.7 : Comparison of LV quality evolution during small scale and bioreactor production at different medium exchange rates.

All experiments were performed using improved production conditions

(A) small scale production at 1 and 2 DMD

(B) bioreactor production at 1 and 2 VVD (corresponding to run # 3 and #4)

When medium exchange rate was increased to 2 DMD in small scale experiments (fig. 4.7 A), a higher LV quality was found from 1-3 dpt than at 1 DMD. Only towards the end of the experiment, LV quality was lower than at 1 DMD. These observations were generally confirmed in bioreactor scale (fig. 4.7 B), although the increase in LV quality was lower compared to what

was observed in small scale. In comparison, the number of total particles (VG titer) was similar in small and bioreactor scale. The difference in LV quality was thus caused by the higher functional titers in small scale experiments.

4.2.2.2 Effect of Sodium Butyrate on LV Quality

After observing that LV quality under improved production conditions was not constant during the experiment, the effect of sodium butyrate addition on the evolution of the ratio of functional to VG titers was investigated. Figure 4.8 presents the analysis of small scale experiments at increasing butyrate concentrations (0, 1 and 5 mM). While both, VG and functional titer increased after sodium butyrate addition, the effect on functional titer was much higher and resulted in an increase in LV quality from around 1 to 4 % at 2 dpt. The evolution of LV quality changed from a maximum at 3 dpt without butyrate to a maximum at 2 dpt when butyrate was added which was in both cases corroborating with the time point of maximum functional LV titer.

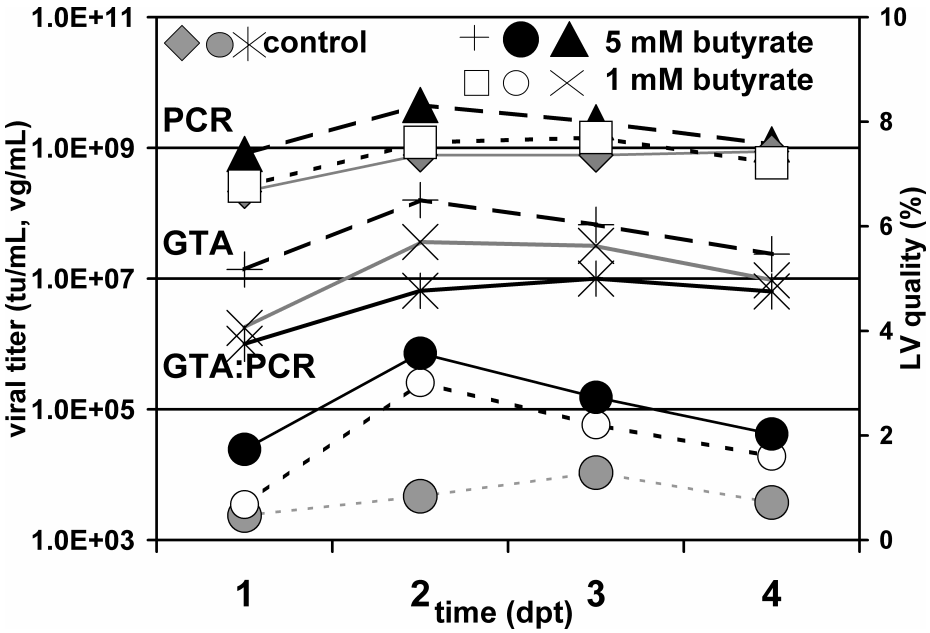

Figure 4.8 : Effect of sodium butyrate addition at 16 hpt at different concentrations (0, 1, 5 mM). Data represent small scale transfections at $5x10^6$ c/mL in HyQ medium

4.3 Discussion

Overall, reproducible production kinetics was observed when butyrate was added to achieve high LV yields, i.e. under improved production conditions. Maximum functional LV titers were found at 2 dpt, followed by an exponential decrease at 3 and 4 dpt. In contrast, VG and VP titer showed smaller differences and almost constant values from 2-4 dpt. Nevertheless, the maximum in VP titer was constantly observed at 3 dpt whereas a reproducible maximum in VG titer could not be clearly identified.

Also, it was demonstrated that LV quality (GTA : VG titer) varied during the production and was higher at increased medium exchange rates. This has, to our knowledge, not been previously reported in the literature.

As demonstrated in chapter 3, butyrate addition significantly increases LV production. With the additional results for VG and VP titer kinetics in hand, additional observations concerning this 'butyrate effect' could be made. It was observed that this effect is not primarily due to an increase of viral RNA/RNA containing particles in the culture supernatant. Although higher VG titers were observed with increasing butyrate concentration, the increase in functional LV was more significant and resulted in higher LV quality of almost 4-fold.

On the one hand, it was found that protein production by transient transfection is generally limited by two main parameters: 1) events regulating the nuclear translocation of plasmid DNA and 2) protein expression, i.e. transcriptional competency and translational/post-translational processes (Carpentier, Paris et al. 2007). For PEI-based transfection, the same authors excluded the delivery of plasmid constructs to producer cells as a bottleneck for production; high numbers of plasmid copies (in the range of tens of thousands per cell) can be detected in producer cells at 1 dpt. In the present case, which employs almost identical PEI-based transfection, it can be assumed that every cell received high amounts of plasmid DNA in the range of tens of thousands of copies per cell for each of the four LV-encoding constructs.

On the other hand, LV production is generally limited by expression of the LV-encoding constructs. Gene silencing has been put forward as a reason for expression loss during LV production after transient transfection and also in stable packaging and producer cell lines (Kafri , van Praag et al. 1999; Ni, Sun et al. 2005; Jaalouk, Crosato et al. 2006; Sun, Goh et al. 2008).

Gene loss, in contrast, has been excluded as explanation for these observations as the *gag-pol* DNA can still be detected in stable packaging cell lines even without butyrate addition (Kafri, van Praag et al. 1999; Sparacio, Pfeiffer et al. 2001). Sodium butyrate is well described as a powerful inducer of LV production leading to, most often temporary, peak titers over a few consecutive days (Gasmi, Glynn et al. 1999; Karolewski, Watson et al. 2003; Jaalouk, Crosato et al. 2006). It acts on several levels, increasing expression in VSV-G, mRNA, CAp24 and vector RNA levels (Kafri, van Praag et al. 1999; Jaalouk, Crosato et al. 2006; Al Yacoub, Romanowska et al. 2007). It is generally observed that butyrate causes hyperacetylation of histones, leading to a higher transcription and an increased expression of transfected DNA (Altenburg, Via et al. 1976; Kruh 1981; Gloger, Arad et al. 1985). Furthermore, it activates several promoter types and reduces effects caused by promoter interference (Gasmi, Glynn et al. 1999; Jaalouk, Crosato et al. 2006). Yet, effects of butyrate in other transfection systems, such as the production of adeno-associated virus (AAV) vectors or recombinant proteins, are not always beneficial and mixed effects on LV titers have also been reported ((Sena-Esteves, Tebbets et al. 2004) and unpublished results from our lab). There only seems to be a major effect on LV titers if the expression efficiency of the affected construct is non-optimal (Sena-Esteves, Tebbets et al. 2004).

Based on our results, it cannot be excluded that butyrate also enhanced nuclear translocation after transfection but this mechanism has not been reported in the literature. Although our analysis was entirely based on quantification methods that only detect LV components in cell-free culture supernatants, a direct action on expression efficiency of the LV-encoding constructs is, in contrast, very likely and in particular supported by reports confirming the positive effect of sodium butyrate on stable packaging and producer cell lines. This suggests that either the volumetric expression in the transfected culture or the intensity of the ‘butyrate effect’ on expression of the LV-related proteins is not constant over time.

This corrorobates with our findings showing that earlier addition of butyrate does not increase total LV yields (fig. AIV.13). This would, however, be expected if butyrate actively increased the translocation of plasmid DNA to the nucleus.

A direct effect of sodium butyrate addition on LV stability can equally be excluded because functional LVs were showing identical half-lifes independent of harvest time and the addition of sodium butyrate (appendix I, fig. AI.1).

In chapter 3, it was hypothesized that the exponential decrease of LV titers at 3 and 4 dpt was caused by a loss of transfected producer cells due to the expression of the toxic LV proteins (in particular VSV-G and *gag*) (chapter 3.2.5). VSV-G has fusogenic activity and its expression in mammalian cells is known to result in scyncytia formation and cell death (Burns et al. 1993). These authors found that VSV-G expression is restricted to a short period before its presumed accumulation on the cell surface leads to cell death. The cytotoxicity of VSV-G is one of the major obstacles for the generation of stable LV packaging and producer cell lines as its constitutive expression prohibited in mammalian cells (Sinn, Sauter et al. 2005; Kafri, van Praag et al. 1999; Sparacio, Pfeiffer et al. 2001). Here, we made several additional observations concerning the 'butyrate effect' and cytotoxicity:

1) without addition of butyrate cell density increased by up to 10-fold after transfection. In parallel, a 3-fold increase in VG titer was found after the maximum in functional titer at 3 dpt (appendix IV, fig. AIV.6 A)

2) although the number of ELISA assays was limited, reproducible VP titer kinetics with a maximum after the functional titer maximum was found at around 3 dpt

3) analysis of protein content in culture supernatants by SDS-PAGE and Western blot confirmed ELISA results and the presence of CAp24 until 4 days after transfection, with a maximum at 2-3 dpt (results not shown); in contrast, while VSV-G was detected at 2-3 dpt, its amount was below the detection limit at 4 dpt

4) the maximum cell density in small and bioreactor scale corresponds to the maximum in viral titer at 2 dpt (bioreactor: fig. 4.5; small-scale: appendix II, fig. AII.3)

For the present system, it is consequently hypothesized that the expression of VSV-G is limiting LV production and that the maximum functional titer is found when expression of this protein is at its maximum. A subsequent loss of producer cells in particular due to the high expression levels of VSV-G is then leading to a decrease in functional LV titer at later time points. Future work will consequently need to analyze in detail if the non-functional particles produced in phase 3 and 4 indeed lacked the VSV-G envelope. If so, it might be a possible avenue for process optimization to increase yields by converting these non-infectious virus-like particles to infectious forms via lipofection agents or VSV-G addition (Sharma, Murai et al. 1997; Abe, Chen et al. 1998).

4.4 Conclusion

Reproducible LV production kinetics was found in all experiments under improved production kinetics. At the same time, LV quality varied significantly during the process, suggesting that expression kinetics of the LV components varied over time. Sodium butyrate addition was a key factor for improved production and the observed ‘butyrate effect’ translated into different kinetics of functional and total LV (VG, VP titer). We propose that the action of sodium butyrate is essentially related to viral protein expression. It would be of interest to further investigate in detail what mechanisms are behind these observations, ideally using methods analyzing cellular mRNA and protein levels.

The observations mainly suggest, however, that alternative avenues at the molecular level should be considered for further process optimization. These could include the regulation of VSV-G or the use of other, less cytotoxic envelopes.

CHAPTER 5 MONITORING LENTIVIRAL VECTOR PRODUCTION KINETICS USING ONLINE PERMITTIVITY MEASUREMENTS

5.1 Article Presentation

This chapter presents the journal article **"Monitoring Lentiviral Vector Production Kinetics Using Online Permittivity Measurements"**. The chapter has been submitted to the ***Journal of Biotechnology***.

To follow the progression of the transient transfection process in real-time, monitoring methods that are able to directly monitor the viral release and production kinetics need to be identified. Such methods would facilitate process development and support process optimization as well as the implementation of PAT strategies for improved process robustness and in-process quality control.

LVs are budding viruses and are thus similar to baculoviruses, causing dramatic physiological changes in the producer cells upon release. Permittivity measurements were thus evaluated as real-time monitoring tool during LV production. Previous studies have demonstrated that online permittivity measurements have great potential in process development of viral vector production processes. To analyze the online signal in detail, it was thus required to propose and validate an offline measurement method for the analysis of permittivity measurements. To that end and for a further understanding of the value and practical applications of online permittivity measurements, the reader is referred to appendix III in which the real-time signal is evaluated in HEK293 batch cultures. It was expected that the combination of the characterization of production kinetics by offline quantification of LVs (chapter 4) and the analysis of online measurements would help in the understanding and determination of critical process events.

More specifically, it was assessed in the present chapter if it is possible to relate real-time monitoring signals and viral production kinetics and yields. It is demonstrated that permittivity measurements are an informative and, to some extent, quantitative tool for LV production monitoring and supervision. A direct use of the signal for process control and decision-making will enable process optimization and increase cost-effectivness of the process. The technology is consequently one of the key tools to consider when implementing PAT strategies.

The bioreactor runs presented in chapter 5 correspond to runs #2-5 (compare table AII.2).

5.2 Monitoring Lentiviral Vector Production Kinetics using Online Permittivity Measurements

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5.2.1 Abstract

Lentiviral vectors (LVs) are effective delivery vehicles that are successfully used in gene and cell therapy. LVs are most commonly produced via transient transfection of several plasmid constructs in adherent cell cultures. Recently, we described an efficient and scalable LV production in serum-free suspension cultures. To further facilitate translation of LV-based interventions to the clinic, robustness of the production processes needs to be documented to ultimately achieve a specified quality and quantity of LV production lots. In this work, *in situ* real-time permittivity measurements were assessed to document LV production. Reproducible process phases that were closely associated with LV production kinetics were identified. The permittivity signal evolution was interpreted exploiting various independent online and offline monitoring measurements. The results suggest that cellular membrane properties and, to a lesser extent, cell size were the main factors contributing to the permittivity variations. It is concluded that the permittivity-related parameters can be used as reliable indicators for determination of the optimum harvest time, thereby allowing real-time assessment of process performance and greatly facilitating process optimization.

5.2.2 Introduction

The most advanced generations of lentiviral vectors (LVs) are safe and potent tools for gene and cell therapy interventions (Cockrell et al. 2007; Pluta et al. 2007). Most LVs are derived from human immunodeficiency virus type 1 (HIV-1) and they possess several advantages over other

virus-based gene delivery vehicles, such as a large packaging capacity, the ability to infect non-dividing cells and the absence of transferred viral coding sequences (Vigna et al. 2000; Cockrell et al. 2007). However, despite recent progress in the field, mass production of LVs remains an important limitation for the translation of LV-based therapeutic strategies to the clinic (Ansorge et al. 2010b). The low production yields of LVs, in combination with a fast decay rate at 37°C (Higashikawa et al. 2001; Merten 2004), require further developments of efficient processes for large-scale LV production. For lentiviral (LV) but also other vectors, such as adeno-associated virus (AAV) vectors, transfection currently remains the most effective option for mass production due to the difficulty and time required to generate stable producer cell lines (Throm et al. 2009; Wright 2009). Large-scale transient transfection has already been used for production of proteins and viral vectors to generate preclinical and clinical material in a timely manner (Park et al. 2006; Baldi et al. 2007; Durocher et al. 2007; Hildinger et al. 2007). Yet, these early generation processes are generally not well characterized due to the lack of detailed key process parameter monitoring and sub-optimal control conditions in bioreactors. One key avenue for optimization of transfection processes is therefore the identification, characterization and control of critical process parameters to ensure robustness and reduce batch to batch variability (Geisse 2009). Thus, to establish industrially viable LV production processes, reliable monitoring tools are required that either directly quantify the vectors and/or reflect their release from the producer cells. Concerning the at-line quantification of recombinant proteins, important progress was made in recent years (Chavane et al., 2008; Jacquemart et al., 2008). These methods might also be applicable to LV production but it remains to be demonstrated that they are industrially viable and able to measure, for example, vector functionality.

In general, reliable monitoring methods are a fundamental element to characterize cell culture operations, facilitate their development and ensure process consistency and robustness during production (Junker et al. 2006). Although there has been a constant interest in monitoring and control technologies since the beginning of the industrial cell culture era, a renewed interest has been generated from the release of FDA's guidance on process analytical technology (PAT). The PAT initiative proposes a shift of biomanufacturing quality control from the laboratory to the process site, ensuring a "Quality by Design", resulting in a pre-defined, i.e. 'built-in', final product quality achieved by design. A successful application of a PAT-based strategy requires technologies and tools operating *in situ*, being non-invasive and generating online information

about multiple key bioprocess and/or metabolic variables (Teixeira et al. 2009). These monitoring tools should provide global, macroscopic bioprocess outputs ensuring their versatile and flexible utilization in different production systems.

The biomass content is the most important variable to monitor and control in any cell culture process (Sonnleitner et al. 1992; Olsson et al. 1997; Kiviharju et al. 2007). Online *in situ* probes based on dielectric spectroscopy (permittivity measurements) are established tools for biomass monitoring. Unlike most other online approaches based upon the correlation of metabolic rates (e.g. OUR) with cell density, permittivity measurements provide a more direct information on the biomass content. They are easily transferable to different bioreactor scales and, as such, hold great promise for application in process development. Moreover, permittivity measurements have the potential to provide meaningful additional real-time information for process control and optimization. Their use to monitor critical transition points in cell culture processes was previously described during the production of baculovirus expression vectors (BEVs) (Zeiser et al. 1999; Zeiser et al. 2000) and, more recently, the technology has been further evaluated in other systems (Cannizzaro et al. 2003; Ansorge et al. 2010a). These transition points were typically related to significant changes in cellular dielectric properties, occurring when cell physiology, morphology or metabolism is altered.

Upon viral infection, the initial release and the production of progeny virus are generally associated with significant physiological changes in the host cells. These physiological changes, in particular when taking place at the plasma membrane level, directly affect the dielectric properties of cells. Several studies describe the monitoring of these changes in single cells using techniques such as electrorotation (Archer et al. 1999; Berardi et al. 2009). Yet, the application of these findings, i.e. the use of permittivity measurements in virus or viral vector production, is to date limited to the BEV system. After infection of insect cell cultures with baculovirus, BEV replication results in changes of the host cell physiology and morphology, most notably a dramatic increase in the mean cell diameter. This change in cell size can be monitored in real-time and used to predict the infection's success and efficiency (Chico et al. 1998; Palomares et al. 2001). The onset of BEV release by budding from the cell membrane is also reflected in the permittivity, in particular when the infection is synchronous (Zeiser et al. 1999; Zeiser et al. 2000). These findings were also helpful in the analysis of more complex systems in which BEV

is used to produce AAV (Negrete et al. 2007; Cecchini et al. 2008), allowing real-time determination of the optimum harvest point.

The goal of this study was to determine whether permittivity measurements could provide meaningful real-time information related to lentiviral vector production kinetics. LVs were produced by transient transfection of suspension-grown HEK293 producer cells using a perfusion process. The online permittivity data were correlated with changes in biovolume and cell diameter and were compared with viral production kinetics, changes in oxygen consumption and GFP expression. Significant changes in permittivity and β-dispersion parameters were indicative of the first viral release and the progress of LV production. Characteristic process phases were identified from the analysis of the pattern of the online data and were reproducibly observed during LV production under different operating conditions. The results suggest that online permittivity signal variations can be correlated with the total viral particle yield. As the signal allows the determination of lentiviral release in real-time, it is concluded that the permittivity measurements could be a useful tool to determine an optimal harvest time and provide an early assessment of process performance.

5.2.3 Materials and Methods

5.2.3.1 Cell Culture

HEK293SF-3F6 cells were grown in suspension in HyQSFM4TransFx293 (HyQ) (Hyclone, Logan, UT) which was supplemented with Cell Boost 5 (CB5) at 5 % (v/v) (Hyclone, Logan, UT) when indicated (HyQ+). Subcultures were passaged every 2-3 days to keep the cells in their exponential growth phase. Hemacytometer counts using erythrosine B dye exclusion were used to assess cellular density (viable, total cell count per mL (*vcc*, *tcc*)) and viability.

5.2.3.2 Bioreactor LV Production

5.2.3.2.1 Transient Transfection

The transfection protocol and process development for high-yield LV production have been previously described in detail (Ansorge et al. 2009). To produce LVs, a GFP-encoding self-inactivating transfer vector (pCSII-CMV5-GFPq), third generation packaging plasmids

(pMDLg/pRRE#54 and pRSV-Rev) and a vector encoding the VSV-G envelope (pSVCMV-IN-VSVg) (Dull et al. 1998; Broussau et al. 2008) were simultaneously transfected in HEK293 cells. A plasmid mass ratio of 1:1:1:2 (VSV-G: Gag-Pol: Rev: Lentiviral Transfer Vector) was used for polyethylenimine (PEI)-mediated transfection according to previous results from our group (Segura, Garnier et al. 2007).

5.2.3.2.2 Bioreactor Cultures

The bioreactor setup was identical to the one previously described (Ansorge et al. 2009). Cultures were grown in batch mode up to ~1-2x10^6 *vcc* when perfusion was started at 1 or 2 volume(s) of medium per reactor volume per day (VVD). After reaching the targeted cell density (5-8 x10^6 *vcc*) in perfusion mode, the culture was transfected by the addition of polyplexes. After transfection, the LV-containing supernatant was kept at 4ºC until clarification and subsequently stored at –80ºC.

Pure oxygen was pulse sparged into the culture at increasing flow rates (depending on the biomass content in the reactor) to control the dissolved oxygen at 40 % of air saturation. From the total oxygen volume sparged into the bioreactor, the oxygen sparging rate (OSR in mL/min) was calculated as an indicator of the volumetric oxygen consumption.

5.2.3.3 Lentiviral Quantification

5.2.3.3.1 Gene Transfer Assay (GTA)

Functional viral titers were determined using a flow cytometry-based gene transfer assay (GTA) (Ansorge et al. 2009). The limit of detection of this assay is ~1×10^5 tu/mL. An in-house LV standard was used in all GTA experiments to minimize inter-assay variability. All final titers were calculated relative to this LV standard, which was found to be stable over a period of more than one year after storage at -80°C in culture medium. Transduction of selected samples was performed in the presence of AZT (SIGMA) to rule out pseudotransduction.

5.2.3.3.2 Total Viral Particle Quantification (VG titer) by Real-Time Polymerase Chain Reaction (RT-PCR)

The VG titer, i.e. the amount of total viral particles expressed as viral genomes (vg)/ml), was determined using a SYBR-Green® I (SGI) quantitative RNA PCR assay (Roche Applied Science, Laval, Qc). An in-house RNA standard was used to quantify LV in supernatants from production runs. After RNA purification from LV-containing supernatants using the High Pure Viral RNA kit (Roche Applied Science, Laval, Qc), samples were treated with DNAse (DNA-free Kit, Applied Biosystems, Foster City, CA). RT-PCR was then performed in a Carousel-based LightCycler (Roche Applied Science, Laval, Qc) or a Mastercycler ep realplex system (Eppendorff, Hamburg, Germany). Primers (P1, P2) targeting a sequence in the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) were selected using clone manager (Sci-Ed Software, Cary, NC). Each reaction had a volume of 20 µL with a concentration for each primer of 0.5 µM (P1: LVWPREF: AGT-TGT-GGC-CCG-TTG-TCA-GG, P2: LVWPRER: AGG-CGA-GCA-GCC-ATG-GAA-AG), amplifying a sequence in the WPRE element of 249 bp. The RNA standard was produced using the MEGASCRIPT kit (Applied Biosystems, Foster City, CA). For this purpose, WPRE from the LV transfer vector (CSII-CMV-GFPq) was cloned into pUC19 T7. The resulting plasmid pUC19 T7-LVWPRE was then linearized and in-vitro transcribed. The transcript (RNA standard) with a length of 260 bases was purified (MEGACLEAR kit, Applied Biosystems, Foster City, CA), quantified by spectrophotometry and aliquoted at the final standard concentrations ($1x10^9$-$1x10^4$ copies/reaction) in DEPC-treated water. Specificity of the PCR reactions was confirmed by melting curve and agarose gel analysis. The lower detection limit of this method is in the range of $5x10^5$ vg/mL.

5.2.3.4 Cell Culture Samples Analyses

5.2.3.4.1 Cell size measurement

The volume weighted arithmetic mean cell diameter (*d*) was determined using a Z2™ Coulter Counter® (Beckman Coulter, Mississauga, ON) with an aperture diameter of 100 µm, followed by analysis of the size distributions with the Accucomp® software package (Beckman Coulter). The upper and lower analysis limits were 7.31 and 23.8 µm, respectively. To calculate *d*, the distributions were plotted as volume (%) against cell diameter (µm). The lower analysis limit was

then manually increased to select only that part of the distribution corresponding to viable cells for the calculation.

Each value of *d* represents the average of at least two distributions. Using *d* and the viable cell count per mL (*vcc*), the biovolume (*bv)* was calculated using equation 5.1, assuming that the distributions were evenly scattered:

$$bv = \frac{4}{3} \times \left(\frac{d}{2} \right)^3 \times \pi \times vcc \qquad (5.1)$$

bv was then considered as an estimate of the volume fraction of cells *P* (equation 5.2 in section 5.3.5).

5.2.3.4.2 GFP measurement

The total expression of GFP was measured on a SpectraMax Gemini EM plate reader (Molecular Devices, Sunnyvale, CA) in 96 well plates. $1x10^5$ cells were distributed in each well and fluorescence was measured at wavelengths of 485/538/495 nm (excitation/emission/cutoff) after fixation with 4 % formaldehyde.

5.2.3.5 Permittivity Measurements

In this study, a Biomass System® (Fogale nanotech, Nîmes, France) as well as a Biomass Monitor 220 (Aber Instruments, Aberystwyth, UK) were employed for the measurement of permittivity across the β-dispersion frequency spectrum of ~0.1-10 MHz. Both systems provide the β-dispersion parameters ($\Delta\varepsilon_{max}$, *fc*, α) after fitting the frequency spectra. In addition, the Biomass Monitor 220 (Aber Instruments, Aberystwyth, UK) measures the dual-frequency permittivity at 0.6 and 19.5 MHz, whereas the Biomass System/Biomass 400 (Fogale nanotech, Nîmes, France) routinely operates at 1 and 10 MHz, providing $\Delta\varepsilon_{FC}$. The calculation of the β-dispersion parameters was performed with commercially available software packages, either offline using AberScan (Aber Instruments) or in real-time with the Biomass 400 (Fogale nanotech).

The underlying theory on the dielectric properties of biological cells has been described in detail elsewhere.(Kell et al. 1985; Harris et al. 1987a; Pethig et al. 1987; Markx et al. 1999) In brief, when placed in an alternating electric field, any given cell suspension shows a characteristic

decrease in permittivity with increasing frequency. This decrease, the β-dispersion, is caused by the polarization of cell membranes (fig. 5.1). The resulting dielectric/permittivity increment ($\Delta\varepsilon_{max}$) is directly correlated to the membrane enclosed volume fraction (P) of the cell suspension. For spherical cells at high viability (and therefore a low membrane conductivity) and for moderate values of P ($P < 0.2$), one can mathematically define this decrease by three parameters ($\Delta\varepsilon_{max}$, f_C, α) and the equation of Schwan (eq. 5.2) (Schwan 1957; Harris et al. 1987b):

$$\Delta\varepsilon_{\max} = \frac{9 \times r \times P \times C_M}{4} \qquad (5.2)$$

with

$\Delta\varepsilon_{max}$: permittivity increment (F m^{-1})

r: cell radius (m)

P: volume fraction of cells (biovolume); $P = \frac{4}{3} \times \pi \times r^3 \times N$ (5.2.1)

N: cell density (cells m^{-3})

C_M: capacitance per membrane area (F m^{-2})

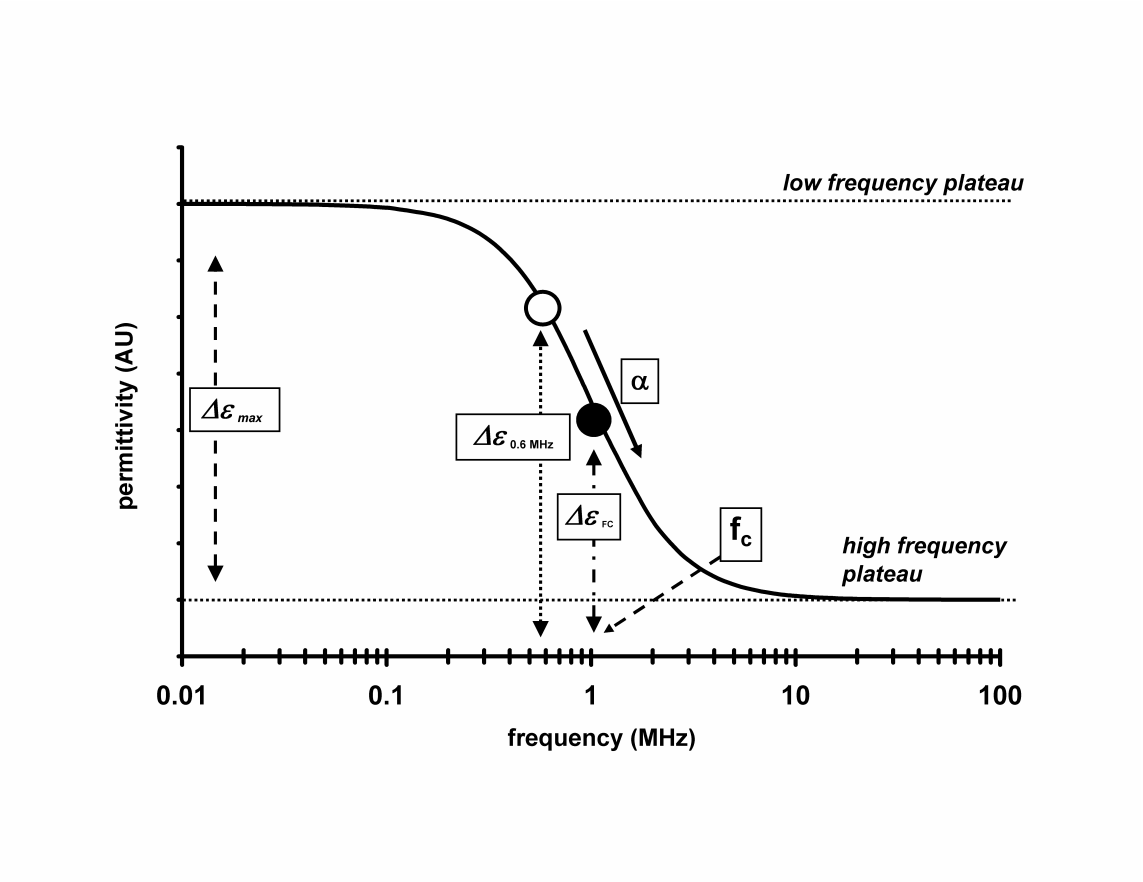

Figure 5.1: Biological cells polarize in electrical fields, resulting in the β-dispersion when frequency is increased in the range of 0.1-10 MHz.

See text in section 2.5 for details and further explanation

The first parameter $\Delta\varepsilon_{max}$ can be measured as the difference in permittivity at the low-frequency and the high-frequency plateau (permittivity increment). The second parameter, the characteristic frequency (f_C), is the frequency at which the permittivity decreased by half. f_C can be defined by the following simplified equation (eq. 5.3) (Schwan 1957; Harris et al. 1987b; Patel et al. 2008b):

$$f_C = \frac{1}{2\times\pi\times r\times C_M\times\left(\frac{1}{\sigma_i}+\frac{1}{2\sigma_m}\right)} \qquad (5.3)$$

with

σ_i: conductivity of the cytoplasm/intracellular conductivity (mS cm^{-1})

σ_m: conductivity of the medium (mS cm^{-1})

As the value of σ_m is generally much higher than σ_i in typical cell culture experiments, this equation can be further simplified by omitting the term $\frac{1}{2\sigma_m}$ (Ansorge et al. 2010a).

A third parameter describing the β-dispersion is α (also called: Cole-Cole α). It is an empirical parameter describing the decrease in permittivity with increasing frequency in the Cole-Cole equation (Cole et al. 1929). α is generally associated to changes in the distribution of cellular electrical properties (Davey 1993).

5.2.4 Results and Discussion

5.2.4.1 LV Production in Bioreactor Runs

5.2.4.1.1 Identification of Process Phases

LVs were produced by transient transfection in bioreactor cultures and the data of a representative experiment are shown in figure 5.2. In this culture, cells were transfected at a *vcc* of ~5x10^6 c/mL. After transfection, cell growth continued at a decreased rate until a maximum *vcc* of ~6x10^6 c/mL was reached ~40 hours post-transfection (hpt).

LVs in the form of functional transducing units (tu/mL) and total viral particles (vg/mL) were first measured in the supernatant around 20 hpt. A maximum LV titer of ~4x10^7 tu/mL in the supernatant was found at around 48 hpt, followed by an exponential decrease. The VG titer profile showed a similar evolution from 20-40 hpt. However, after the maximum in functional viral particles was reached, the VG titer further increased to achieve a maximum at ~96 hpt.

The small dilution of the cell suspension resulting from the addition of the polyplexes was reflected in the values of the permittivity $\Delta\varepsilon_{FC}$ and its increment ($\Delta\varepsilon_{max}$) Both values then reached a maximum around the time of the first viral release (~24hpt) with $\Delta\varepsilon_{max}$ exhibiting a more distinct maximum compared to $\Delta\varepsilon_{FC}$. In contrast, the characteristic frequency (*fc*) achieved a minimum at the time of the maximum in $\Delta\varepsilon_{FC}$ and $\Delta\varepsilon_{max}$.

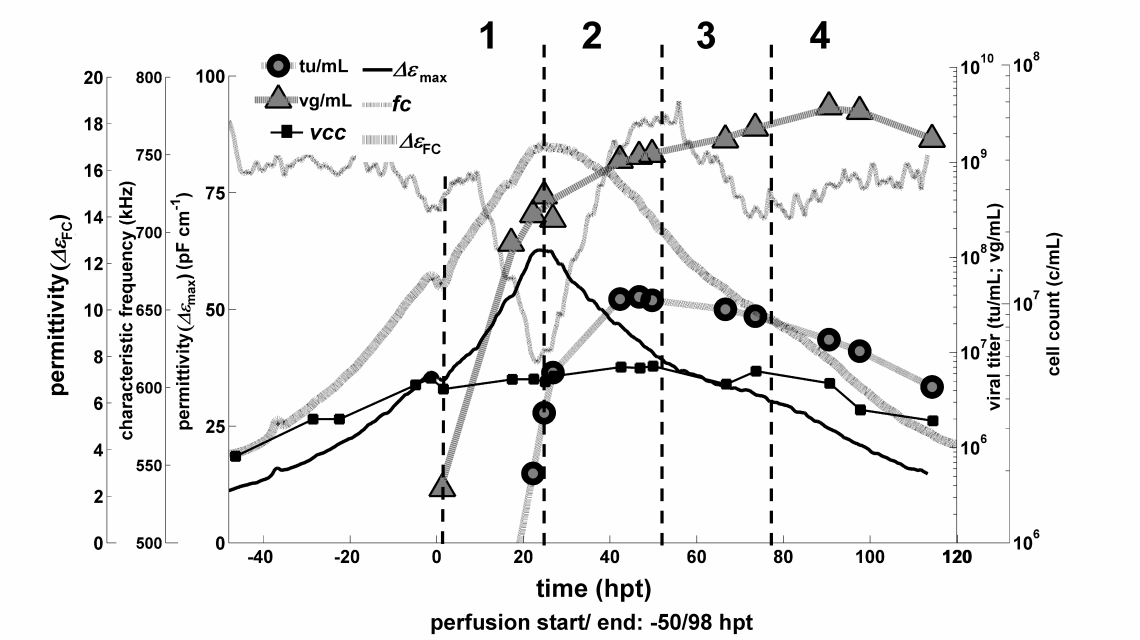

Figure 5.2: High-yield LV production in bioreactor scale.

Dashed lines and numbers indicate characteristic process phases which were identified according to changes in permittivity and the related β-dispersion parameters

The overall LV production profile was thus broken down into four distinct process transition phases, defined according to the observed changes in the aforementioned online permittivity signals (β-dispersion parameters).

Phase 1 (0-24 hpt) was characterized by a relative increase in $\Delta\varepsilon_{max}$ of 80%, whereas the characteristic frequency (*fc*) decreased by 13 % and reached a minimum at the time of the maximum in $\Delta\varepsilon_{max}$. During the second phase (24-50 hpt), $\Delta\varepsilon_{max}$ was found to decrease by ~38 % while *fc* increased by ~28 %. This phase ended when *fc* reached its maximum which coincided with a slope change in the permittivity increment. During phase 3 (50-75 hpt), the values of $\Delta\varepsilon_{max}$ and *fc* both decreased gradually. The onset of the last phase (75-120 hpt) corresponded to a second minimum in *fc*, paralleled by a slope change in $\Delta\varepsilon_{max}$.

Although LV release in the form of transducing particles could be detected at low levels at the end of phase 1, significant amounts of viral titers, i.e. 10-100 % of the functional viral peak titer and up to 30 % of the VG peak titer in the bioreactor supernatant, were not detected before

the beginning of phase 2. It was therefore hypothesized that the extrema in $\Delta\varepsilon_{max}$ and *fc* at the transition from phase 1 to phase 2 were related to this initial viral release after transfection. The subsequent changes also appeared to correspond with events related to LV production, such as the transition into phase 3 which coincided with the maximum in functional viral titer in the reactor. Finally, at the beginning of phase 4, functional LV titers had significantly decreased (<30 %). In contrast, the VG titer remained stable, indicating that a large number of non-functional viral particles containing viral RNA were constantly produced after transfection.

5.2.4.1.2 Analysis of Biovolume and Multifrequency Permittivity Measurements

Only a few of the variables that affect the permittivity of the cell suspension can be measured by standard cell culture methods. These include the mean cell diameter (*d*) and the cell density (*N*), from which the biovolume (*bv*) can be calculated (equation 1). The biovolume can be used as an offline estimate of the online permittivity value, in particular during the exponential growth phase and when viability remains high (Zeiser et al. 1999; Ansorge et al. 2007). The value of *bv* reflects either the dual-frequency permittivity ($\Delta\varepsilon_{FC}/\Delta\varepsilon_{0.6MHz}$) or, if multiplied by the cell radius (*bv***r*) the permittivity increment ($\Delta\varepsilon_{max}$) (see equation 2). Before transfection, a good correlation (R^2 = 0.93) was observed between *bv***r* and the permittivity increment $\Delta\varepsilon_{max}$ (fig. 5.3 A). However, the samples taken after transfection, i.e. during process phase 1 and phase 2 did not follow the same trend. In contrast, the samples from process phases 3-4 were again in-line with the correlation with the exception of those corresponding to the last day of the cultivation (90-114 hpt).

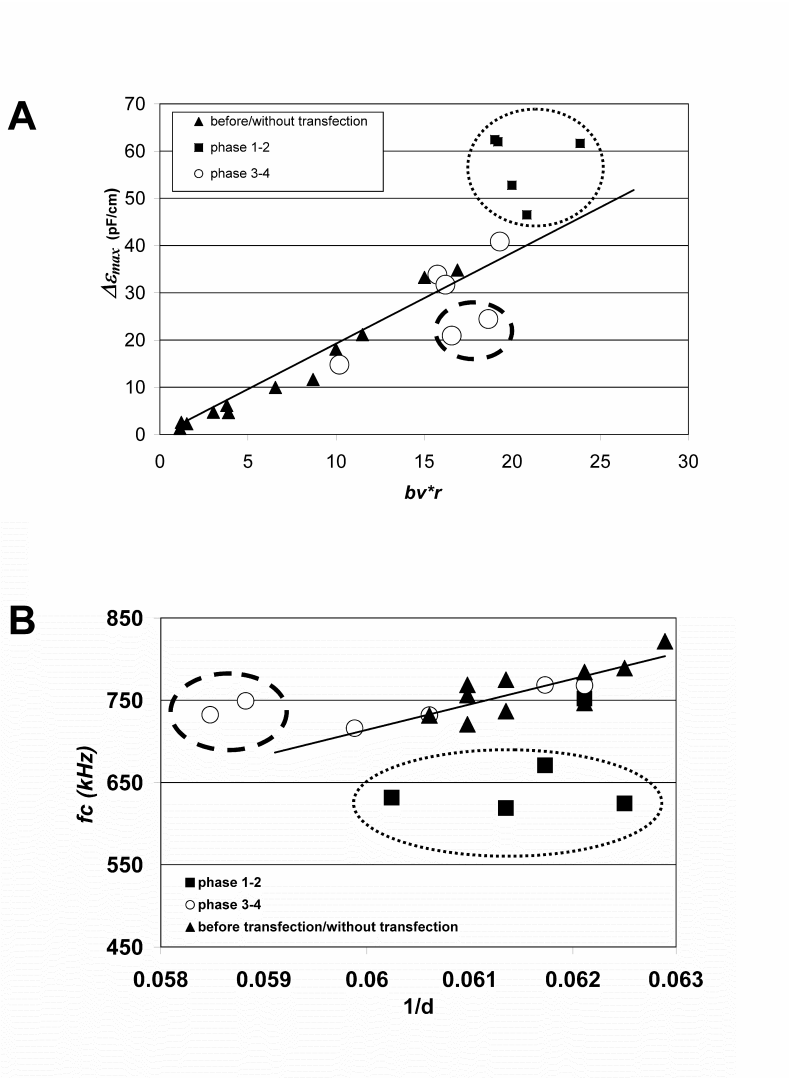

Figure 5.3: Regression lines for $\Delta\varepsilon_{max}$ and *bv*r* (A) and f_C and the inverse of the mean cell diameter (B); see equations 5.1-3 for the theoretical relationship of the variables.

Regressions were in both figures calculated based only on samples from the exponential growth phase in batch and before transfection in perfusion (triangles); regression coefficients were: R^2 = 0.93 (A) and R^2 = 0.6 (B). Dashed circles mark samples at the end of the culture; whereas dotted circles mark the samples after transfection in process phase 1/ the beginning of phase 2. The values from the perfusion culture correspond to the cultivation shown in 5.2 and 5.4

The characteristic frequency (*fc*) is directly affected by changes in cell diameter, intracellular conductivity and membrane capacitance (equation 5.3). *fc* was consequently plotted against the

inverse of the offline measured cell diameter $\left(\frac{1}{d}\right)$ measured offline (fig. 5.3 B). Although a linear relationship between the two variables is apparent, the regression coefficient for this cultivation was rather low ($R^2 = 0.6$) even when the values obtained from process phases 1 and 2 and the late stages of the culture were excluded from the regression, as is further discussed later.

To further investigate the changes in β-dispersion parameters and a possible relationship with initial LV release and production kinetics, the permittivity spectra were analyzed after mean centering and variance scaling. Attributing the same weight to the permittivity signals at the different frequencies allowed inferring which of the dielectric parameters most likely caused the observed changes in the spectrum (fig. 5.4).

Before transfection, the permittivity showed the same response at all frequencies within the β-dispersion range. After transfection, changes in the permittivity spectra occurred at 8-10 hpt when a first inflection point in the data became apparent. At that time, permittivity measured at low frequencies (0.3-1 MHz) increased at a much faster rate than measurement performed at high frequencies (2-10 MHz). A second inflection point was then observed at ~32-40 hpt, after which the permittivity at lower frequencies decreased more rapidly than at high frequencies. These inflection points were observed before the minimum in *fc* and after the maximum in $\Delta\varepsilon_{max}$, respectively.

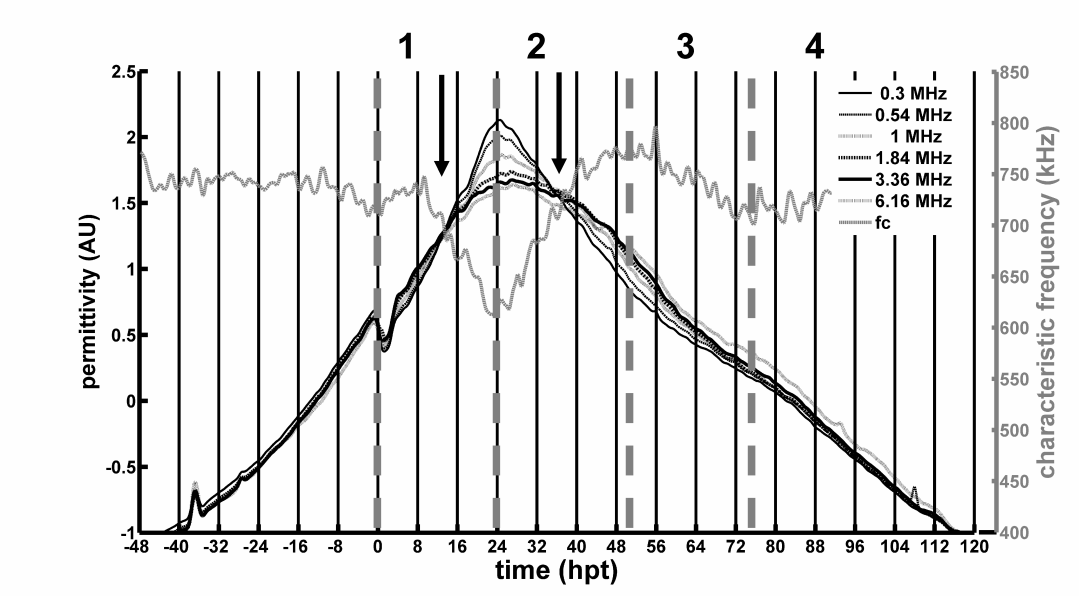

Figure 5.4: Inflection points (marked by arrows) in the mean-centered and variance-scaled permittivity spectrum indicate changes in the dielectric properties of producer cells after transfection; grey dashed lines mark process transition phases 1-4

The results from fig. 5.3 A and B indicate that neither a higher cell density with a slight increase in phase 1 and 2 only nor differences in cell diameter were solely responsible for the observed changes in the permittivity increment and characteristic frequency after transfection. That is, during phases 1 and 2, $\Delta\varepsilon_{max}$ increased and *fc* decreased to a larger extent than was expected from the respective changes in *vcc* or *d* (fig. 5.3 A and B). Values for samples from the very end of the cultivation also deviated from the correlations, as the remaining cells showed a lower permittivity per biovolume. Although cell viability remained high until the end of the run (> 80 %), a considerable amount of cell debris was observed during phase 4, accompanied by an attachment of biomass to the inner surface of the bioreactor/shake flask and increased cell aggregation. Others reported that permittivity measurements cease to predict the biovolume at decreasing viability (Ducommun et al. 2001; Ducommun et al. 2002). In fact, in the case of decreasing viability, membrane conductance typically increases resulting in a lower cell-specific permittivity (Patel et al. 2008b). One also needs to take into account that cell viability is assessed differently in the three methods used to measure the biovolume (dielectric spectroscopy, dye exclusion for

viability measurements and cell size measurement). Deviations might therefore be expected towards the end of the cultivation.

The analysis of permittivity spectra gave indications as to which of the dielectric parameters is responsible for the changes in the permittivity spectrum and the β-dispersion parameters. If no significant changes in cell diameter, intracellular conductivity and membrane capacitance occur, the response at all frequencies should be identical (equations 2 and 3). This is the situation observed prior to transfection, where only the amplitude of the β-dispersion ($\Delta\varepsilon_{max}$), but not its shape (*fc*) changed. In principle, a decrease in σ_i, accompanied by constant values of C_M and *r*, would result in a drop in permittivity at high frequencies and a decrease of *fc* and $\Delta\varepsilon_{FC}$ without affecting $\Delta\varepsilon_{max}$. Here, a variation in σ_i alone during phase 1 and 2 can consequently be excluded. Yet, it is still possible that σ_i changed in parallel to the variations in cell diameter and C_M. In contrast, and as was observed here, an increase in C_M or *r* would result in a higher permittivity signal at low frequencies without a significant effect on the response at high frequencies. Differences in the permittivity spectrum and in turn the dielectric properties of the producer cells, were monitored as early as ~10-12 hpt, with permittivity measured at the lower frequencies increasing at a faster rate than at higher frequencies. Based on the results presented in fig. 5.3A and B, the initial LV release causes an increase in the value of C_M of 30-70 %. These variations were possibly caused by the LV budding process and responsible for the characteristic profile of the permittivity parameters observed during production.

It was reported earlier that the production of HIV-forming proteins is initiated as early as 5-6 h after transfection (Jouvenet et al. 2008). This is consistent with the early changes observable in the permittivity spectra (fig. 5.4). C_M was originally reported to be a biological constant with values in the range of 1 ± 0.5 μF/cm^2 and described as a measure of the 'wrinkliness' of the cell membrane (Schwan 1957; Harris et al. 1987b; Patel et al. 2008b). More recent studies found, however, that C_M changes after viral infection and overexpression of cation channels (Archer et al. 1999; Zimmermann et al. 2008). It therefore seems to be particularly affected by processes involving the cellular membrane properties, such as, for example, exocytosis (loss of microvilli), viral release, and apoptosis (Patel et al. 2008a; Patel et al. 2008b). Reductions in C_M of 40-60 % were reported for several cell types after infection with different viruses, such as herpes simplex and polyomavirus. C_M is affected as early as a few hours post-infection as a result of the loss of

membrane blebs and a more uniform membrane conformation or a membrane surface with more irregularities (Archer et al. 1999; Berardi et al. 2009). For HIV-1, from which LV are derived, it is hypothesized that the release of virus by membrane budding and fission takes place very shortly after the formation of particles (Goto et al. 1990).

In contrast, studies on BEV-infected insect cell cultures have mainly focused on the ‘charging’ phase right after infection during which an increase in cell volume results in higher biovolume and permittivity values. Only two of the studies describing permittivity measurements in the BEV system used multifrequency technology for monitoring and only one of those analyzed changes in *fc*, which increased dramatically at the point of first baculovirus release (Ansorge et al. 2007). However, a key difference to the present LV system is that BEV-infected cells loose viability rapidly after the first viral release.

5.2.4.1.3 Reproducibility and Comparison of Off- and Online Methods for Monitoring of LV Production

Consistently, similar LV production kinetics and monitoring results were observed in several bioreactor perfusion cultures under different operating conditions (fig. 5.5) and could be described using the same phase distribution pattern. During process phase 1 (0-24 hpt) and the transition into phase 2 (24-50/70 hpt), a single maximum value for the oxygen sparging rate (OSR) was observed, coinciding with the maximum in biovolume and permittivity. In contrast, the total GFP expression attained high levels only in phases 3 and 4, with the culture at 1 VVD resulting in higher values than the cultivations at 2 VVD.

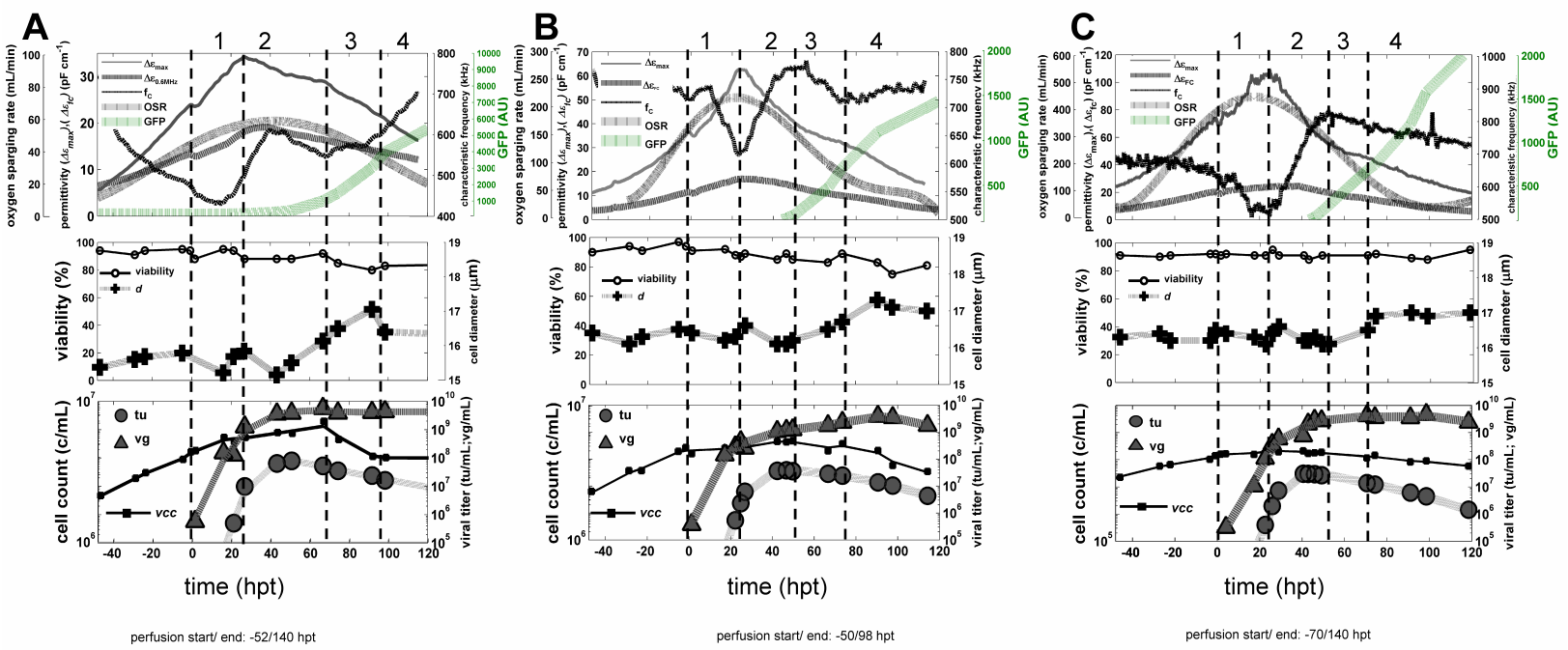

Figure 5.5: Monitoring results for high-yield LV production runs.

Three independent experiments are shown for comparison. The culture in (B) is identical to the one presented in fig. 5.2 and 5.4. Operating conditions varied with:

(A): perfusion rate in volume(s) of medium per reactor volume and day (VVD), *vcc* at time of transfection (Lao et al.): 1 VVD, 4.4 x10^6 *vcc*; B: 2 VVD, 4.7 x10^6 *vcc*; C: 2 VVD, 8x10^6 *vcc*; for cultivations shown in A and B the same medium composition (HyQ) was used, culture shown in C included additional supplementation with CB5 (HyQ+)

In all the cultures, the cell diameter was found to initially decrease after transfection, but eventually increased during the transition into phase 2. During the second phase, decreases in cell size, $\Delta\varepsilon_{max}$, $\Delta\varepsilon_{FC}/\Delta\varepsilon_{0.6MHz}$ and OSR were also observed while viability remained high in all cases. The diameter returned to its value before transfection during phase 2 and increased significantly thereafter (until phase 4). For all cultures, similar permittivity spectra were obtained and the correlations between *bv*r*, *bv* and $\Delta\varepsilon_{max}$, $\Delta\varepsilon_{FC}/\Delta\varepsilon_{0.6MHz}$ and between cell diameter and *fc* were, respectively, similar (regressions not shown for all cultures).

Also, the changes in cell diameter (*d*) clearly played a role in the evolution of $\Delta\varepsilon_{max}$ and *fc*. A first maximum in *d* was observed from the transition of phase 1 to phase 2 at the beginning of the initial viral release and was in-line with the maximum in $\Delta\varepsilon_{max}$/the minimum in *fc*. The second inflection point in the permittivity spectrum (fig. 5.5) coincided with the minimum in cell diameter and/or C_M.

The maximum in OSR might also be used as an indicator of the initial lentiviral release and the onset of the production phase. Changes in oxygen consumption after viral infection are well documented in the literature (Kussow 1995; Kamen et al. 1996; Lecina et al. 2006). During LV production however, monitoring of OSR alone seemed to provide considerably less direct information specific to the titer kinetics than $\Delta\varepsilon_{max}$ and *fc*.

If GFP is the transgene encoded by the viral vector construct, monitoring GFP expression can also provide indication on the infection and production kinetics of viral vectors (Henry et al. 2004; Kamen et al. 2004). This also holds for the production of VSV-G pseudotyped LVs in which self-infection of the producer cells results in high transgene expression once LV are released (Farson et al. 2001; Bagnis et al. 2009). In small scale experiments, the peak in production after 48 hpt and the GFP expression measured at 72 and 96 hpt correlated well over a wide titer range (results not shown). In bioreactor-scale cultivations, a lower perfusion rate resulted in less GFP expression in the producer cells, possibly due to an increased residence time of functional particles in the vessel. The use of *in situ* probes could therefore be explored during LV production (Gilbert et al. 2000). However, the value of these measurements will be most likely limited because information on functional viral titer would only be available with a delay of 1 or 2 days. More importantly, once GFP will be replaced by a different transgene of interest, this method will be inapplicable.

5.2.4.1.5 Changes in Online Permittivity Signals as Indicators of LV Yield

The ratio of functional to total particles is generally low during LV production with the expression of LV proteins such as *gag* or VSV-G being sufficient for the release of non-functional enveloped particles (Haglund et al. 2000; Göttlinger 2001; Higashikawa et al. 2001; Sherer et al. 2003; Mitta et al. 2005; Pichlmair et al. 2007). All of these species bud off from the membrane and would hence affect its properties. They were also found to contain viral or cellular DNA/RNA (Pichlmair et al. 2007; Zhao et al. 2008) and lead to an overestimation of VG titer. We therefore evaluated if the relative changes in the two key β-dispersion parameters, *fc* and $\Delta\varepsilon_{max}$, could give indications on the total LV yield from bioreactor runs. We found a good correlation of the relative changes in *fc* and $\Delta\varepsilon_{max}$ (during phase 2) and the total VG yield from bioreactor LV productions (fig. 5.6). However, these results will need to be confirmed by future cultures with a wider range in viral yield. In contrast to the VG titer evolution, the permittivity signal and the production of functional particles continuously decreased starting in phase 2. In control experiments, SDS-PAGE and western blot revealed that 4 days after transfection, the expression of VSV-G had dramatically decreased in LV-containing culture supernatants (results not shown). Cytotoxic effects of the envelope protein VSV-G were observed by others (Li et al. 1993; Quinonez et al. 2002; Pichlmair et al. 2007; Zhao et al. 2008). Lysis of producer cells induced by these toxic effects might explain why the kinetics observed for VG titer and functional viral particles varied in particular during phase 3 and 4 (section 5.2.4.1.1, fig. 5.2 and 5.5).

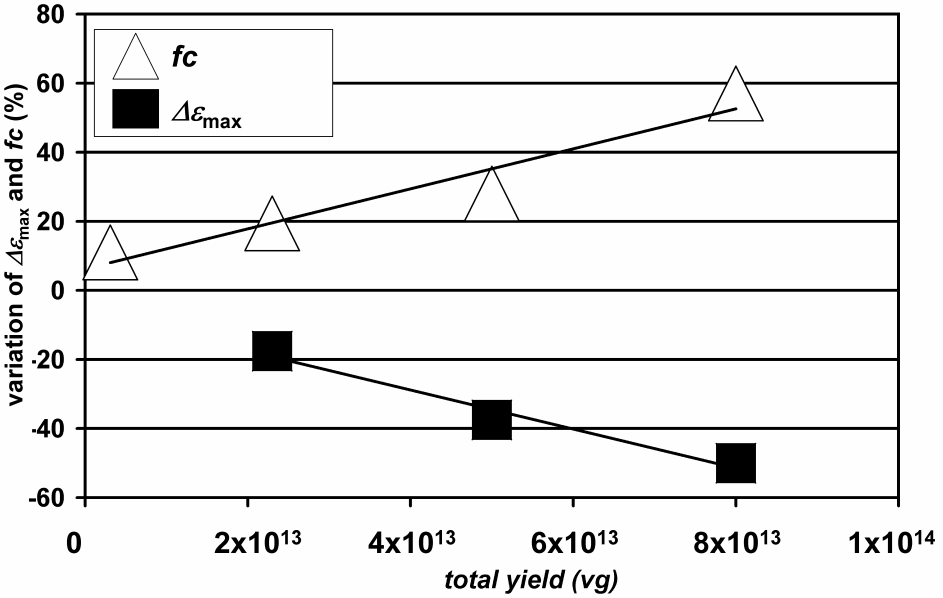

Figure 5.6: Relative changes during phase 2 in permittivity-related parameters ($\Delta\varepsilon_{max}$ and f_C) indicate total process yield.

$\Delta\varepsilon_{max}$: R^2 = 0.9741; f_C: R^2 = 0.9332. Similar high regression coefficients were found when plotting the total yield after 2 dpt (not shown); one value for $\Delta\varepsilon_{max}$ for a culture at low yield in which the maximum was related to a cease in cell growth was omitted in the graph

5.2.5 Conclusions

To our knowledge, this study is the first presenting permittivity measurements as online monitoring tools for the detection of viral release after transfection. Previous reported observations were made for systems in which viral vectors are produced after infection of producer cells. Based on online permittivity data, four distinct and reproducible process transition phases were identified during LV production and were shown to be related to the initial release of LVs and the kinetics of functional viral particle titers. Extrema and slope changes in the β-dispersion parameters ($\Delta\varepsilon_{max}$, *fc*) indicated the first viral release at the end of phase 1 (~24 hpt), the maximum functional viral titer in the culture supernatant during phase 2 (~48 hpt) and the optimal harvest time after phase 3/during phase 4 (>72-96 hpt). These characteristic features were

observed for different operating conditions, including differences in perfusion rate, cell density at transfection and media composition (fig. 5.5).

Before transfection, a good correlation was found between offline biovolume measurements and online permittivity signals (R^2>0.9). In contrast, samples taken during the initial viral release phase (phase 1-2) exhibited higher permittivity values per biovolume and a decreased characteristic frequency (*fc*) per cell diameter. After analysis of the permittivity spectra and comparison with other off- and online measurements, it was inferred that membrane capacitance (C_M) must have increased significantly after transfection and around the time of first viral release. The measured changes in cell size alone were, on the other hand, not sufficient to explain the differences in $\Delta\varepsilon_{max}$, $\Delta\varepsilon_{FC}$ and *fc*, suggesting that LV budding could be detected by permittivity measurements. However, the changes of cell membrane properties (C_M) during LV production were not directly measured and it is consequently possible that other parameters (such as the intracellular conductivity) were also affected. The use of methods such as dielectrophoresis or electrorotation should be of interest in this context to shed light on the events occurring at the cellular level and to better understand the impact of LV release on the dielectric properties of cells. Yet, it is not necessary to unambiguously identify the dielectric parameter(s) affected during LV production to use our observations for process characterization and control.

Compared to other methods (oxygen consumption, total GFP expression), dielectric spectroscopy appeared to be the most sensitive and informative tool for online monitoring of LV production. The use of changes in $\Delta\varepsilon_{max}$, $\Delta\varepsilon_{FC}$ and *fc* as guiding variables for the real-time identification of action points or as a direct tool for perfusion rate control and final harvest should allow optimizing LV production processes.

Interestingly, the relative changes in $\Delta\varepsilon_{max}$ and *fc* correlated with the yield in total viral particles (VG titer), suggesting that the method might be used for the real-time estimation of process yield. These findings should not only facilitate LV production process characterization and modelization but also enable advanced process control. The monitoring of parameters that are indicative of process efficiency in real-time should greatly facilitate and speed up process development and allow prediction of process performance and eventually failure. Such tools are therefore invaluable to further support the establishment of robust lentiviral vector production processes in large-scale.

Also, it is expected that the findings reported herein could be translated to other viral vector and vaccine production processes based on transient transfection and infection.

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CHAPTER 6 OVERALL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

Lentiviral vectors are currently under investigation as gene delivery vehicles in more than 20 clinical trials. Meanwhile, the molecular architecture of the vector system remains under development. Improvements and possible modifications of the system will depend on the acceptance and success of these ongoing clinical trials.

For LV mass manufacturing of final commercial material, it can be anticipated that stable packaging and producer cell lines will be employed. However, it is questionable if the efforts to produce a stable packaging or producer cell line are beneficial in early stages of an LV-based therapy development when different or modified vector components often need to be evaluated. To that end, transient transfection has advantages in terms of flexibility and shorter time to product. To date, yields from both production strategies are equivalent. It is therefore expected that both technologies will be used in parallel in the foreseeable future.

Although time-consuming and subject to large variability, current production protocols using T-flasks or culture dishes provide sufficient yields for small scale research applications. Improvements in benchscale LV production protocols could increase yields significantly but the approaches almost exclusively used adherent cell lines. A comparison of results from different groups is generally difficult due to the variety of production protocols and titration methods found in the literature. Optimization approaches guided by bioprocess engineering principles are only rarely reported.

However, to avoid production constraints in later evaluation phases, robust and readily scalable suspension based approaches need to be in place for pilot and large-scale productions, ideally before preclinical studies and phase 1 clinical trial are being contemplated.

This thesis deals with the production of LVs by transient transfection and describes their scalable production. Some of the parameters that are critical for efficient LV production were identified and monitored or controlled to improve process yields. Using a suspension-grown cell line for production in perfusion mode, the issue of low LV stability was successfully addressed and previous constraints concerning the scalability of production methods were solved. More specifically, the present work demonstrated that LV production is amenable to the 3 L bioreactor

scale. Beyond that, it is hypothesized that production in larger scales is feasible without major changes in the production setup.

As a result of the perfusion strategy, the cell density limitation of common transfection protocols was circumvented by producing LVs at high cell density (HCD) of $5x10^6$ c/mL. This strategy should be transferable to other systems and advantageous for unstable products for which the production process needs to be performed in perfusion.

Optimization of transfection conditions further increased LV titers. In our hands, best results were obtained for a PEI:DNA mass ratio of 2:1 and when 0.4-0.6 µg of total DNA/10^6 cells was used for transfection in HyQ medium. This strategy reduces toxic effects of PEI and increases the cost-effectiveness of the method.

The addition of sodium butyrate had the most significant impact on LV production. At a concentration of 5 mM, sodium butyrate altered the production kinetics and increased total yields by 15-fold.

The LV production kinetics after transient transfection in perfusion mode was analyzed in detail. It was found that productivity of functional LVs and the ratio of functional to total LV particles (LV quality) were changing during the process. Production kinetics and the ratio of functional (GTA) and total LV particles (VG/VP titer) were found to differ from intial conditions when using improved production conditions. Optimum expression of all four plasmid constructs for LV production took in the latter case place at 2 dpt, resulting in highest functional titers and LV quality. This ‘butyrate effect’ was attributed to an increase in the overall expression of the LV-encoding constructs. VSV-G seemed to be critical under improved production conditions and it was hypothesized that its expression is limiting production yields and confines the generation of functional LV at high yield to transient production of a few days. If these findings hold true, this might also have implications for the development and use of stable cell lines, as these systems would then also be intrinsically limited to short LV production windows.

Enriched medium formulations (LC-SFM GL, HyQ, HyQ+) outperformed standard medium formulations (LC-SFM L, HyQ). LV production is thus also a function of the nutrient availability of the culture. Further work is however required to understand the influence of the cell’s physiological state on production yields. Preliminary results did not allow determining if further

process improvements were limited by metabolic constraints or the efficiency of transfection at high cell density.

Overall, the optimization strategy resulted in improved production conditions delivering 100-150 fold higher total yields compared to routine small scale conditions. These conditions were then validated at the 3-L bioreactor scale, resulting in an increase of ~75-fold compared to a bioreactor production performed under initial conditions. A continuous harvest strategy and storage of LV at low temperature outside the reactor allowed for the efficient rescue of functional LV.

Even when using improved production conditions, the cell-specific productivity of functional viral vector remained low (60-120 tu/cell). This can in part be explained by the low ratio of functional to total LV particles (GTA:VG titer) of 1-4 %. Due to differences in production protocols, titration methods and plasmid constructs, a direct comparison of cell-specific yields with other groups is not always possible. The yields observed here, with a maximum of $>1x10^8$ tu/mL in non-concentrated supernatants should nevertheless be superior to what has been so far reported by other groups.

We note that the process strategy developed in this work is flexible and can consequently be easily modified to rapidly respond to the requirements defined by the final application for which LV are generated. As a general recommendation for efficient LV production it is pointed out that the choice of the ideal production strategy is a function of several parameters including:

- total LV quantity needed
- downstream processing (DSP) strategy
    - are functional LVs separated from total particles/is this required for the final application?
    - production yield (volume and titer) vs DSP capacity per day
- the importance of LV quality for the final application

In small-scale, i.e. for a total targeted production yield of $0.5\text{-}1x10^{10}$ tu (for the model system) the optimum production strategy would consist of LV production at $5\text{-}10x10^6$ c/mL at 2 DMD, ensuring high total yields and LV quality. Culture supernatants could then either be frozen for subsequent purification or directly concentrated by ultracentrifugation.

In bioreactor scale, i.e. for a targeted production yield of $>1x10^{11}$ tu, the choice of medium exchange rate highly depends on the capacity and resources of the downstream facilities and the importance of LV quality. 2 VVD resulted in higher LV quality than production at 1 VVD. However twice the amount of LV-containing supernatant is generated and LV-containing supernatants can, due to the low LV stability, only be stored for limited periods (at 4°C) whereas freezing of large volumes is challenging. The DSP strategy is thus determining if production at high medium exchange rates is advantageous.

It must be noted that total LV yields are known to be a function of the LV system and the transgene of interest; absolute yields might therefore differ significantly from the values observed for the model system used throughout this work. Our described LV production platform should be ideal for the testing of improved LV constructs and their evaluation from bench to bioreactor scale. The scalable perfusion strategy will also facilitate the development of LV-related production and manufacturing technologies, such as large scale downstream processing which was previously limited by the inability to rapidly generate large volumes of highly concentrated LV supernatants.

Current LV quantification methods are an important constraint for process development. Most methods are time-consuming, standardization is urgently needed and assay result variability is generally high. Further development of novel quantification methods for lentiviral vectors and other enveloped viruses is consequently needed. In that context, precision and time-to-result of the different methods is instrumental to facilitate process development. To date, the quantification of VG titer by RT-PCR seems to be the most advantageous direct method to assess total particle numbers. Concerning indirect methods, our results indicate that it will be of particular importance to control the passage numbers of target cells as this might significantly decrease variability of functional LV titers.

Optimized operation and LV yield maximization of the bioreactor perfusion process require tools that convey information on vector production in real-time. For that purpose, we sought to link LV production kinetics to online measurement signals. As a first step, suitable real-time tools for the monitoring of LV production needed to be identified. Despite the, in comparison to other viral vectors, low productivity of functional lentiviral vectors after transfection, online *in situ* permittivity measurements were identified as the most informative tool for the rapid

characterization of bioreactor productions. Based on the real-time permittivity data, four distinct and reproducible process transition phases were identified during LV production. The monitoring results consistently reflected LV production kinetics during several bioreactor perfusion cultures under different operating conditions. Changes in the signals were related to the initial release of LVs and the kinetics of functional viral particle titers. In addition, relative changes in the β-dispersion parameters (*fc* and $\Delta\varepsilon_{max}$) correlated with the total VG yield from bioreactor LV productions. In conclusion, the technology is of great value for the screening of different production conditions and process development. It is also recommended as a PAT tool for process characterization which is in turn a prerequisite for performance optimization.

Despite the significant increase in LV yield when using the improved production conditions reported here, viral vector titers were low compared to what is reported for other viral constructs and production systems. For capsid virus-based vectors, such as adenoviral and adeno-associated viral vectors, cell-specific yields in the range of 10^3-10^4 infectious viral particles per cell are generally observed in HEK293 cells (Kamen, Henry 2004; Aucoin, Perrier et al. 2008). Baculovirus expression vectors, which are similar to LVs budding from the producer cells can be produced in insect cells at yields of up to 10^4 infectious particles/cell (Carinhas, Bernal et al. 2009). Such comparisons are, however, generally difficult because of differences in viral (capsid vs budding virus) and/or host cell (insect vs mammalian cells) biology. Nevertheless, even for production of influenza virus which is, supposedly, the closest system compared to LV production, resulted in higher infectious particle yields of 10^2-10^3 IVP/cell in non-optimized bioreactor batch productions (Le Ru, Jacob et al. 2010).

It is thus expected that there is still a possibility to further improve current LV production processes significantly. On the other hand, it is concluded that the dramatic effect of butyrate on production yields and kinetics is at the same time a limitation for further process improvement. The overexpression of LV-related proteins limits production windows. This is supported by our findings that the time point of butyrate addition has no positive effect on total LV yields. The extension of production windows seems to be a promising strategy to achieve higher yields. This would be possible if the loss of functional titers in later process stages could be avoided. For example, sequential transfection with the VSV-G plasmid might be explored as well as the reversible inhibition of the fusogenic properties of VSV-G during production.

At this point, most significant improvements can, however, be expected from modifications at the molecular level, such as an increase in functional LV stability, a decrease in envelope cytotoxicity or regulation strategies for VSV-G.

Alternative LV envelopes have been described and their use would likely result in higher functional vector yields (Verhoeyen and Cosset 2004; Cronin, Zhang et al. 2005). Yet, LVs that are not pseudotyped with VSV-G are limited to specific applications and target cells.

Beyond these improvements, another avenue for process optimization is a further analysis of the HEK293 metabolism in HyQ medium with the goal to identify and develop a) strategies to further increase cell density at the time of transfection without loss in specific productivity and b) optimized medium formulations for the production of LVs.

Therefore, more work integrating recent advances in metabolic engineering and medium optimization is required to further increase LV production yields. Only preliminary data are available on the impact of the physiological state and the nutrient availability at the time of transfection on LV production yields. An example is that lactate concentrations at inhibiting concentrations were observed in some of the bioreactor perfusion cultures. Due to the limitations of LV quantification methods, it is recommended to perform this evaluation using suitable transfection model systems for which the protein product can be more reliably quantified.

Specific to LV production is an analysis of the importance of lipids and lipid metabolism. Extensive work related to the production of retroviral vectors suggests that cellular lipid content and metabolism play a critical role in vector production (Merten 2004; Coroadinha, Alves et al. 2006; Coroadinha, Ribeiro et al. 2006; Coroadinha, Silva et al. 2006). As lipid content in the produced vectors appears to determine their stability, it will be particularly in that context instrumental to deconvolute effects on LV production and stability (Beer, Meyer et al. 2003; Carmo, Faria et al. 2006; Coroadinha, Alves et al. 2006).

Further work and in particular the demonstration of improved process performance when using the real-time signals for process control and decision-making is required. For example, the permittivity signals could be used to directly control the harvest rate and for further advanced process control or supervision to optimize the process and maximize yields. Preliminary results also suggest that the feed could be controlled based on real-time permittivity signal before production, which would minimize medium consumption.

Overall, it is difficult to anticipate to what degree these strategies and their combination would further improve LV production yields using the model system. Another avenue for future work would thus be to evaluate if the critical parameters identified here are applicable and transferable to other LV systems.

This work demonstrates that mass production of LV using suspension cultures is feasible in large scale. Our findings will make the application of these vectors cost-effective, facilitate their experimental and clinical evaluation and eventually render LVs more accessible to patients. The strategies developed in this work provide unprecedented yields of 10^{10}-10^{11} tu/L corresponding to 10^{12}-10^{13} vg/L of production supernatant. Although many aspects require further research and development and process optimization efforts, the progress in LV production described here should enable the cost-effective and simple mass production of clinical LV material under GMP conditions in the near future.

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APPENDICES

The appendix contains preliminary and additional experimental observations that facilitate the understanding of the results presented in the main part of this thesis.

In appendix I, the stability of LV is assessed in cell culture supernatants. Appendix II summarizes the efforts undertaken to further improve LV yields in the production system beyond the yield improvements described in chapter 3. This part points out the limitations of the current production strategy. Finally, complementary experiments for the respective chapters as well as additional information are presented in appendix III.

APPENDIX I- A Preliminary Study on the Stability of LVs in Culture Supernatants

As was summarized earlier (section 1.2.4, 2.1.3), the stability of functional LV is generally low, with a half-life of only 3-18 h at 37 °C (Higashikawa and Chang 2001; Watson, Kobinger et al. 2002; Croyle, Callahan et al. 2004). Yet, literature does not provide sufficient data on the stability of lentiviral vectors in cell culture supernatants. In most cases, pre-purified or concentrated LVs are used to evaluate LV stability in final preparations. Here, it was of particular interest to assess the viral decay during production. According to the results presented here, the harvest strategy was modified, i.e. higher medium exchanges rates of 2 DMD/VVD were tested to further increase process yields (appendix II and chapter 5).

AI.1 Stability of functional LV produced in small-scale experiments

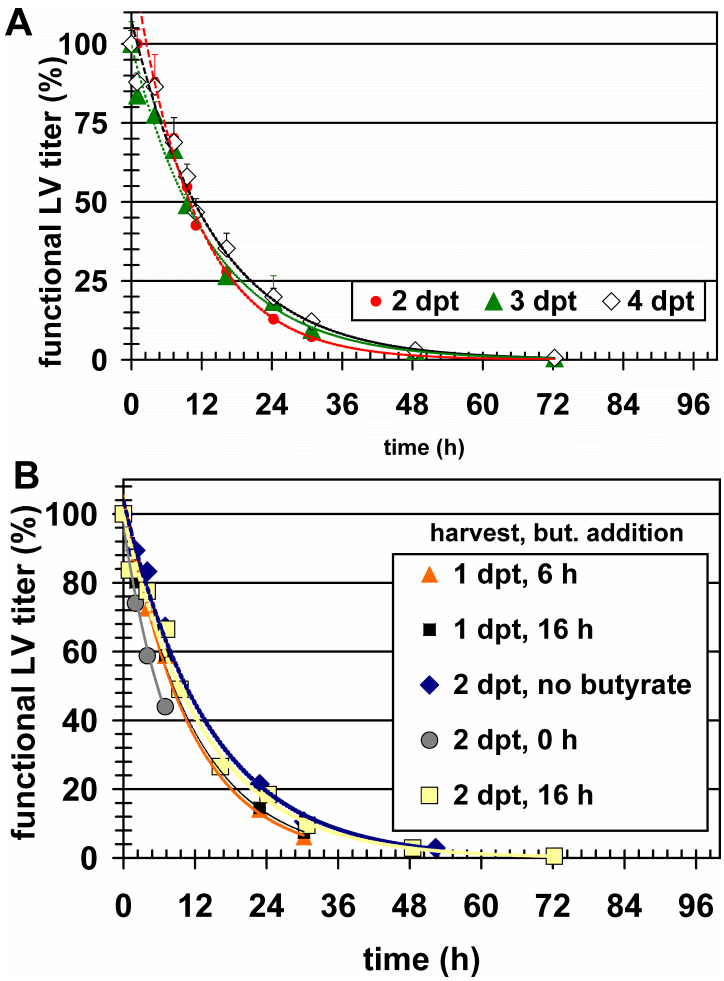

Figure AI.1: LV stability in small scale culture supernatants at 37°C

(A) LV decay in supernatants harvested from cultures transfected using improved production conditions and 2 DMD
(B) Modification of production conditions (sodium butyrate addition) does not significantly affect LV stability

The stability of functional LVs was evaluated after harvest of supernatants from cultures using improved production conditions in small scale experiments. Figure AI.1 presents the kinetics of LV decay after incubation at 37°C. No significant differences in LV stability were found for harvests at varying production days (2-4 dpt) (fig. AI.1 A). The time point of butyrate addition

did not affect LV stability (fig. AI.1 B). In all cases, the loss in transducing units at 37°C followed an exponential decrease. The half-life ($t^1/_2$) of LVs was in most conditions between 8-10 h (refer to table AI.1 for half-life values). Variations in $t^1/_2$ were, however, quite high (standard deviations not shown), due to the fact that the analysis was based on GTA. Subsequently, LVs harvested during a bioreactor perfusion culture (run #3) were analyzed (fig. AI.2). A higher number of samples allowed for a more reliable estimation of $t^1/_2$ which was calculated at ~12-13 h. No difference was found for $t^1/_2$ of LVs in supernatants harvested on 2 and 3 dpt, respectively. At a reduced temperature of 4°C, the degradation of LVs was slowed down and followed a linear decay ($t^1/_2$ of ~ 100 h).

In contrast, the viral decay based on VG titer was similar at 4 and 37°C ($t^1/_2$ of 40-50 h). This indicates that viral RNA degradation was not responsible for the rapid loss in the decay of functional LVs. Similar kinetics for viral genome degradation of VSV-G pseudotyped LVs were observed by others. The same authors excluded enzyme inactivation and viral degradation as responsible for the fast loss in LV infectivity and hypothesized that the loss in functional LV is associated to a loss in the capacity to perform reverse transcription (Carmo, Panet et al. 2008).

In conclusion, the $t^1/_2$ of functional LVs was found to be in the range of 8-13 h. Higher variations were observed during the analysis of supernatants produced in small scale cultures but these could not be attributed to the modifications in production conditions. It also needs to be noted that lower $t^1/_2$ values for PCR analysis compared to GTA results were found at 4°C indicating that further experiments are required to assess decay kinetics with higher confidence. Nevertheless, the results provide a good indication on viral degradation during the process and suggest production at increased medium exchange rates for yield optimization (appendix II). In contrast, as these studies were performed in cell-free supernatants, the results do not allow conclusions on the loss of functional LV due to retransduction of producer cells. Those events do, however, most likely occur, as GFP expression during production attained high levels and correlated with maximum LV titers (chapter 4). The significance of retransduction events for production yields would thus need to be evaluated in additional studies.

AI.2 Stability of functional LV produced in bioreactor cultures

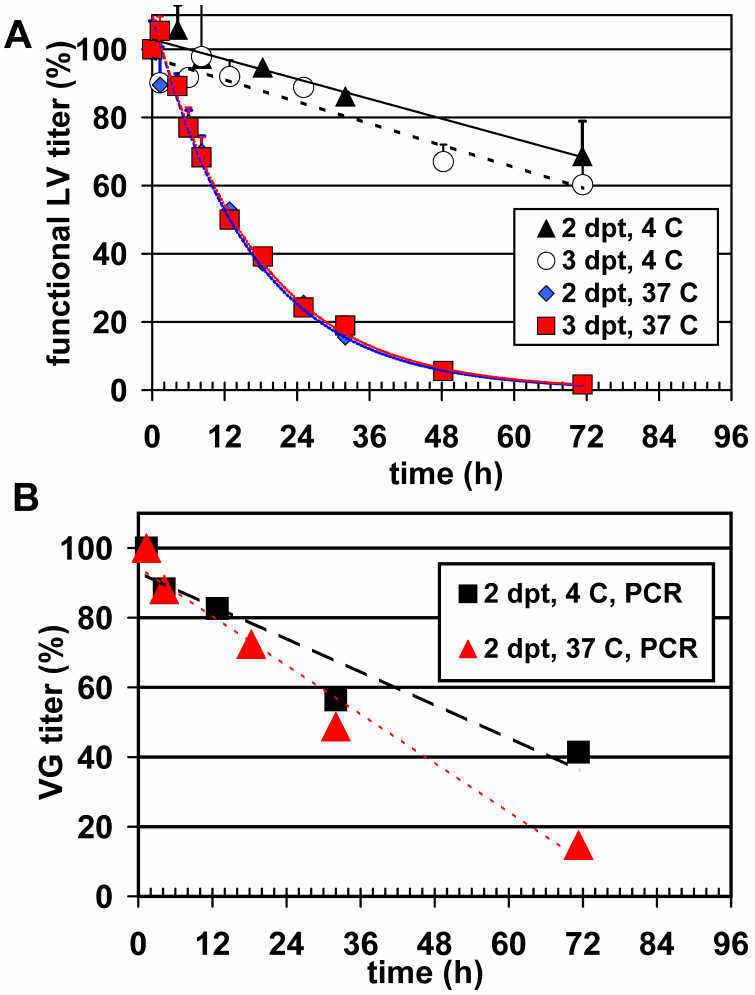

Fig AI.2: LV stability at 37°C in cell free harvest from bioreactor supernatants; supernatants were harvested from continuous bioreactor cultures at 2 and 3 dpt, bioreactor run corresponds to run # 3; (A) decay analyzed by GTA at 4 and 37 °C, note that this figure is identical to fig. 2.2; (B) decay at 4 and 37 °C analyzed by PCR

Table AI.1: LV stability in different production scales and conditions

Experiment[I]/ *Harvest Day[II]*	**Improved Production Conditions**				**Modified Production Conditions**			
	Small Scale (GTA, 37°C) Exp #1[IV]	**Bioreactor Scale[III] (GTA, 37 °C), run #3**	**Bioreactor Scale[III] (GTA, 4 °C), run #3**	**Bioreactor Scale[III] (PCR 4/37 °C), run #3**	**Harvest (hpt), butyrate addition (hpt)**	**Small Scale (GTA, 37 °C) Exp #2[V]**	**Harvest (hpt), butyrate addition (hpt)**	**Small Scale (GTA, 37 °C) VI**
1 dpt (starting titer, (tu/mL) $t^1/_2$, R^2)	$8.1x10^6$, **8.4**, 0.99.	n.a.	n.a.	n.a.	16, 0	$1.3x10^7$ **6.35**, 0.99		
*2 dpt (starting titer, (tu/mL/**vg/mL**) $t^1/_2$, R^2)*	$7.1x10^8$, **10**, 0.99	$5.2x10^7$, **12.85**, 0.99	$4x10^7$, 109.2, 0.96	**$1.5x10^9$**, **54**, 0.91 **$9.5x10^8$**, **38**, 0.97	40, 0	$2.14x10^8$, **5.66**, 0.99,	48, no butyrate	$2.3x10^7$, **10.44**, 0.99
3 dpt (starting titer, (tu/mL) $t^1/_2$, R^2)	$1.8x10^8$, **9.3**, 0.99	$7.6x10^7$, **13.34**, 0.99	$8.66x10^7$, **88.46**, 0.91	n.a.	24, 6	$7.3x10^7$, **8.1**, 0.99	72, no butyrate	$4x10^7$, **15.3**, 0.99
4 dpt (starting titer, (tu/mL) $t^1/_2$, R^2)	$2.85x10^7$, **10.7**, 0.99	n.a.	n.a.	n.a.	40, 6	$2.14x10^8$, **9.66**, 0.89		

$t^1/_2$: half-life

I: scale (quantification method, temperature, experiment #)

II: harvest time (LV starting titer, half-life, regression coefficient)

III: harvest from cell-free supernatant (perfusion harvest), run #3, improved production conditions in bioreactor, 1 VVD

IV: experimental conditions: improved production conditions, 2 DMD

V: experimental conditions: improved production conditions, butyrate addition at different time points

VI: transfection at 5x106 c/mL, no butyrate addition

APPENDIX II- Exploring Process Limits in LV Production:

Operation Windows for High Cell Density Transfection in Small and Bioreactor Scale

AII.1 INTRODUCTION

The production of viral vectors is typically performed at low cell densities only (refer to references in chapter 1 and 3). In other viral vector production systems, productivity highly depends on the physiological state of the producer cells at the time of infection (Henry, Dormond et al. 2004; Kamen and Henry 2004; Henry, Kamen et al. 2007b). This phenomenon is now commonly described as the cell density effect which was first identified during the production of adenoviral vectors (Garnier, Coté et al. 1994; Nadeau, Garnier et al. 2003). Perfusion strategies have been demonstrated to alleviate unidentified nutrient limitations and byproduct inhibitions, thereby enabling the production at higher cell densities (Henry, Kamen et al. 2007a). Similarly, routine transfection protocols for the production of recombinant proteins employ low cell densities only (Durocher, Perret et al. 2002; Derouazi, Girard et al. 2004; Pham, Kamen et al. 2006). Transfection at high cell density in perfusion mode has now also been described (Sun, Hia et al. 2008). However, for most recombinant products, production in perfusion is not economically justifiable. Perfusion processes are generally associated to a higher contamination risk, are more complex to operate, more difficult to validate because of longer process times and require dedicated facilities and skilled personnel to ensure a robust operation. In contrast, fed-batch cultures can now be considered the industry standard, mainly due to the success of monoclonal-antibody based therapeutics which are almost exclusively produced in this process mode (Wurm 2004). Consequently, production in perfusion mode is generally avoided as long as product stability and titers are sufficiently high to allow for product accumulation in the reactor over several days. In the case of LVs, however, the low stability of functional particles requires production with frequent harvest and medium replacement ((Merten 2004); refer to appendix I for LV stability data). As it is the case for other labile biologicals, such as FVIII but also for some monoclonal antibodies (Chu and Robinson 2001), continuous production in perfusion mode is therefore the strategy of choice for LVs.

In chapter 3, a perfusion strategy for the high-yield production of LV was described. Frequent harvest of LVs by medium replacement allowed operating at increased cell densities of up to $5x10^6$ c/mL, superior to what was described earlier in routine transfection protocols.

This part of the appendix will summarize our efforts undertaken to further improve LV yields in the production system. It was the goal to explore and define process limits for LV production at high cell density. The first part of the results (appendix II.3.1) presents results for LV production in small-scale experiments. It was then evaluated to what extent these process conditions could be transferred to bioreactor scale (appendix II.3.2).

AII.2 Materials and Methods

All materials and methods used in this chapter were described previously (chapter 2)

AII.3 Results and Discussion

AII.3.1 LV production in small-scale experiments

AII.3.1.1 LV Production at 1 DMD

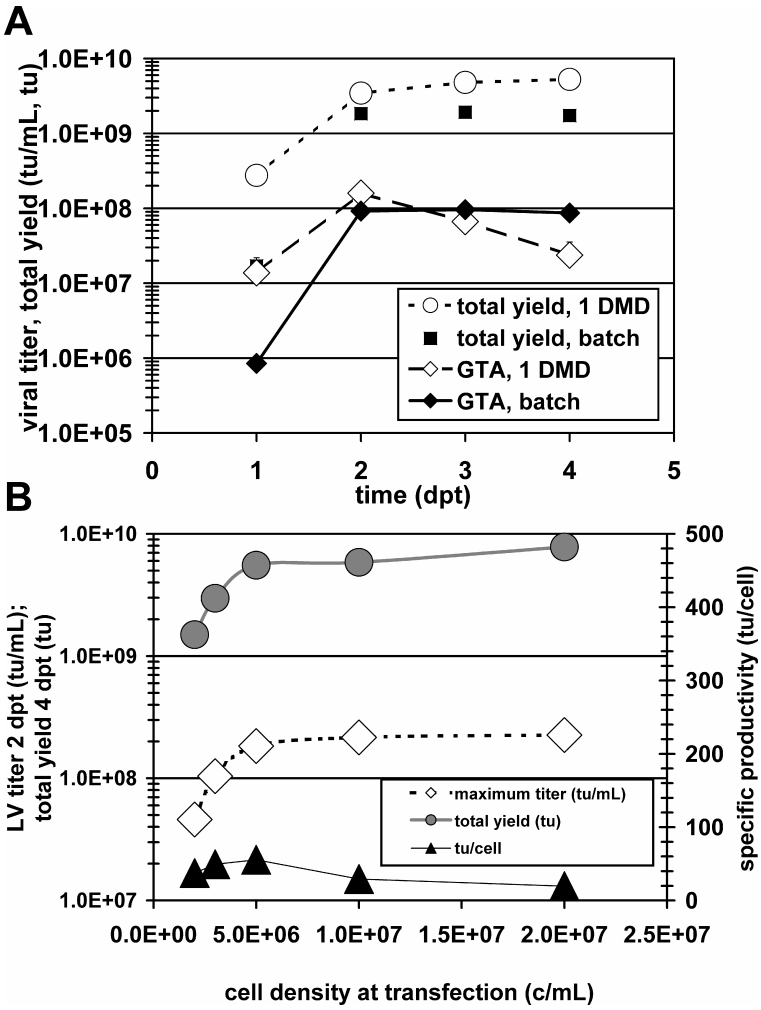

Figure AII.1: LV production in small scale at 1 discontinuous medium exchange per day (DMD).

(A) LV production at $5x10^6$ c/mL with 1 DMD leads to a total LV yield of $>5x10^9$ tu at 4 dpt (average of 3 independent experiments is shown); LV production in batch mode was performed after transfection at the same cell density of a culture taken from bioreactor run #3 (see chapter 4 and section appendix II.3.2; data shown in figure are identical to what is shown in fig. 4.1)

(B) Increase in cell density at 1 DMD; productions were performed using the same transfection (polyplex) mix for all cultures at 1 and 2 DMD, respectively, i.e. the volume of polyplex amount

varied from 4-40 % of the final culture volume; values represent data from 3 independent experiments

LV production employing improved conditions at 1 discontinuous medium exchange per day (DMD) identified in chapter 3 resulted in reproducible production kinetics with high total yields of >$5x10^9$ tu after 4 dpt per 20 mL shake flask (fig. AII.1 A). After transfection at a cell density of $5x10^6$ c/mL, the maximum functional titer was observed 2 dpt, followed by an exponential decrease thereafter. In contrast, transfection at the same cell density without subsequent medium exchange, i.e. in batch mode, gave total yields which were at least 2-3 fold lower.

Subsequently, a further increase in cell density was explored at the time of transfection (fig. AII.1 B). Cell densities of up to $2x10^7$ c/mL were tested but resulted in only slightly increased functional titers with similar kinetics at 1 DMD. At the same time, specific productivity decreased dramatically from ~60 to ~20 tu/cell (fig. AII.1 B).

AII.3.1.2 Production at 2 DMD Results in Higher Total LV Yields

The study on the stability of infectious LVs in culture supernatants revealed that their half-life is in the range of 8-13 h, a value independent of the time of harvest during production (see appendix I for details). Accordingly, the optimum harvest rate for LV should be about twice a day. It was therefore investigated if production at an increased medium exchange rate of 2 DMD resulted in higher total yields of functional LV (fig. AII.2). Operation at 2 discontinuous medium exchanges per day (DMD) was expected to result in constant functional titers (tu/mL) and increased total yield due to the doubling of the harvested volume. LV production kinetics were indeed similar, increasing the total yield to >$1x10^{10}$ tu corresponding to a specific productivity of ~120 tu/cell.

In comparison to production at 1 DMD, 2 medium exchanges per day resulted in a drop in LV titers on 3 and 4 dpt. It is possible that the discontinuous exchange of medium by centrifugation and resuspension had detrimental effects on or led to a loss in producer cells. Furthermore, in particular at cell densities >$5x10^6$ c/mL a high degree of cell clumping made it difficult to resuspend the cell pellet after centrifugation on 3 and 4 dpt (see fig. AIV.16-17 for pictures). At 1 and 2 DMD, the viable cell density increased slightly after transfection at $5x10^6$ c/mL and

reached a maximum at ~7x10^6 c/mL at 2 dpt (fig. AII.3). The cell density then decreased until the end of production at 4 dpt. Count errors for these samples were high, resulting in a high standard deviation for the average of several experiments, probably because of the observed significant cellular aggregation.

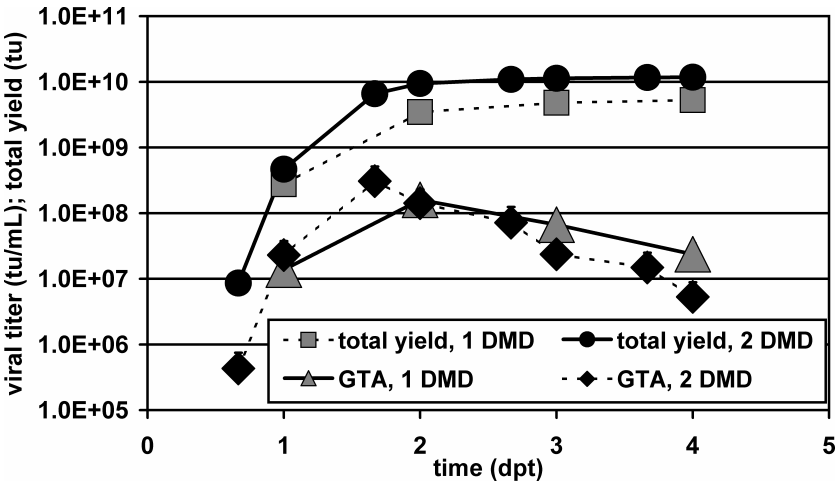

Figure AII.2: LV production using improved production conditions in small scale at 1 and 2 DMD

The values correspond to average values from n = 3 independent experiments at 1 DMD and n = 5 at 2 DMD; error bars represent the respective standard deviations.

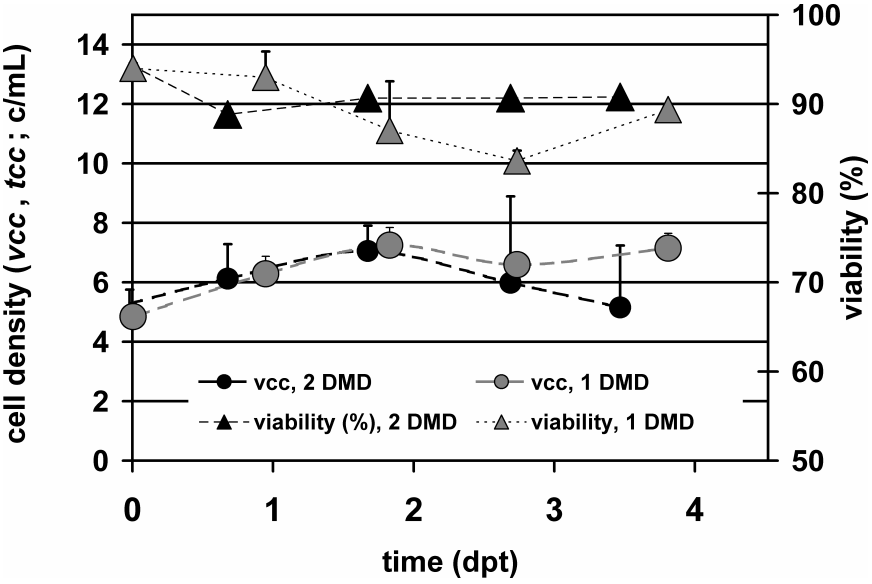

Figure AII.3: Cell density and viability during small-scale LV production at 1 and 2 DMD. Improved production conditions with transfection at $5x10^6$ c/mL were used. Error bars represent standard deviations from independent experiments (1 DMD: n = 3; 2 DMD: n = 5).

As a higher medium exchange rate also increases the nutrient supply to the culture, a cell density increase at the time of transfection was then explored at 2 DMD. Higher titers and total yields were achieved at cell densities superior to $5x10^6$ c/mL (fig AII.4 A). Yet, when increasing the cell density beyond $5x10^6$ c/mL, a decrease in specific productivity by ~30 % was observed. During these experiments, the polyplex volume varied (similar to what was previously presented in fig. AII.1 B), corresponding to 40 % of the final culture volume for transfection at $2x10^7$ c/mL.

AII.3.1.3 Effect of Medium Supplementation on LV Production and Constraints for Scalability

Previously, it was observed that the use of an enriched medium composition (HyQ) had a positive effect on LV production. It was hypothesized that this was due to an improved nutrient availability and physiological state of the culture at the time of transfection (see chapter 3, fig. 3.2). It was consequently tested if additional supply of nutrients could further improve productivity and LV yields. A commercial medium supplement (cell boost 5 (CB5 reg)), developed for fed-batch cultivations, was used in subsequent experiments. In previous work and preliminary experiments (unpublished results from our group; fig. AIV.11), it could be shown

that the addition of CB5 reg at a concentration of 5 % (v/v) significantly improved growth of the HEK293 cell line. A significant increase in proliferation rate during the post-exponential growth phase was observed, thereby reaching higher maximum cell densities in batch cultures. As supplementation of the medium with up to 10 % (v/v) of CB5 reg did not lead to a further increase in cell density, supplementation with 5 % (v/v) of CB5 reg was evaluated for LV production.

For this part of process yield optimization, the comparison of total yields and specific productivity turned out to be challenging due to the intrinsic variability of the GTA assay (see section 2.2, table 2.2). Due to the high standard deviation of this assay, it was difficult to detect effects of production conditions that resulted in differences smaller or equal to 2-fold. For subsequent experiments, an internal standard, i.e. a shake flask which was transfected using improved production conditions at 2 DMD (HyQ medium, 0.4 µg of total DNA per $1x10^6$ cells, addition of 5 mM butyrate at 16 hpt) was therefore included. In all experiments, the results were then normalized to this condition.

When CB5 reg was added post-transfection, i.e. starting with the first medium exchange at 16 hpt, the increase in yield was small compared to the regular HyQ formulation (fig. AII.4 A) Supplementation of HyQ with CB5 reg before transfection was not feasible because transfection was dramatically inhibited, resulting in no functional virus particles that were detectable by GTA after transfection (data not shown). This inhibition was overcome by using CB5 mod and the HyQ+ medium formulation (see methodology). Subsequent experiments were then performed at increasing cell densities and 2 DMD (fig. AII.4 B).

In addition, to ensure scalability of the production conditions to the bioreactor scale, the polyplex volume was subsequently kept constant for all conditions at 10 % of the final culture volume. In particular due to the specifications of the level control device, the bioreactor system used in this work allows robust operation in perfusion only in a limited culture volume range. This means that an increase in 10 % of culture volume by polyplex addition is considered as maximal if the small scale conditions should be readily scalable.

Using HyQ+ and a constant polyplex volume of 10 %, transfection at cell densities of up to $1x10^7$ c/mL resulted in higher total yields although specific productivity decreased when transfection took place at densities beyond $5x10^6$ c/mL (fig. AII.4 B).

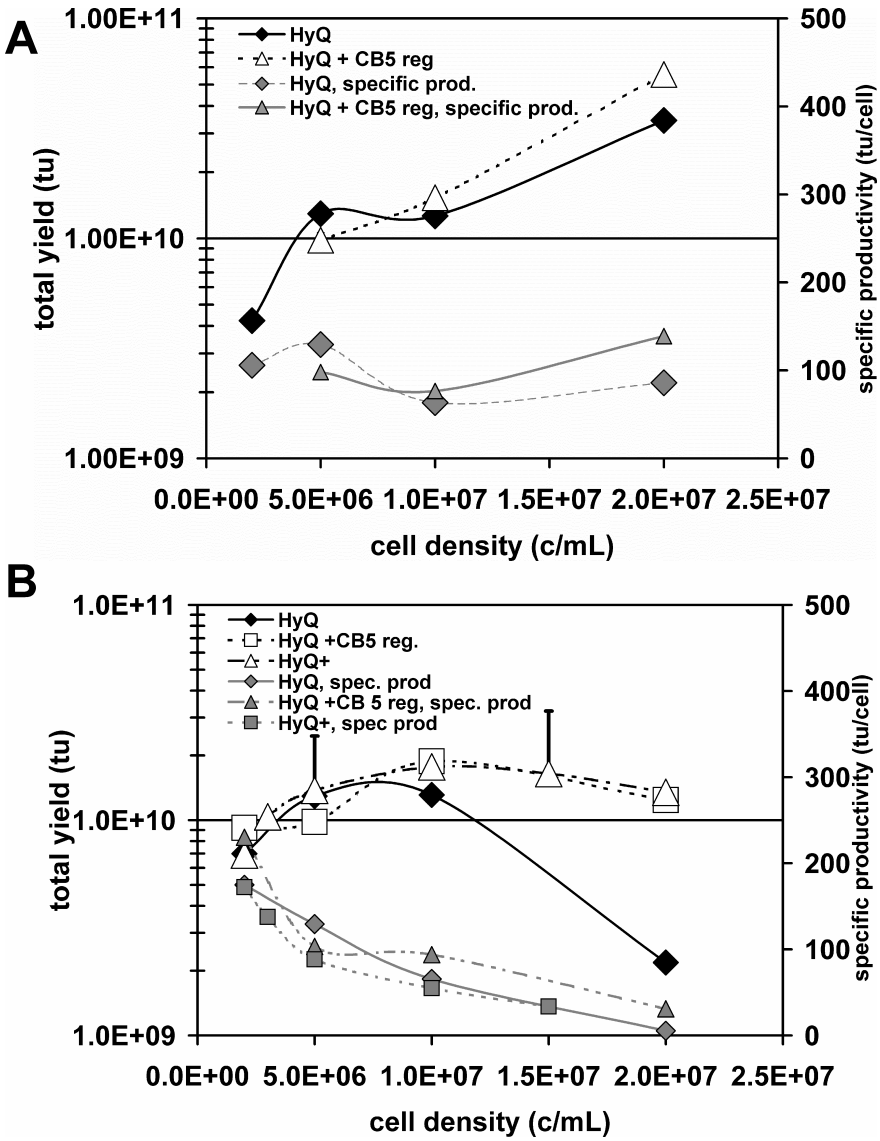

Figure AII.4: LV production in small-scale at 2 DMD is a function of the cell density at the time of transfection; CB5 reg.: regular formulation of cell boost which is inhibitory for transfection; HyQ+: HyQ medium supplemented with CB5 mod, a modified formulation of cell boost that does not inhibit transfection

(A) LV production at increased cell density at 2 DMD using variable polyplex volumes which were increased as a function of the cell density and correspondend to 4-40 % of final culture volume; CB5 reg was added only post-transfection

(B) LV production at increased cell density at DMD and constant polyplex volume (10 % of the final culture volume)

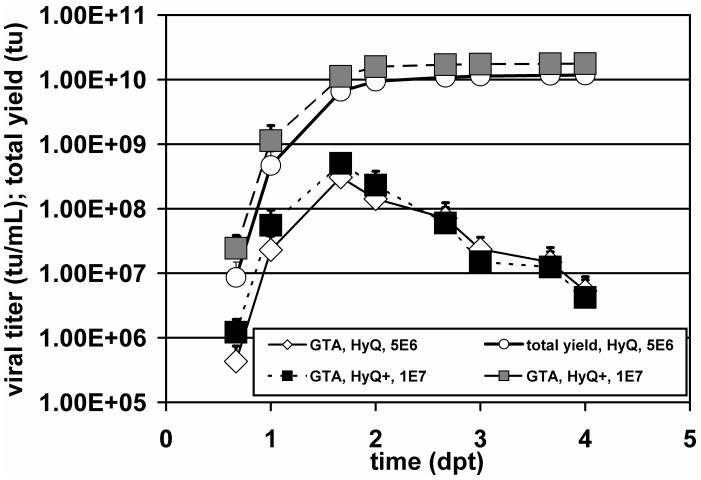

Figure AII.5: Comparison of LV production at 2 DMD in HyQ (at $5x10^6$ c/mL) and HyQ+ (at $1x10^7$ c/mL) medium

However, when comparing transfection at $1x10^7$ in HyQ+ and $5x10^6$ c/mL in HyQ, the gain in viral titer and total LV yield did not formally reach statistical significance (fig AII.5). On the one hand, this means that under these production conditions and with the described constraints, the process limit was reached. On the other hand, LV production based on transfection was reproducibly leading to functional LV titers that were superior to $1x10^8$ tu/mL in small scale, corresponding to $>1x10^{10}$ tu per experiment. The reasons for why these limitations were observed remain unknown. Two major hypotheses need to be discussed in this context.

First, it is possible that nutrient limitations were responsible for the decrease in specific productivity. Nevertheless, major nutrients (such as glucose and amino acids) were not depleted in any of the small-scale experiments. Similarly, byproducts such as lactate and ammonium were far below or at the lower limit of what has been reported as inhibiting concentrations, with the exception of lactate concentration 1 day-post transfection at 1 DMD (see fig. AIV.8).

Second, according to our results, in particular the concentration range in which polyplexes are efficiently formed seems to require a further evaluation. Strategies that use high cell densities to increase productivity after transient transfection are not often described in the literature. Transient transfection in medium that supports high transfection efficiencies at cell densities as high as evaluated here has not yet been reported. Only one study described that the culture volume in which the polyplexes are prepared does affect transfection efficiency during transfection at $1x10^7$

c/mL (Sun, Hia et al. 2008). The operation windows of these processes are generally not well defined. We note that high cell density transfection with polyplexes prepared at routine concentrations resulted in higher LV yields (fig. AII.4 A). However, the cell suspension was also significantly diluted with the addition of polyplexes. An isolated analysis of this concentration effect was therefore impossible. More work is thus needed to establish robust transfection protocols at high cell density. The present LV system, with its intrinsic constraints and variations of viral quantification methods, does not seem to be the ideal system to further investigate this problem.

With respect to scalability, one of the constraints of the present bioreactor system was that the polyplex volume could not exceed 10 % of the final culture volume. Perfusion reactors which allow for higher flexibility might therefore be advantageous for LV production.

Alternatively, direct addition of DNA to the culture, followed by PEI addition could be another option for transfection at high cell density. This method has first been described as one way to overcome inhibitory properties of culture media that are not suitable for transfection (Backliwal, Hildinger et al. 2008). Although the direct sequential addition of DNA and PEI simplifies the production protocol by avoiding *a priori* polyplex formation, large-scale reproducibility of the method remains to be demonstrated. Another major drawback of this strategy is that the associated costs would be significantly increased, as higher amounts of purified DNA are needed to achieve comparable transfection efficiencies (Backliwal, Hildinger et al. 2008, and unpublished results from our group).

AII.3.1.4 Comparison of Functional LV Yields and Direct Particle Quantification Methods

Table AII.1:LV yields from productions at high cell density ($5x10^6$ c/mL) at different operating conditions in small-scale

	Functional titer (GTA)			***VP titer (ELISA)***			***VG titer (PCR)***		
operating condition/ *parameter*	**1 DMD** [I]	**2 DMD** [II]	**2 DMD, HyQ+** [III]	**1 DMD** [IV]	**2 DMD**[IV]	**2 DMD, HyQ +** [IV]	**1 DMD**[V]	**2 DMD**[VI]	**2 DMD, HyQ +**
maximum titer (LV/mL)	$1.6x10^8$	$3.1x10^8$	$6x10^8$	$2x10^{10}$	$9x10^9$	$1.4x10^{10}$	$4.5x10^9$	$4.2x10^9$ [VII]	n.a.
Total yield (LV)	6.8 +/- 1.6 $x10^9$	1.17 +/- 0.75 $x10^{10}$	2.14 +/-1.08 $x10^{10}$	$1x10^{12}$	$6.4x10^{11}$	$1.1x10^{12}$	1.8 +/-0.6 $x10^{11}$	3 +/- 0.45 $x10^{11}$	n.a.
Spec. prod. (tu/cell)	70 +/-16	117 +/- 77	214 +/- 108	10300	6400	11000	1800 +/- 600	3000 +/- 450	n.a.

n.a.: results not available

I: average values from n = 3 experiments

II: average values from n = 6 experiments

III: average values of n = 2 experiments

IV: values were based on a small number of data points; VP titers for 1 dpt were not available

V: average values from n = 3 experiments

VI: average values from n = 2 experiments

VII: constant values from 2-5 dpt

Table AII.1 summarizes the results for LV yields as assessed by direct (GTA) and indirect (CAp24 ELISA, PCR) quantification methods. An increase in specific productivity of functional LVs was found as result of an increased medium exchange rate (from 1 to 2 DMD) but also the improvement of nutrient availability (from 2 DMD (HyQ) to 2 DMD (HyQ+). VG titer yields were higher at 2 DMD while there was a decrease in VP titer from 1 to 2 DMD. The number of experiments for which ELISA results were available was, however, too low to allow for a final conclusion on the differences in VP titers. In general, the improvements in titer were lower than what was observed during the first part of process improvement (chapter 3). As a consequence, some of the modification in production conditions did not formally reach statistical significant titer differences.

In summary, higher cell densities at the time of transfection resulted in increased total LV yields. Beyond $1x10^7$ c/mL, however, no increase in LV titer was observed when transfection was performed in readily scalable conditions at 2 DMD (constant polyplex volume at 10 %). This observation seems to correspond to previous results for other systems (cell density effect) (Garnier, Côté et al. 1994; Nadeau, Garnier et al. 2003; Henry, Kamen et al. 2007). Nevertheless, in the present production system, an advanced medium formulation combined with a high medium exchange rate for LV harvest allows operating at significantly increased cell density. Our results do not allow to infer the reasons behind the limitations in production yield. LV production might be either limited by lower transfection efficiencies at densities beyond $1x10^7$ c/mL and/or unidentified nutrient limitations and inhibitions.

The operation window for efficient LV production was thus defined to be in the range of 5-$10x10^6$ c/mL at the time of transfection. This value served as starting point for the transfer of the production conditions to bioreactor scale.

AII.3.2 Transfer of LV production to bioreactor scale

The second part of this section presents the analysis of LV production in bioreactor cultures. Based on batch growth results (appendix III), it was in general concluded for the subsequent LV productions in bioreactor scale that initial batch growth should not be extended beyond a cell density of ~$2x10^6$ c/mL. Growth in perfusion mode should then ensure that the culture remains in its exponential growth phase and in a favourable metabolic state before transfection. In chapter 3, the scalability of production conditions has already been demonstrated by two bioreactor perfusion cultures (using starting and improved production conditions corresponding, respectively, to run #2 and #3). Here, it was sought to further characterize LV production in bioreactor scale by comparing total yields from small and bioreactor scale experiments as well as changes in growth and metabolic rates.

In addition to previously discussed data, LV production in bioreactor scale employing improved production conditions was evaluated in a total of three perfusion cultures (see table AII.2 for operating and production conditions).

AII.3.2.1 Comparison of Yields from Small and Bioreactor Scale Experiments

When comparing the LV yields from small-scale and bioreactor experiments, it was found that yields from all bioreactor experiments were lower (table AII.2 B). This finding was already briefly discussed for run #3 in chapter 3 (section 3.2.5). Taking the volume difference to shake flask cultures (20 mL) into account, bioreactor cultures were expected to generate 1-$3x10^{12}$ tu per culture. In contrast, although all bioreactor cultures gave consistently high total LV yields (>$3x10^{11}$ tu), the total functional titers were 2.5-10 fold lower than in the comparable small scale conditions. On the other hand, the VP and VG titer yields were closer to the values observed in small-scale and increased with each improvement in production conditions from run #2-5 (fig. AII.5).

Table AII.2: Comparison of bioreactor runs conducted in this work

(A) Experimental conditions for bioreactor scale cultures in which HyQ medium was used

(B) LV yields from bioreactor cultures and comparison to corresponding small scale experiments

A **Culture**	**Operation (mode, perfusion rate)**	**Transfection at cell density of (c/mL), after cultivation time of (h)**	**Growth rate[I] (μ(*vcc*), μ(perm); (batch, pre-transfection) (d^{-1})**	**Medium, Remarks**
Run #1	batch	- , -	0.67[II] +/- 0.18; 0.6 +/- 0.15	HyQ, no transfection; batch culture; see appendix III for process evolution
Run #3	perfusion, 1 VVD	$4.4x10^6$, 96	0.73 +/- 0.07, 0.36 +/- 0.03; 0.69 +/- 0.16, 0.42 +/- 0.16	HyQ, process kinetics shown in chapter 3 (fig. 3.5) and 4 (fig. 4.2-4 B and 4.5 (1 VVD))
Run #4	perfusion, 2 VVD	$4.7x10^6$, 96.5	0.72 +/- 0.08, 0.34 +/- 0.05; 0.83 +/- 0.16, 0.56 +/- 0.15	HyQ, see chapter 4 (fig. 4.2-4 B and 4.5 (2 VVD) for process kinetics
Run #5	perfusion, 2 VVD	$8x10^6$, 120.5	0.61 +/- 0.04, 0.55 +/- 0.09; 0.74 +/- 0.08, 0.56+/- 0.08	HyQ+ (medium composition included 5 % (v/v) of supplement mixture), see chapter 5 for kinetics

Refer to chapters 3 and appendix II.2 for the identification of production conditions

I: average values in d^{-1}; pre-transfection refers to phase of process after perfusion start and before transfection

II: average value for exponential growth phase only (in batch culture)

One bioreactor culture performed in starting conditions using medium composition LC-SFM L (run #2) is not shown in the table; refer to fig. AII.5, AIV.6, as well as chapter 3, fig. 3.4 for functional titer kinetics

B	***Functional titer (GTA)***			***VP titer (ELISA)***			***VG titer (PCR)***		
Operating condition/ *Parameter*	**1 VVD, run #3**	**2 VVD, run #4**	**2 VVD, HyQ+, run #5**	**1 VVD, run #3[III]**	**2 VVD, run #4**	**2 VVD, HyQ+, run #5**	**1 VVD, run #3**	**2 VVD, run #4**	**2 VVD, HyQ+, run #5**
Maximum titer (LV/mL)	$7.7x10^{7}$	$3.0x10^{7}$	$3.1x10^{7}$	$2.2\ x10^{10}$	$2.85x10^{9}$	n.a.	$5.61x10^{9}$	$3.7x10^{9}$	$4.6x10^{9}$
Total yield	$3.6x10^{11}$	$4.25x10^{11}$	$3x10^{11}$	$1.4x10^{14}$	$2.4x10^{13}$	n.a.	$2.3x10^{13}$	$5x10^{13}$	$8x10^{13}$
Spec prod (tu/cell)	30	33	14	11500	1900	n.a.	2000	3900	3600
Relative to total expected yield (%) [IV]	38.5	26.5	10.2	100.2	27.3	n.a.	93	121	n.a.

III: ELISA results possibly overestimated but from same assay as values for run #3

IV: based on total yields from small scale experiments

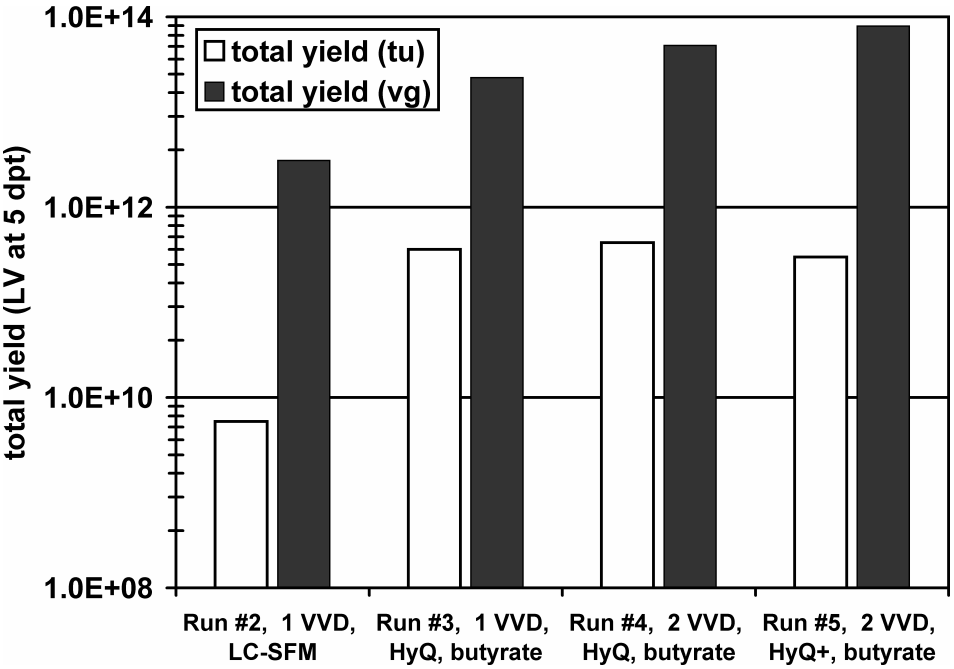

Figure AII.5: Functional (tu) and total particle (vg) yield from bioreactor scale LV production runs

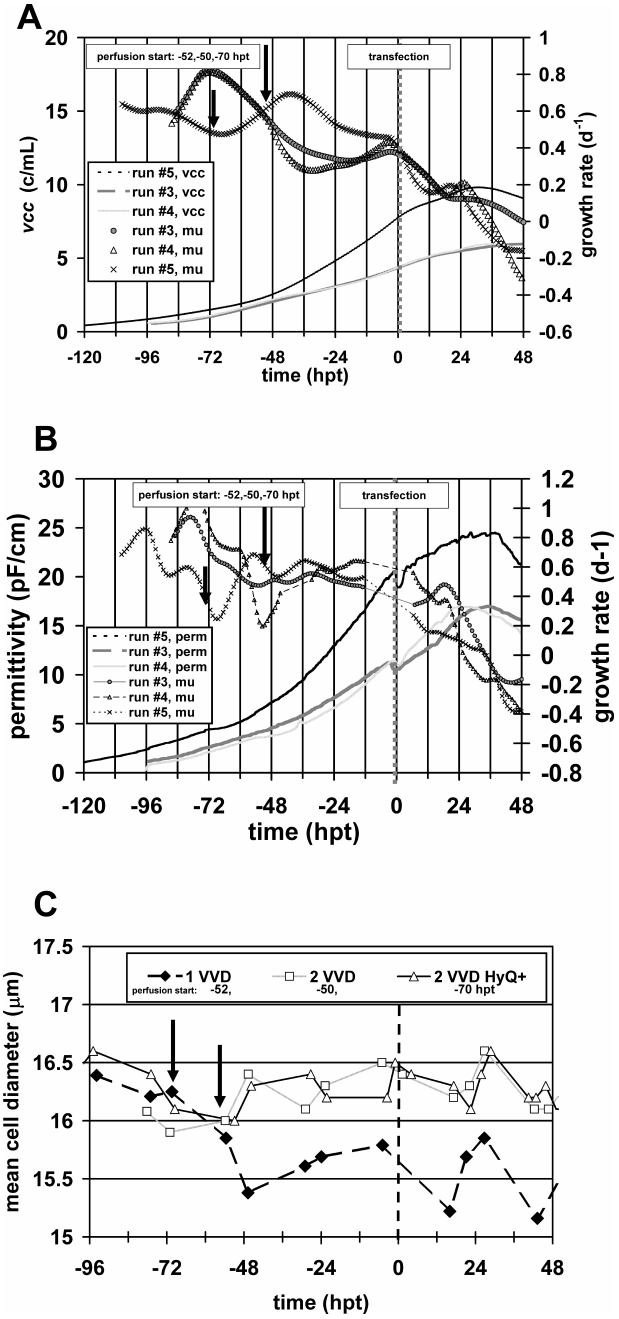

Figure AII.6: Viable cell density, permittivity, cell diameter and growth rates during LV production in bioreactor scale perfusion cultures. Values for three independent cultures are shown (see runs #3-5 in table AII.2). None of the values (cell counts, growth rates, permittivity) were corrected for loss of cells when perfusion was started.

(A) growth rate and *vcc*
(B) growth rate (based on biovolume)
(C) mean cell diameter

AII.3.2.2 Growth rates and evolution in cell diameter

The observation that all bioreactor cultures gave lower functional yields than expected based on small-scale results suggested that there was a systematic reason behind this finding. In contrast to small-scale cultures, in which producer cells were concentrated in fresh medium before transfection, the cultures were in bioreactor conditions were grown in batch and perfusion mode (at the targeted LV harvest rate (1 or 2 VVD)) to the targeted cell density without discontinuous medium exchange (see chapter 2). It can thus be assumed that nutrient availability and byproduct concentrations differed at the time of transfection. The importance of the cell's physiological state at the time of infection during viral vector production has been demonstrated by others (Henry, Dormond et al. 2004; Henry, Kamen et al. 2007). The same authors also reported increased metabolic rates during the viral vector production phase.

We therefore analyzed growth and, as the continuous medium exchange allowed for a reliable calculation, the metabolic rates in the bioreactor cultures. The goal was to identify potential limitations in the current process and propose strategies for further improvement of bioreactor scale LV production.

Similar values were found for the average growth rate calculated using offline cell counts (μ(*vcc*) or the online permittivity signal μ(perm) in perfusion mode (table AII.2). The apparent growth rates during perfusion were in all cases lower than during initial batch growth (table AII.2, fig. AII.6), which was partly due to a small loss of cells during the onset of perfusion. We also note that cell size was larger compared to batch cultures after perfusion was started and cell diameter thus remained rather constant during the perfusion phase (compare to batch culture in appendix III, fig. AIII.1 A). The cell size increase was also reflected in higher growth rates based on the permittivity signal (μ(perm)) compared to μ(*vcc*).

During two of the three perfusion cultures (run #3 and #4), the growth rates (μ(*vcc*) and μ(perm) reached their maximum (of ~0.8 d^{-1} and 1 d^{-1}, respectively) during batch growth (fig. AII.6). The situation was similar for (μ(perm) of run # 5 while the maximum in μ(*vcc*) was reached here after perfusion was started. (μ(perm) remained almost constant in the pre-transfection phase in all cultures (~0.5-0.6 d^{-1}) while a local maximum was in all cases reached for μ(*vcc*) after the perfusion start. At the time of transfection, μ(*vcc*) was in all cases at around 0.4-0.5 d^{-1}, corresponding to the values found at the end of exponential growth phase in batch cultures (see

appendix III, fig. AIII.3). All cultures were, except run #5, transfected at the maximum in growth rate in perfusion mode (μ(*vcc*)).

Although operated at different medium exchange rates, run #3 and 4 were showing very similar growth rate profiles. Nevertheless, differences in the cell size evolution were observed between the two cultures (fig. AII.6 C). In addition, more cells were lost in the harvest during the start of perfusion of run #4 resulting in a drop in growth rate from -60-50 hpt. Run # 5 was showing a very similar cell size evolution than run #4, with a cell diameter increase after perfusion start and subsequent constant values before transfection. The evolution of metabolic rates was then analyzed during the bioreactor cultures. Because of the observed differences in cell diameter, i.e. the increase in cell size when perfusion was started at 2 VVD, those rates were calculated based on the permittivity (qperm; refer to chapter 5 for qperm values in batch mode).

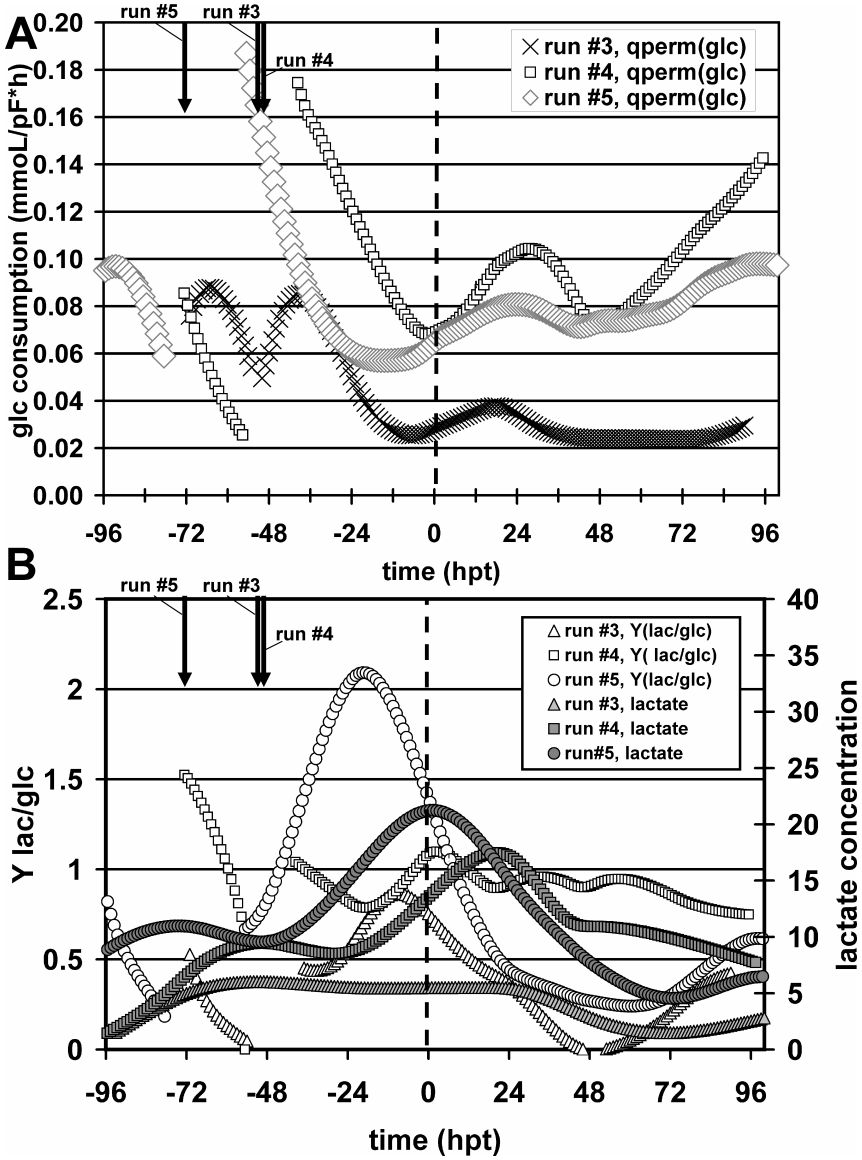

Figure AII.7: Metabolic rates for LV production in bioreactor cultures; arrows indicate the time of perfusion start for runs #3-5; dashed lines mark the time of transfection

(A) glucose consumption rate
(B) molar ratio lactate/glucose and lactate concentration

During the batch growth phase, the glucose consumption rate was during all three cultures at ~0.08- 0.1 mmol*cm/pF (corresponding to 0.2-0.3 μmol/10^6 cells*h) during all three cultures (fig. AII.7). Those values were similar to what was observed in batch cultures (see appendix III, table AIII.1). After the perfusion start, the specific glucose consumption rate (qperm (glc))

increased by almost 2-fold at 2 VVD while similar values compared to batch growth were observed during run #3 (at 1 VVD). At the time of transfection, qperm (glc) had then decreased to ~0.07 mmol*cm/pF (runs # 4 and 5) while it was significantly lower at 1 VVD (0.03 mmol*cm/pF). After transfection, a maximum was then reached at 16-30 hpt in all three cultures.

At 1 VVD, the lactate concentration during the complete culture remained at low levels with a maximum at ~5 mM (fig. AII.7 B). Much higher concentration were reached at 2 VVD, with maxima at 15 (run #4) and ~20 mM (run #5).

As the specific production rates of lactate were showing different kinetics than glucose consumption, the molar ratios of lactate and glucose Y(lac/glc) varied significantly during the three cultures. Maxima in the molar ratio were observed either before (runs # 3 and 5) or at around the time of transfection (run #4). For run #3 and 5, minima in the molar ratio were observed at around 48 hpt while run #4 was showing an almost constant value from 24- 72 hpt. Y(lac/glc) was in all cultures lower after transfection than in the growth phase, indicating a more efficient metabolism with less lactate production from glucose. Overall similar observations were made for all cultures for the metabolic rates and ratios of glutamine and ammonium (results not shown).

In summary, the metabolic profile of run #3 suggests that perfusion was started after the beginning of the metabolic shift (compare chapter 5) because lactate production ceased before the perfusion start. This was confirmed by the decrease in cell diameter during batch growth that was indicating reduced metabolic activity in batch cultures (compare appendix III, fig. AIII.1). We also note that the response to the increased glucose concentration (due to the start of perfusion) was lower in that culture. On the other hand, perfusion start for run #4 and 5 took place before the metabolic shift with Y(lac/glc) at positive values of 0.6 and 0.2, respectively. In these two cultures, a significant response (increase in qperm(glc)) was observed after perfusion start. Yet, during run #4 and 5, lactate concentrations of 15-20 mM were reached. This means that in particular for the case of run #5, this concentration could have been inhibiting before transfection. Only in run #3, the metabolic rates were low compared to initial batch growth at the time of transfection. In the two subsequent cultures (run #4 and 5), the rates were similar to maximum values during batch growth. If the observations from batch cultures are directly applicable to these two cultivations in perfusion mode, online (*fc*) and offline monitoring (cell size) indicated

constant metabolic activity after the perfusion start as both values remained unchanged in the pre-transfection phase (see chapter 5 for a further discussion on the relationship of these variables in HEK293 cell cultures).

AII.4 Overall Summary, Discussion and Recommendations

In small-scale experiments, maximum functional LV titers and total yields were found after increase of the cell density at transfection to 5-10x10^6 and 2 DMD with HyQ or HyQ+ medium. The cell suspension was concentrated by centrifugation and precultures were resuspended in fresh medium before transfection. After transfection, none of the analyzed nutrients (glucose, amino acids) was limiting in these conditions and byproduct concentrations (ammonium, lactate) were below inhibiting concentrations. A further increase in cell density did not result in higher LV yields. The reasons for the limitations of production beyond 1x10^7 c/mL remain unknown, with transfection efficiency or (unidentified) nutrient limitations or inhibitions as possible explanations.

In bioreactor scale, cells were initially grown in batch mode and perfusion was used to reach the targeted cell density without discontinuous medium exchange or concentration. It was generally expected that continuous and controlled process conditions in perfusion would result in higher LV yields. The total functional yields from these experiments were, however, 2.5-10 fold lower than in the comparable small-scale conditions. In contrast to small-scale LV production, specific productivity decreased with ‘improvement’ in production conditions from run #3-5. This was suggestive for systematic metabolic limitations or inhibitions that occurred at high cell density.

Comparing small-scale and bioreactor cultures, we noted first that specific growth rates were similar during batch growth in both cases (fig. AIII.3 and supplementary figure AIII.1/AIV.12). The concentrations of byproducts were significantly lower during small-scale LV production compared to the bioreactor cultures (continuous vs discontinuous medium exchange). Whereas no depletions of nutrients could be identified in small-scale, the continuous operation in bioreactors allowed the calculation of specific metabolic rates. Interestingly, a local maximum in glucose consumption rate was observed after transfection and at around the time of the first viral release, indicating increased metabolic acitivity of the culture at that time. As they were observed for qperm(glc), these changes were not related to increases in cell size but seemed to be rather

attributed to production of LV-related proteins. This should be further investigated to uncover the specific requirements of cells producing LVs.

It was also assumed that the metabolic rates are indicative of the physiological state of the cultures at the time of transfection. Only for the production at 1 VVD (run #3), a significantly reduced metabolic activity was observed in comparison to batch cultures. In contrast, no significant decrease in metabolic activity was observed at increased medium exchange rates of 2 VVD. It is thus hypothesized that the lower LV yields in bioreactor scale were due to a combination of byproduct inhibition (at 2 VVD) and a lower nutrient availability (at 1 VVD).

A straightforward explanation for why lower titers were found in bioreactor scale remained nevertheless difficult. In other studies, extracellular accumulation of byproducts and factors that would down regulate the overall metabolic activity was put forward as limitation for cultivation at high cell densities (Carinhas et al.). It is consequently concluded that further optimization of LV production in bioreactor scale, based on the analysis of HEK293 metabolism, is required.

To that end, our results can provide general recommendations for the development of high-yield HEK293 perfusion processes at high cell density in the current medium formulation (HyQ/HyQ+):

1. a rational approach is needed to improve the understanding on the impact of physiological state of the cell line on LV yields after transfection; this should be ideally done employing a less complex system for which quantification methods with a lower intrinsic variability are available (e.g. production of GFP or secreted alkaline phosphatase (SEAP) only)
2. in the present work, small-scale conditions (including the concentration of cells before transfection) were set to ease the practical execution of experiments and increase experimental throughput; however, the resulting differences in production strategy, i.e. concentration in small-scale and continuous cultivation of cells in bioreactor perfusion made a direct translation of production conditions (at constant productivity) difficult
3. the perfusion start at a high medium exchange rate of 1 or 2 VVD is possibly non-optimal and leads to high byproduct concentrations; lower medium exchange rates should be tested in small-scale or (see 4.)
4. a reduction in glucose concentration in the feed could be tested to avoid high glucose concentrations and high lactate production before transfection

5. the findings from chapter 5 concerning the online monitoring of the metabolic activity could be helpful during the development of improved process strategies

Beyond the observations concerning the physiological state of the cultures, further work is needed to establish robust transient transfection protocols for production at high cell density and in bioreactor scale. Several independent experiments (n>10) resulted in consistently high LV titers and total yields. Using improved production conditions, a maximum functional titer of $>1x10^8$ tu/mL with total yields of $0.5\text{-}2x10^{10}$ tu was found in all experiments. Yet, it could not be excluded that transfection efficiency at high cell density was a constraint when cell densities were higher than $1x10^7$ c/mL in small-scale. In addition, the number of cultures was limited in bioreactor scale and inter-experimental variations in the transfection process might have contributed to yield differences from the different cultures. It is generally possible that transfection in bioreactors and at cell densities beyond $1x10^7$ c/mL is consistently less efficient than in small-scale or routine conditions. On the other hand, we exclude events associated to the distribution of polyplexes in the reactor or inactivation of virus by bioreactor-specific cultivation conditions (such as the acoustic filter or agitation), as shake flask controls, which were taken from the bioreactor before/after transfection and transfected outside/inside the reactor with the identical polyplex mix, resulted in similar LV titers than the respective mother culture (data not shown). For a further rational optimization of high-cell density transfection, several challenges will need to be addressed:

1. whereas the preparation in small-scale appears to be highly reproducible, mixing of large volumes for bioreactor cultures (in particular when larger scales than studied here are targeted) needs to be evaluated for its consistency; ideally, this would include the use of well-defined conditions in a stirred external unit (in contrast to formation of polyplexes by manual shaking as performed here)
2. the optimum concentrations at which polyplexes are formed needs to be evaluated; this is of particular importance for cultures that are transfected at cell densities beyond $1x10^7$ c/mL

3. the identification of a suitable method for evaluation of polyplex quality would thus be of great value (for 1. and 2.); one of the most promising parameters to evaluate and which should be also easily accessible is polyplex size (Guillem et al. 2004)
4. transfection of high cell density cultures according to the ‘direct method’ (direct addition of DNA followed by PEI without *a priori* polyplex formation) would circumvent several of these challenges and should consequently be evaluated

AII.4 Final Remarks and Conclusion

It is concluded that the developed process for LV production is readily scalable (see chapters 1 and 3 for a further discussion on the subject). For a further improvement in process yields, optimization of bioreactor production is, however, needed. This should comprise further analysis of the HEK293 cell metabolism in HyQ medium and optimization of high cell density transfection.

APPENDIX III: Practical Applications of Online Permittivity Signals: Characterization of HEK293 Batch Growth and Identification of Metabolic Shifts

This chapter presents the characterization of HEK293 growth in batch cultures and introduces permittivity measurements as an online monitoring tool. It is presented as a draft manuscript for submission as a short communication in a peer reviewed scientific journal.

Following FDA's Process Analytical Technology (PAT) initiative, real-time monitoring tools gained considerable momentum within the biotech community in recent years (Read, Park et al. 2010). Evidently, identification and evaluation of suitable PAT tools are the first step towards a successful application of this strategy which pursues the goal to develop well-defined and controlled processes that deliver products of predefined quality. Ideal PAT tools provide macroscopic data at-line, i.e. in the case of bioprocessing use *in situ* online probes.

Within this section, the growth of the producer cell line HEK293SF (clone 3F6) is characterized in preliminary batch experiments using online permittivity measurements. This cell line is used with the goal to produce LVs by transient transfection throughout the entire work related to this thesis. As the HyQ medium formulation allowed attaining high LV yields, this composition was chosen to characterize bioreactor batch cultivations employing the same methods later used to analyze the cultures during LV production. We first point out the practical value of permittivity measurements in batch cultivations and conclude that the technology provides information on the biovolume content and, at the same time, the physiological state of the HEK 293 cell cultures.

In this context, the experiments presented here had the following specific objectives:

In contrast to LVs, most of the viral vectors, vaccines and proteins produced in HEK293 have a sufficient stability to favour production in batch or fed-batch mode. Fed batch mode has been reported to dramatically increase yields after transient transfection (Sun, Goh et al. 2006). Feeding strategies for the HEK293 system thus need to be developed to reach higher cell densities and improve volumetric productivity. As a starting point for rational process and feeding strategy optimization in future work, a detailed characterization of the producer cell metabolism is hence needed. Recent literature reports demonstrate that the permittivity signal is a tool that can be used beyond the simple estimation of biomass as it also reflects the physiological

state of cell cultures (Ansorge, Esteban et al., 2010 a and b). As an online tool indicating the cellular state in real-time would largely facilitate the development of feeding strategies, it was explored if growth and metabolic rates are reflected in the online permittivity signal. More specifically, we assessed if there is a link between cellular metabolism and the online permittivity parameters.

In addition, the offline measurement method for the analysis of the permittivity signal needed to be validated. The goal was to use this method for comparison and analysis of the permittivity signal during LV production to monitor viral production kinetics (chapter 5).

Finally, for the establishment of an efficient process for LV production in perfusion mode, it was fundamental to characterize growth and metabolic behaviour of the producer cell line in batch cultures. As perfusion is in this work primarily used to harvest LV, the main parameter that needed to be determined was the cell density at which perfusion needs to be started to ensure exponential growth until the time of transfection.

The bioreactor batch cultivation presented in this chapter corresponds to run #1 in appendix II.

It needs to be noted that this part of the appendix contains only a shortened section on the background on permittivity measurements to avoid repetition with chapters 2 and 5. A final version of this draft manuscript will contain a short paragraph on the theory behind permittivity measurements (similar to section 5.2.3.5).

PRACTICAL APPLICATIONS OF ONLINE PERMITTIVITY SIGNALS: CHARACTERIZATION OF HEK293 BATCH GROWTH AND IDENTIFICATION OF METABOLIC SHIFTS

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AIII.1 Abstract

Permittivity measurements are becoming a routine tool for cell culture process monitoring. Their advanced use for process control is, however, still limited by a lack of understanding of the complex information reflected in the permittivity-related parameters. Permittivity measurements monitor the evolution of biovolume content in real-time and can in parallel also reflect the metabolic activity of mammalian cells.

To identify how permittivity measurements could be practically used, the technology was evaluated for the characterization of HEK293 in bioreactor batch cultivations. The analysis of batch growth gave insights on what information can be obtained from the permittivity signals. The dual-frequency permittivity signal and the dielectric increment correlated well with the offline measured biovolume (from offline cell counts and cell diameter measurements). In addition, the characteristic frequency was correlated with the decrease in metabolic activity and its online signal could be used for the early identification of a metabolic shift at the end of exponential growth. The technology should thus be considered as a monitoring tool to support the design and implementation of improved feeding strategies.

AIII.2 Introduction

Routine cell culture experiments and production runs include sampling from the bioreactor once a day only. Parameters that are then commonly measured offline comprise cell density, viability and metabolite concentrations. This significantly prolongs the response time to process variations and events. Process-related actions, such as feed additions, are therefore mostly scheduled in a strictly time-based manner. This also holds for most industrial fed-batch cultivations and can be also explained by the fact that current real-time sensor technology does not allow uncovering the intracellular state of cell cultures (Henry et al. 2007b). Rational, i.e. process/event-based control strategies are thus challenging. Online monitoring offers a virtually unlimited sample number and provides a continuous signal that reflects the status of the bioprocess. This holds great advantages compared to classical offline sampling. It can be assumed that an approach based on the continuous signal from one or, ideally, several combined online monitoring measurements on when to perform an action critical for the production will result in a more robust and reproducible process. Efficient monitoring techniques have therefore been described as instrumental for the rapid development and optimization of bioprocesses (Kamen et al. 2004; Lecina et al. 2006; Henry et al. 2007b). In that context, in particular the direct online monitoring of the biomass content and the metabolic activity would allow for early fault detection of cultures and improved process control strategies.

In part due to the renewed interest in process analytical technology (PAT), online *in situ* permittivity measurements are on the way to become a routine tool to monitor cell culture processes. The technology holds particular promise for the monitoring and characterization of viral vector production (Zeiser et al. 1999; Zeiser et al. 2000; Negrete et al. 2007). It has also been reported that permittivity measurements indicate the metabolic activity of mammalian cells, in particular when physiological changes occur during the cultivation (Noll et al. 1998; Cannizzaro et al. 2003; Ansorge et al. 2010a). An advanced exploitation of the signal, beyond a basic descriptive monitoring of cultures is, however, still limited by a lack of understanding of the complex information reflected in the permittivity-related parameters. This can, in part, be explained by the difficulty of the assessment of the cellular dielectric properties by routine cell culture methods.

To that end, the significance of permittivity measurements was evaluated as a monitoring tool in batch cultures of HEK293 cells to identify the practical value of the technology beyond simple biomass estimation. The suspension-grown HEK 293 cell line, in combination with HyQ medium, has already demonstrated promising potential for the high-yield production of recombinant proteins and viral vectors by transient transfection (Durocher et al. 2007; Tom et al. 2007; Ansorge et al. 2009). Other viral vectors and viruses, such as AdV and influenza, can also be efficiently generated using this platform (unpublished results from our group, (Le Ru et al. 2010). The transfer to further systems and subsequent process optimization to further increase yields is therefore required. Real-time monitoring tools indicating metabolic activity online would facilitate process development (e.g. feeding strategies). It was thus of particular interest to investigate if changes in cellular metabolism are reflected in the online permittivity parameters.

AIII.3 Materials and Methods

AIII.3.1 Cell Culture

HEK293SF-3F6 cells were grown in suspension in HyQSFM4TransFx293 medium (HyQ) (Hyclone, Logan, UT). Subcultures were passaged every 2-3 days to keep the cells in their exponential growth phase. Hemacytometer counts using erythrosine B dye exclusion were used to assess cellular density (viable, total cell count per mL (*vcc*, *tcc*)) and viability.

AIII.3.2 Cell Culture Sample Analysis

The volume weighted arithmetic mean cell diameter (*d*) was determined using a Z2™ Coulter Counter® (Beckman Coulter, Mississauga, ON) with an aperture diameter of 100 µm, followed by analysis of the size distributions with the Accucomp® software package (Beckman Coulter). The upper and lower analysis limits were 7.31 and 23.8 µm, respectively. To calculate *d*, the distributions were plotted as volume (%) against cell diameter (µm) and only the part of the distribution corresponding to viable cells was considered for the calculation.

Each value of *d* represents the average of at least two distributions. Using *d* and the viable cell count per mL (*vcc*), the biovolume (*bv)* was calculated using equation AIII.1, assuming that the distributions were evenly scattered:

$$bv = \frac{4}{3} \times \left(\frac{d}{2} \right)^3 \times \pi \times vcc \qquad \text{(AIII.1)}$$

bv was then considered as an estimate of the volume fraction of cells *P* (equation 5.2 in section 5.3.5).

AIII.3.3 Bioreactor Setup

The bioreactor setup was identical to the one previously described (Ansorge et al. 2009). In brief, cultures were performed in in a controlled 3.5 L Chemap CF-3000 bioreactor (Mannedorf, Switzerland). Agitation rate was set to 85 rpm, pH was controlled in the range of 7.1-7.2 by addition of CO_2 via the surface or sodium bicarbonate addition. The vessel (working volume 2.7 L) was equipped with probes to measure and control pH, DO, temperature. Permittivity was acquired with a Biomass 400 system (Fogale nanotech, Nîmes, France). The DO was controlled at 40 % of air saturation by sparging pure oxygen in pulse mode.

AIII.3.4 Permittivity Measurements

In this study, a Biomass 400 system (Fogale nanotech, Nîmes, France) was employed for the measurement of permittivity across the β-dispersion frequency spectrum of ~0.1-10 MHz. The system measures the dual-frequency permittivity signal $\Delta\varepsilon_{FC}$ at 1 and 10 MHz and additionally provides the β-dispersion parameters ($\Delta\varepsilon_{max}$, *fc*, α) in real-time after fitting the frequency spectra.

AIII.3.5 Calculation of Growth and Metabolic Rates

Growth and metabolic rates were calculated using standard equations (Henry et al. 2004) after filtering of the raw data (online and offline measurements). Offline raw data were fitted using the smoothing spline regression function of the kyplot software. Online data were treated with Matlab (The MathWorks, Natick, MA). The calculation or specific rates was based either on the viable cell count (vcc) or the online dual-frequency permittivity signal ($\Delta\varepsilon_{FC}$).

AIII.4 Results and Discussion

AIII.4.1 Characterization of HEK293 Batch Growth

When HEK293 were grown in bioreactor batch cultures, the exponential growth phase started after a short lag phase, lasting for ~90 h until a viable cell count (*vcc*) of ~ 2.5-3x10^6 c/mL was reached (fig AIII.1 A; see also table AIII.1). Cell growth continued subsequently at a reduced rate and a slightly lower viability over a period of 125 h until death phase started and viability decreased rapidly. The maximum *vcc* was reached at 1.3x10^7 c/mL after 215 h of culture. The

cell diameter (*d*) remained almost constant during exponential growth and then decreased significantly from 16.5 to 13.5/14.0 µm during the post-exponential growth and death phase.

The concentrations of metabolites and byproducts (glutamine, glucose, lactate, ammonium) for this batch cultivation are shown in figure AIII.1 A. Glucose and glutamine concentrations decreased during exponential growth phase, until a slope change in their consumption was observed at residual concentrations of 22 mM and 1.5 mM (glucose and glutamine, respectively). Both metabolites were then further consumed until glucose was depleted at ~220 h whereas the concentration of glutamine stabilized at ~0.6 mM. The concentrations of the byproducts lactate and ammonium increased until around 100 h after incubation. Subsequently, the concentration of ammonium decreased from 1.6-1 mM before it slightly increased towards the end of the cultivation. Lactate concentration levelled/reached a plateau for 2-3 days (95-160 h) at 18 mM after the end of the exponential growth phase, further increased up to 26 mM and finally decreased during the last two days of the cultivation.

Overall, similar observations were made in control shake flask cultures which were directly drawn from the bioreactor culture (representative culture shown in supplementary figure AIII.1/Figure AIV.12).

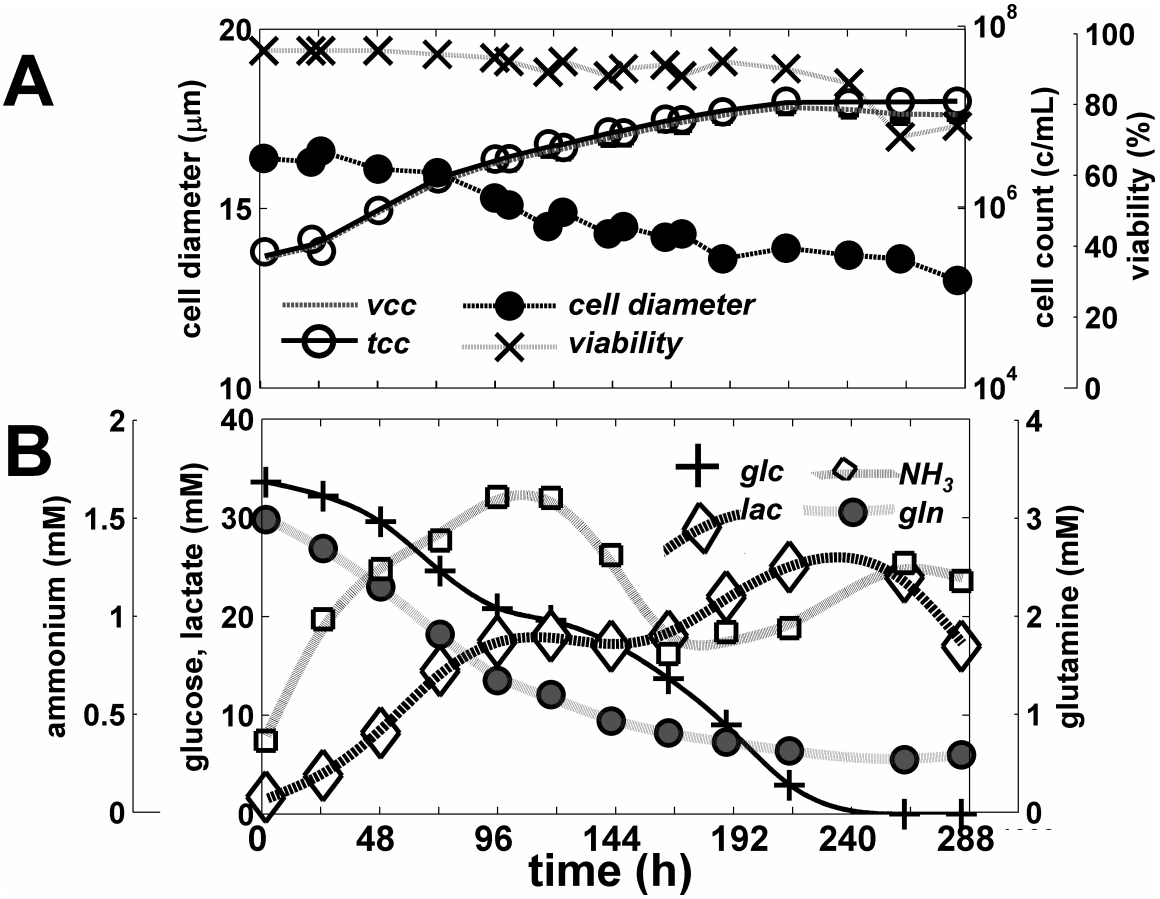

Figure AIII.1: Batch cultivation of HEK293 cells in 3L bioreactor scale

(A) viable cell count (*vcc*), total cell count (tcc), cell diameter, viability

(B) metabolite and byproduct concentrations

AIII.4.2 Biovolume Measurements Are Reliable Indicators of Online Permittivity Measurements

It was subsequently investigated if offline measurement of the biovolume (from *vcc* and *d*) were correlating with the online permittivity signal during batch cultures. The biovolume (*bv*) includes variations in cell density and cell diameter, providing a value which can be correlated to either the dual-frequency permittivity ($\Delta\varepsilon_{FC}$) or, if multiplied by cell radius with the permittivity increment ($\Delta\varepsilon_{max}$). In this batch cultivation, the permittivity signal ($\Delta\varepsilon_{FC}$ and $\Delta\varepsilon_{max}$) followed the biovolume well, resulting in a high regression coefficient, in particular for the values of the exponential growth phase and when viability was high (figure AIII.2, table AIII.1). Yet, the slope and the regression coefficient decreased with increasing culture time. Samples from late stages of

the process showed comparatively smaller permittivity values. This observation was attributed to changing dielectric properties of the remaining viable cells with decreasing viability (Ansorge et al. 2010c). It seems to be a general finding that the accuracy of permittivity measurements in predicting *vcc* is lower when viability decreases (Opel et al. 2010). Nevertheless, all samples at high viability fell on the same trend line being thus independent of variations in nutrient and byproduct concentrations and cell diameter.

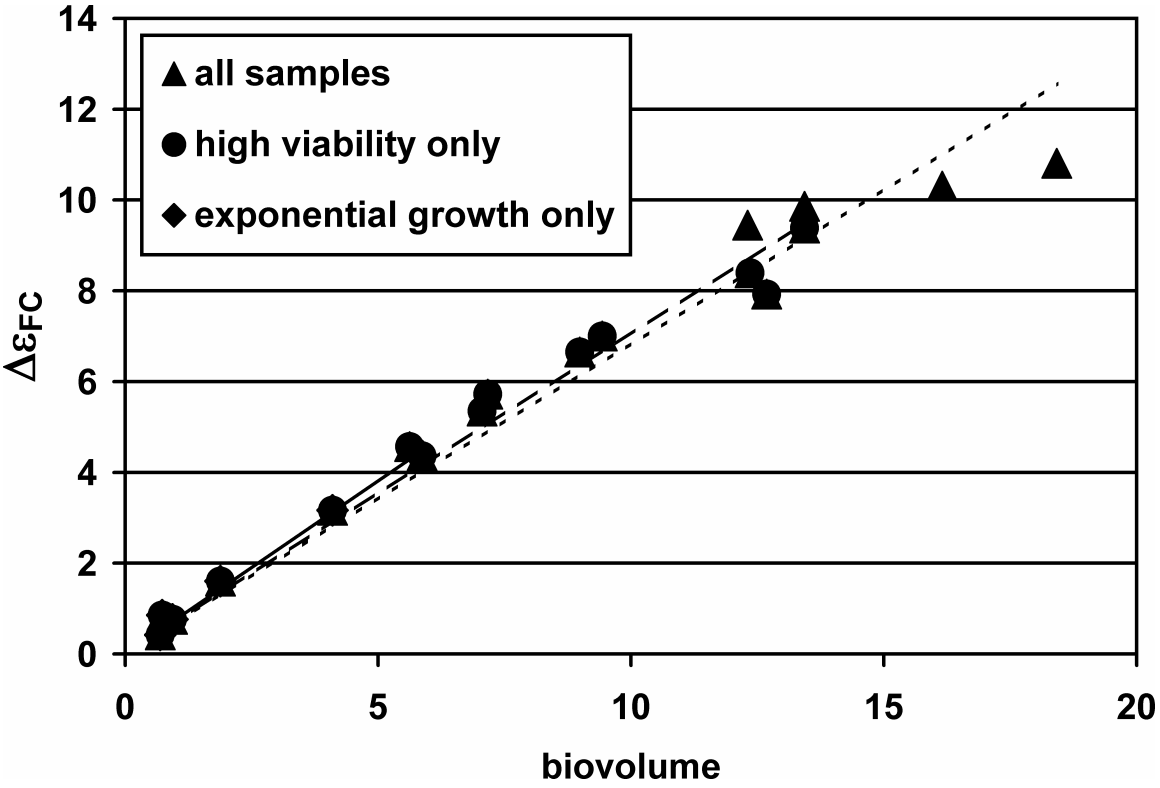

Figure AIII.2: Correlation of biovolume and permittivity in HEK293 bioreactor batch culture; note that the values for $\Delta\varepsilon_{max}$ are not shown in the figure (refer to table 5.1 for regression parameters); thick line: regression for exponential growth; dashed line: regression for high viability only; dotted line: regression for all samples

Table AIII.1: Correlation of permittivity signal ($\Delta\varepsilon_{FC}$)/dielectric increment ($\Delta\varepsilon_{max}$) and biovolume(*bv*)/*bv**r

	$\Delta\varepsilon_{FC}$ vs bv, ◆●▲ (Y) (X)		$\Delta\varepsilon_{max}$ vs bv*r (Y) (X)	
culture phase/ regression parameter	R^2	slope (pF/cm/µl/ml)	R^2	slope (pF/cm/um*µl/ml)
exponential growth ◆	0.99	0.76	0.99	1.60
high viability●	0.98	0.71	0.97	1.68
complete culture▲	0.96	0.68	0.94	1.54

In all cases, the regression was calculated with the constraint that the trend line had to pass through the origin

The strong correlation of offline biovolume measurements (from hemacytometer cell counts mean cell diameter) and online values ($\Delta\varepsilon_{max}$, $\Delta\varepsilon_{FC}$) validated the approach for the analysis of online permittivity data. This is consistent with findings by others according to which the biovolume measured by the electrical sensing zone method (Coulter principle) follows, in comparison with other methods, the evolution of permittivity measurements best. This seems to be a finding which holds for several cell types, in particular for the exponential growth phase (Zeiser et al. 1999; Zeiser et al. 2000; Ansorge et al. 2007b; Ansorge et al. 2010a).

AIII.4.2 Monitoring of the Characteristic Frequency Allows Identification of Metabolic Shifts

AIII.4.2.1 Metabolic Shift Identification at the End of Exponential Growth

The end of the exponential growth phase at ~90 h was identified after computing the growth rate from the offline cell counts (fig. AIII.3 A). The decrease in growth rate from 0.6 d^{-1} to 0.29 d^{-1} was consistent with results obtained from online monitoring. No rapid changes were observed in the permittivity signal ($\Delta\varepsilon_{FC}$) (fig. AIII.3 B). The value increased until the beginning of death

phase, reaching a maximum at around 220 h. At around 90h, $\Delta\varepsilon_{FC}$ continued to increase at a reduced slope.

Two approaches were then used to calculate the specific growth rates: either the calculation was done specific to the viable cell count (μ(*vcc)*) (fig. AIII.3 A) or, alternatively, to obtain a value which includes possible variations in cell size, specific to the online dual-frequency permittivity signal ($\Delta\varepsilon_{FC}$) that represents the biovolume (μ(*perm)*). Both approaches resulted in similar growth rate values and trends. As expected due to the decrease in cell diameter (fig. AIII.1 A), μ(perm) decreased faster towards the end of the exponential growth and was additionally showing a lower value of 0.18 d^{-1} compared to μ(*vcc*) (0.29 d^{-1}) during the post-exponential growth phase (fig. AIII.3 A and B, table AIII.1).

Accordingly, similar observations were made for the specific lactate and glucose production/consumption rates calculated using either *vcc* (q(lac); q(glc)) or permittivity ($\Delta\varepsilon_{FC}$) (qperm(glc); qperm). These values showed a similar decrease than the growth rates from 50 h until the beginning of death phase (see table 5.1 for average values), resulting in a strong linear correlation (R^2>0.95; regressions not shown). The consumption/production rates for glutamine/ammonium (NH_3) showed an almost constant decrease during exponential growth, followed by values close to zero in later stages of the cultivation .

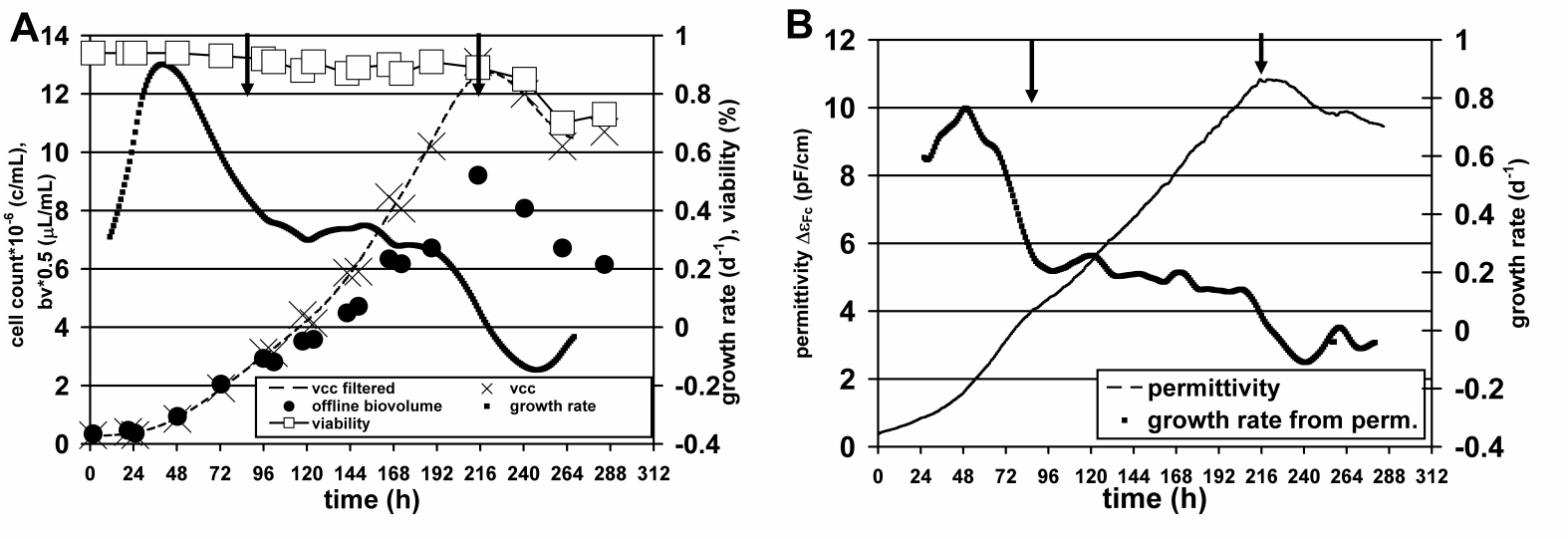

Figure AIII.3: Growth for batch cultivation of HEK 293 cells. Arrows indicate end of exponential growth and beginning of death phase, respectively

(A): growth rate calculated based on *vcc*; offline *bv* values (circles) shown for comparison

(B): calculation of growth rate from the online permittivity signal $\Delta\varepsilon_{FC}$

Table AIII.2: Growth and metabolic rates for HEK293 batch culture; average values for each culture phase are shown

parameter	***exponential growth***	***post-exponential growth***	***death phase***
time (h)	***0-90***	***90-215***	***215-285***
μ(*vcc*) (d^{-1})	0.67 +/- 0.18	0.29 +/- 0.07	-0.064 +/- 0.018;
μ (perm) (d^{-1})	0.6 +/- 0.15	0.18 +/- 0.04	-0.04 +/- 0.04
qglc (μmol/10^6* h); qperm(glc) (μmol*cm/pF*h)	0.15 +/- 0.05; 0.07 +/- 0.03	0.02 +/- 0.005; 0.02 +/- 0.005	0.003 +/- 0.001; 0.004 +/- 0.004
qlac (μmol/10^6* h); qperm (μmol*cm/pF*h)	0.22 +/- 0.10; 0.12 +/- 0.05	0.007 +/- 0.01; 0.007 +/- 0.009	0.01 +/- 0.01; -0.01 +/- 0.01
qgln (μmol/10^6* h); qperm(gln) (μmol*cm/pF*h)	0.02 +/- 0.013; 0.010 +/- 0.06	0.001 +/- 0.0013; 0.001 +/- 0.001	0; 0
qNH3; qperm(NH3) (μmol*cm/pF*h)	0.02 +/ - 0.02; 0.01 +/- 0.01	-0.001 +/- 0.001; -0.001 +/- 0.001	0; 0

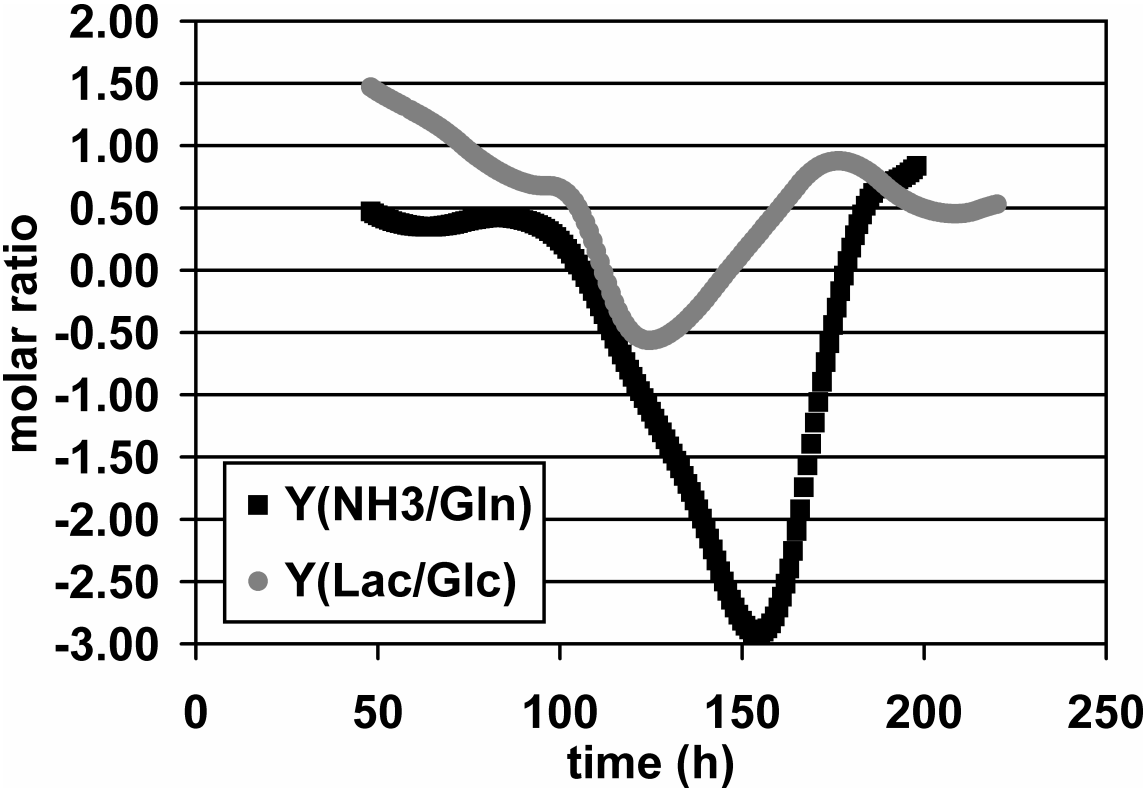

Figure AIII.4: Evolution of the molar ratios for lactate/glucose and NH_3/glutamine

Significant changes in the lactate/glucose and ammonium/glutamine molar ratios confirmed a metabolic shift starting at around 90 h or when 12 mM/1.5 mM of glucose or glutamine were consumed and 15 mM/1.2 mM of lactate and ammonium were produced, respectively (fig. AIII.4). This change in cellular metabolism was most clearly observable for the values of the lactate/glucose molar ratio which gave negative values from 100-170 h.

The end of exponential growth was thus in this culture marked by a metabolic shift, when the consumption of glucose and glutamine decreased while lactate and ammonium production ceased for several days (table AIII.1, fig AIII.1). The change in metabolism was linked to the significant drop in growth rate from 50-90 h. The specific metabolite consumption and production rates, in particular of glucose and lactate were indicative for the reduced metabolic activity and were strongly correlating with growth rate until the beginning of the death phase. The depletion of glucose finally resulted in the beginning of death phase after 215-220 h of cultivation. It is also

noted that the byproduct concentrations of lactate and ammonium at the time of the metabolic shift were below or at the lower end of the concentrations that are reported to be limiting for HEK 293 cultures (>20 mM for lactate and 1-2.5 mM for ammonium (Hassell et al. 1991; Nadeau et al. 1996). It is therefore likely that other, unidentified medium components or byproducts were limiting or inhibiting at the end of the exponential growth phase.

AIII.4.2.2 Changes in Characteristic Frequency (fc) as Indicator for Metabolic Activity

Online permittivity measurements provide primarily a measure of the biovolume ($\Delta\varepsilon_{FC}$). Multifrequency permittivity measurements give additional real-time information on the state of the cell suspension via the β-dispersion parameter *fc*. In most cases, *fc* is used as an indicator for cell size changes, assuming that both the intracellular conductivity (σ_i) and cell membrane capacitance (C_M) remain constant during the cultivation. For most mammalian cell culture experiments, f_C can be defined by a simplified form of equation 5.3 (Schwan 1957; Harris et al. 1987; Patel et al. 2008a; Patel et al. 2008b; Ansorge et al. 2010a):

$$f_C = \frac{1}{2 \times \pi \times r \times C_M \times \frac{1}{\sigma_i}} \qquad \text{(AIII.1)}$$

fc is therefore a function of cell radius, membrane capacitance and intracellular conductivity.

During the present cultivation, *d* and *fc* did, however, not correlate following one single linear relationship (fig. AIII.5). In contrast, smaller cell diameters seemed to correlate with the drop in *fc* during the first part of the cultivation. This correlation was thus contradictory to the theoretical relationship according to which *fc* increases with decreasing diameter if it is assumed that C_M and σ_i remain constant (eq. AIII.1). In general, we noted a decrease in cell diameter with increasing culture time. This decrease corresponded to a reduction in cellular volume of up to 30 % and started towards the end of the exponential growth phase. Reduction of cell size for the same and other cell lines during batch cultures has been reported in other studies (Ramirez et al. 1990; Ansorge et al. 2007a; Ansorge et al. 2010a). Several studies describe cell size to be one of the parameters reflecting the physiological state of a culture (Fantes et al. 1977; Frame et al. 1990 ; Stocker et al. 2000) (Lloyd et al. 2000; Berdichevsky et al. 2008). For example, multifrequency permittivity measurements in CHO cell perfusion cultures revealed that large cells disappeared once nutrient availability decreased (the feed was stopped), causing changes in the permittivity at

low frequencies (Cannizzaro et al. 2003). The cell diameter during batch cultures of HEK293 cells might therefore be considered as an indicator of cellular metabolic activity.

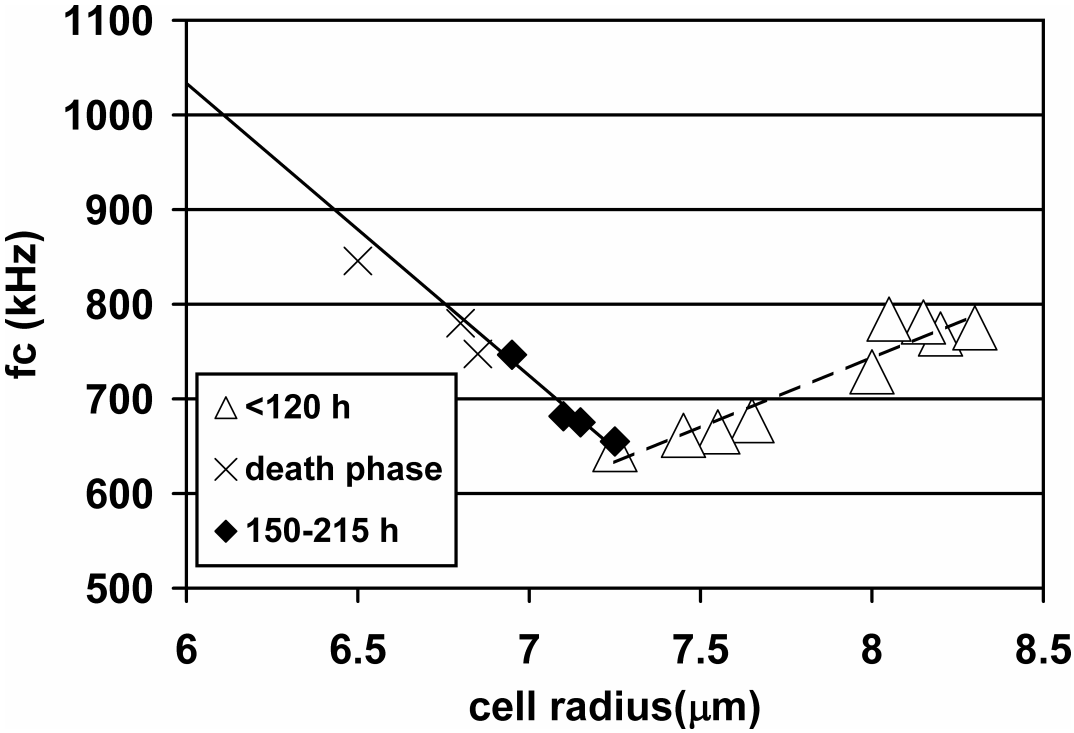

Figure AIII.5: Characteristic frequency (*fc*) and cell diameter correlated following two distinct linear correlations.

Regression coefficients were high in both cases with R^2 = 0.91 during the entire growth phase (<120 h) and R^2 = 0.95 during post-exponential growth phase (150-215 h)

Concerning the relationship of characteristic frequency and cell diameter, only the values for *d* and *fc* during the post-exponential growth, i.e. from 150-215 h, were consistent with the theoretical relationship (fig. AIII.5). For the regression, values from death phase (>215 h) were not included because of the decreased viability during that phase (see AIII.1 A). Taken together, these observations suggested that the mean cell diameter was not the only variable that changed during the exponential growth phase.

A recent literature report could link the occurrence of a metabolic shift in CHO cell batch cultures, as a result of the depletion in glutamine, with a dramatic decrease in characteristic frequency (*fc*) which was caused by a drop in intracellular conductivity (σ_i) (Ansorge et al.

2010a). σ_i was thus described to be linked to the physiological state and the nutrient availability of the cell suspension. These findings were also confirmed in fed-batch cultivations of the same and a second CHO cell line during which responses to discontinuous feed additions in OUR and *fc* were monitored (Ansorge et al. 2010b). The authors did, however, not directly correlate the changes in *fc* with a specific metabolic parameter.

The evolution of *fc* was further analyzed in the present cultivation. During the exponential growth phase, the value of *fc* decreased starting at around 50 h, reaching a minimum at ~115 h and increased thereafter until the end of the culture. (see fig AIII.6 A). In comparison, the growth, metabolic rates and *fc* showed a similar decline from 50-100 h, the values (fig. AIII.6 A), correlating with high regression coefficients (R^2 >0.95; regressions not shown). Even when the changes in cell diameter towards the end of the exponential growth rate were taken into account (see equation AIII.2 in the figure legend and fig. AIII.6 B), *fc* correlated with the metabolic rates (lactate and glucose) with a high regression coefficient (R^2>0.95) until 90 h. During the post-exponential growth and death phase, the values for the metabolic rates were almost constant at very low levels (see table AIII.1). For that time, a good correlation of cell diameter (1/*d*) and *fc* was observed (see fig. AIII.5).

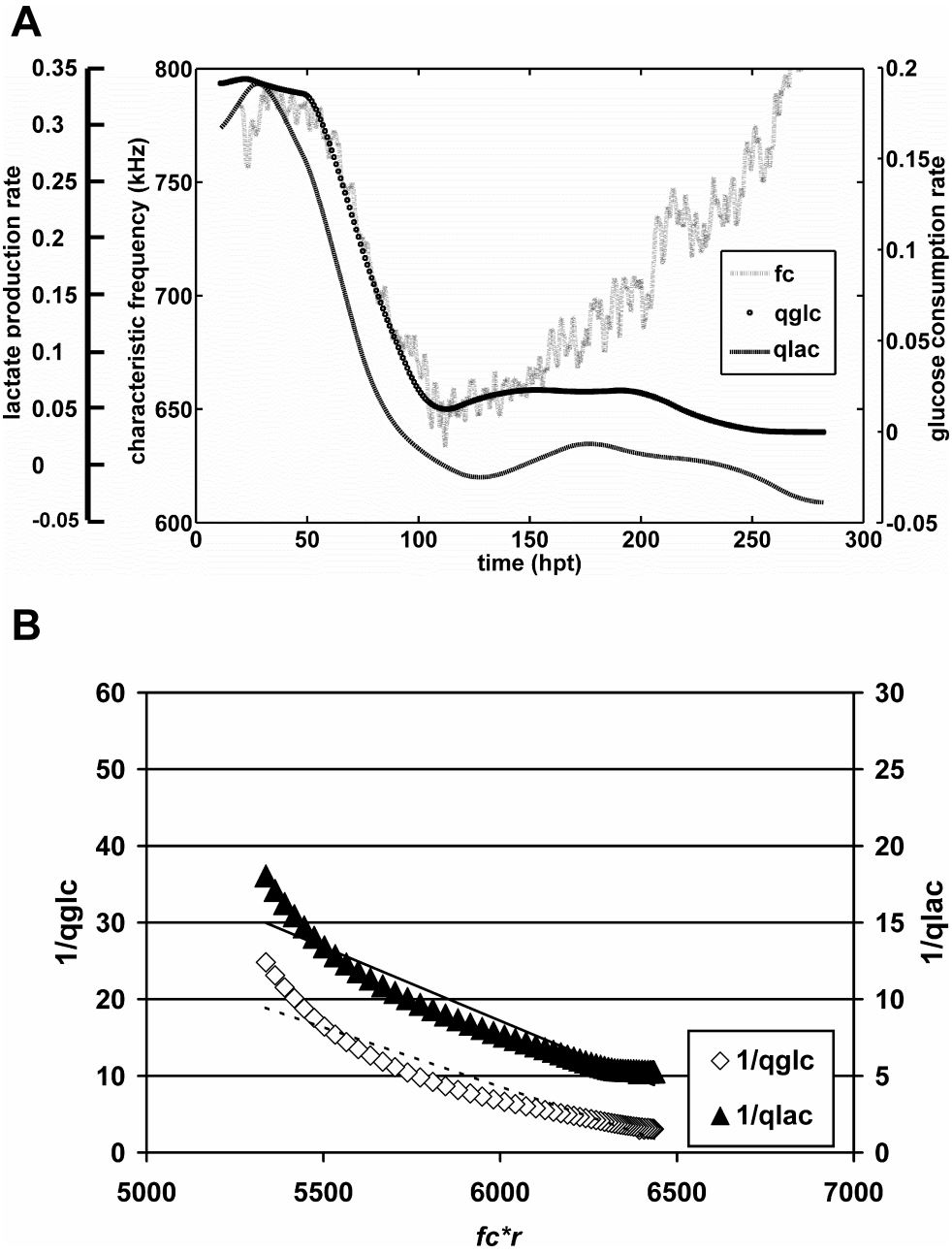

Figure AIII.6: During the exponential growth phase, the characteristic frequency correlates with the metabolite consumption and production rates.

(A): metabolic rates (qglc and qlac) and characteristic frequency show similar evolutions until the end of exponential growth phase

(B) Correlation of *fc**r and the inverse of glucose consumption and lactate production show that *fc* is an indicator of the metabolic activity, most likely because of changes in intracellular conductivity (σ_i). Data were plotted according to equation AIII.1 which can also be written:

$f_C \times r = \frac{\sigma_i}{2 \times \pi \times C_M}$ (equation AIII.2) and assuming that C_M remained constant during exponential growth. Regression coefficients were as follows:

1/qlac: $R^2 = 0.94$; 1/glc: $R^2 = 0.92$

The characteristic frequency, which can be directly acquired in real-time, is thus a parameter providing information on the metabolic activity of the cell suspension. If it is assumed that the membrane capacitance (C_M) was constant during the exponential growth phase, changes in intracellular conductivity (σ_i) must have been responsible for this correlation. This confirms that previous findings for CHO cells (Ansorge et al. 2010a; Ansorge et al. 2010b) are transferable to other cell types. Whereas depletion in glutamine resulted in that case in a levelling of the biovolume and a decrease in permittivity ($\Delta\varepsilon_{FC}$), the situation for the batch cultivation of HEK293 cells was in the present study less dramatic, i.e. metabolic rates decreased but cell growth and biovolume increase continued, although at reduced rates. Therefore, it cannot be excluded that C_M also changed, although its value was in several reports found to be mainly affected during processes involving trafficking through the membrane or other dramatic morphological changes of the cell membrane (Archer et al. 1999; Patel et al. 2008a; Patel et al. 2008b; Zimmermann et al. 2008). In addition, Ansorge et al. (2010) refuted earlier assumptions according to which C_M varies with changes in metabolic rates (Noll et al. 1998). Instead, variations in intracellular conductivity were put forward as reason for the correlation of *fc*, permittivity and nutrient availability. To date, a direct demonstration for why the intracellular conductivity varies with nutrient availability has however not been reported, mainly because this parameter is not accessible with routine cell culture methods.

AIII.4 Summary and Conclusion

The HEK293 cell line which was studied here grows to high cell densities of up to $1x10^7$ c/mL in batch mode. Exponential growth lasts, however, only up to a cell density of 2.5-3 $x10^6$ c/mL when a metabolic shift occurs, accompanied by, as the most characteristic change, a cease in lactate production. The specific metabolic rates of glucose and lactate were indicative for the reduced metabolic activity. Online measurement of the permittivity ($\Delta\varepsilon_{FC}$ and $\Delta\varepsilon_{max}$) allowed

monitoring of cell growth in real-time, calculation of the growth rate based on the online values and thereby identification of the exponential growth phase. Offline biovolume measurements were evaluated during the bioreactor batch culture and identified as a suitable tool to follow the evolution of the permittivity signal. In combination with the values for cell size, this information enables the analysis of permittivity variations in detail. In contrast to routine offline analyses of culture samples, online *in situ* permittivity measurements offer the advantage to monitor the evolution of the biovolume in real-time with a quasi-continuous signal. Growth rate (based on the biovolume) is then directly accessible from this online signal. However, the use of this 'online growth rate' (as derivate of $\Delta\varepsilon_{max}$ or $\Delta\varepsilon_{FC}$) for process control (e.g. feed additions) would face the challenge that its value is likely to be highly affected by noise on the online signal. In addition, the dual-frequency signal can also be affected by changes in the dielectric properties (C_M and σ_i) of the cells.

The β-dispersion parameter characteristic frequency *fc*, on the other hand, also provides information related to the metabolic activity and correlated with metabolic consumption and byproduct production rates during the exponential growth phase. These findings will need to be validated in other systems. In contrast to metabolite analysis, *fc* is available in real-time. The virtually unlimited 'sample' number of permittivity measurements consequently results in a higher degree of confidence concerning the state of the culture. Yet, as the permittivity signals provide complex information and several parameters may affect the dielectric properties of a cell suspension, the use of the technology should be supported by offline cell size measurements. This is required for a first characterization of the system, to uncover the mechanisms behind the signal variations and an in-depth analysis of the signal if various operating conditions are investigated. Cell size measurements are the most useful parameter to determine offline as they allow calculating the biovolume and the analysis based on dielectric theory (see equations 5.1-3). In addition, cell size seems to be indicative of the metabolic activity and belongs to the set of the parameters that define the physiological state of a culture. Nevertheless, cell size appears to be a parameter that is often overlooked when analyzing cell culture processes with literature on the subject being scarce.

It is concluded that our observations might be of value for the development of fed-batch strategies or for transfer time determination in production seed trains.

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APPENDIX IV: Complementary Experiments, Supporting Information and Experimental Observations

ADDITIONAL DATA FOR CHAPTER 2

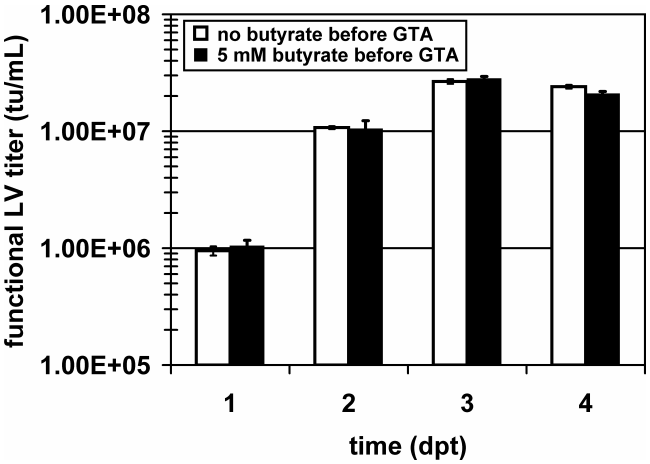


Figure AIV.1: Sodium butyrate does not compromise results from GTA assays. LVs were produced without butyrate addition using HyQ medium. Before titration, the LV containing supernatants were subjected to 5 mM of sodium butyrate and incubated for 15 min at 37°C

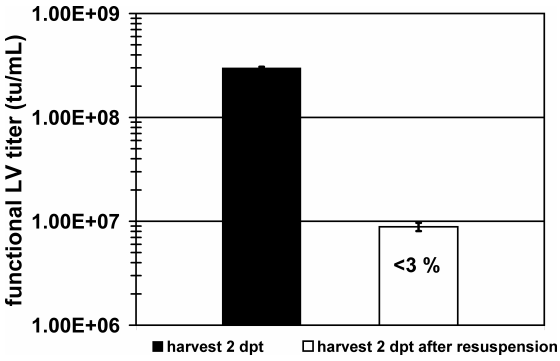


Figure AIV.2 : The number of functional LV is low (< 3% of LV titer before harvest) immediately after resuspension of the cell pellet during small-scale experiments. LVs were produced using improved production conditions, comparison was done 2 dpt. These data were submitted as supporting information to Ansorge et al. (2009)

ADDITIONAL DATA FOR CHAPTER 3

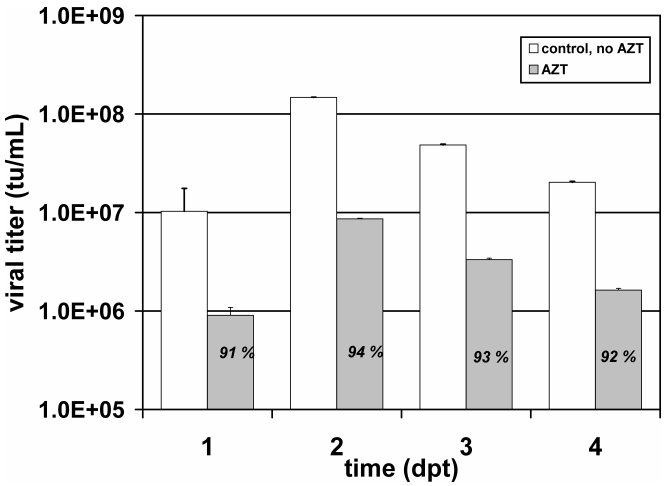


Figure AIV.3 : Control titrations in the presence of 100 μM AZT result in significantly reduced functional LV titers. LV was produced using improved production conditions at 1 DMD

ADDITIONAL DATA FOR CHAPTER 4

Alternative LV Quantification Methods

This section briefly summarizes additional alternative methods that were evaluated for LV quantification/titer estimation within this work.

LV Quantification by HPLC

Current protocols for the quantification of lentiviral vectors (GTA, RT-PCR, ELISA) are time-consuming (Sastry et al. 2002) (chapter 2, table 2.2). These methods are thus not suitable for a rapid at-line monitoring of LV titers. In addition, none of the previously described methods is based on the direct quantification of physically intact lentiviral particles. In contrast, anion-exchanged high-performance liquid chromatography (HPLC) can specifically and efficiently resolve viral particles from other contaminants. The LV-containing fraction can then be precisely quantified. The method has been proven to have large potential for the quantification of retroviral total particle numbers (Transfiguracion et al. 2004). The detection limit of the method can be increased by the use of fluorescent dyes such as SYBR® Green I to label the viral genomic DNA or RNA. The feasibility of this approach has been demonstrated for the most commonly used viral vectors (Transfiguracion et al. 2005). An adaptation of this method to the LV system has been successful. Detection of the intact LV particles takes place after labeling the viral RNA

genome with SYBR® Green I followed by HPLC separation from sample contaminants. A full description of the developed method would go well beyond this scope of this work and a publication concerning this subject is ongoing work in our group (Transfiguracion, 2010, manuscript in preparation).

Preliminary HPLC quantification results were made available (fig AIV.4). Analysis of bioreactor supernatants from run #3 at 1 VVD resulted in almost constant concentrations in the range of $1x10^{10}$-$1x10^{11}$ total viral particles (tp/mL) from 2-5 dpt (see table AII.2 for summary of bioreactor runs). Viral release could also be observed 1 dpt. However, before this method can be routinely used for LV quantification, further optimization of the method is required (ongoing work and unpublished results).

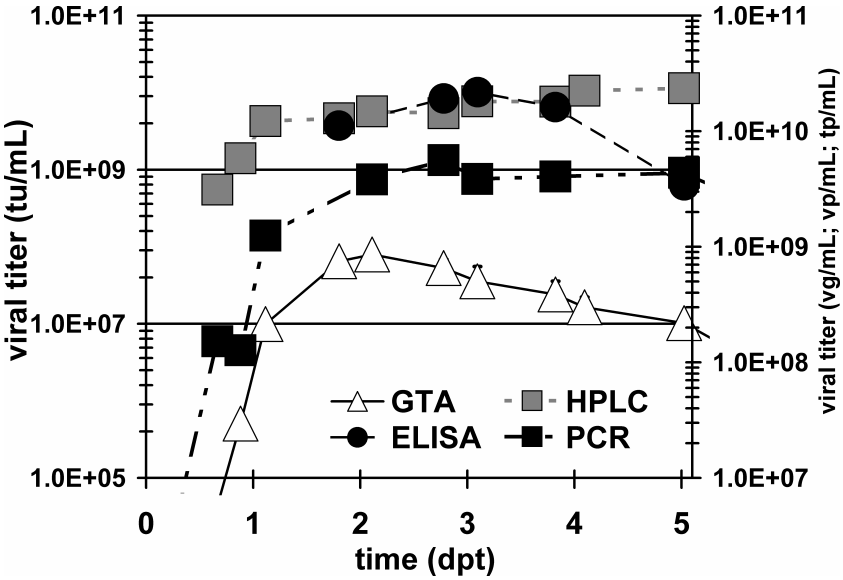


Figure AIV.4 : Comparison of LV quantification methods for a bioreactor perfusion culture at 1 VVD (run #3)

Data obtained so far are promising but challenges such as a high detection limit and specificity to LV related particles need to be overcome (see table AIV.1).

Table AIV.1: Novel LV quantification method based on HPLC

LV Quantification Assay/ *assay parameter*	**HPLC**
method	in-house, Transfiguracion et al manuscript in preparation
Assay standard	in-house, 50x concentrated, semi-purified LV preparation
Inter-assay-variability[I]	<2 %
Intra-assay variability[II]	<10 %[VI]
Time to result/throughput per day[III]	1-3 h, ~50
Labor intensity[IV]	++/+++[VII]
Flexibility[V]	++
Detection limit (DL)	$5x10^8$ tp/mL
Remarks	method still under development

VI: variability if a large number of samples is analyzed and labelling time is increased up to 24 h

VII: depending on sample pre-treatment

Refer to table 2.2 for further explanations and remarks I-V

Measurement of Total GFP Expression in Producer Cells During LV Production

During the production of VSV-G pseudotyped LVs, a high level of transgene expression in producer cells is typically observed (Farson et al. 2001; Bagnis et al. 2009). It was thus assessed if the total GFP expression at 3 and 4 dpt would indicate the amount of functional LV particles. In small scale experiments, a linear trend between LV titer and GFP expression was found, although the regression coefficients were quite low and inter-experimental variations were high (fig. AIV.5).

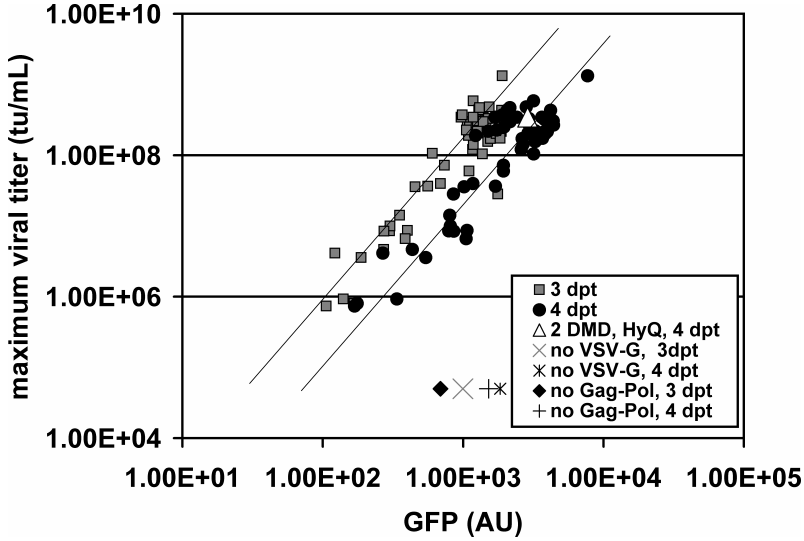


Figure AIV.5 : Total GFP expression on 3 and 4 dpt is indicative for the maximum functional LV titer.

Results represent 61 data points for 3 and 4 dpt, respectively, corresponding to 15 independent experiments at 1 or 2 DMD and in different medium compositions. Regressions for samples from 3 and 4 dpt gave: R^2= 0.7 and R^2 = 0.6, respectively

One of the limitations of the method was that the total GFP expression signal is a function of the number of producer cells distributed in the well prior to measurement. Any variation in cell density (obtained from manual counting) is thus reflected in the GFP signal (unpublished observation). In addition, control cultures which were transfected either without the VSV-G or the *Gag-Pol* encoding plasmid and did not generate functional vector particles, also resulted in significant GFP expression, suggesting that the transgene is also directly expressed of the transfer vector plasmid (plasmid construct CSII-CMV5-GFPq alone; refer to section 2.1.2 for details on plasmid constructs) after transfection.

Whereas the value of the method, in particular with respect to LVs carrying other transgenes is limited, the advantage of the GFP expression measurement during LV production is, however, that it value is rapidly available during the experiment. The method is therefore a valuable indicator for process development.

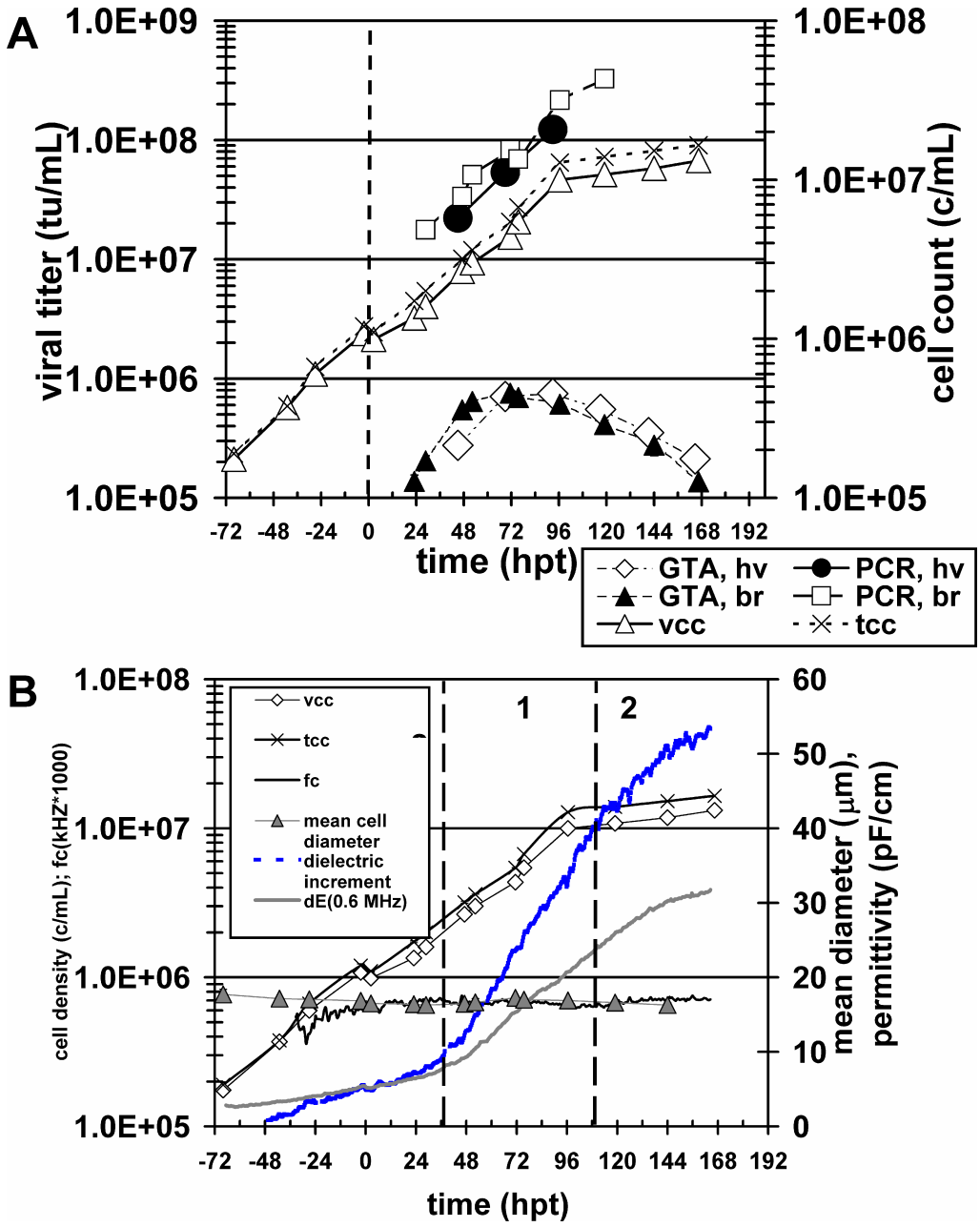


Figure AIV.6 : Viral production kinetics and permittivity monitoring for bioreactor run #2; (A) cell, density, functional LV and VG titer, dashed line indicates time of transfection; (B) monitoring results (permittivity), dashed lines mark process phases 1 and 2; (compare chapter 6 (bioreactor run in starting conditions), appendix II, figure AII.5 and table AIII.1)

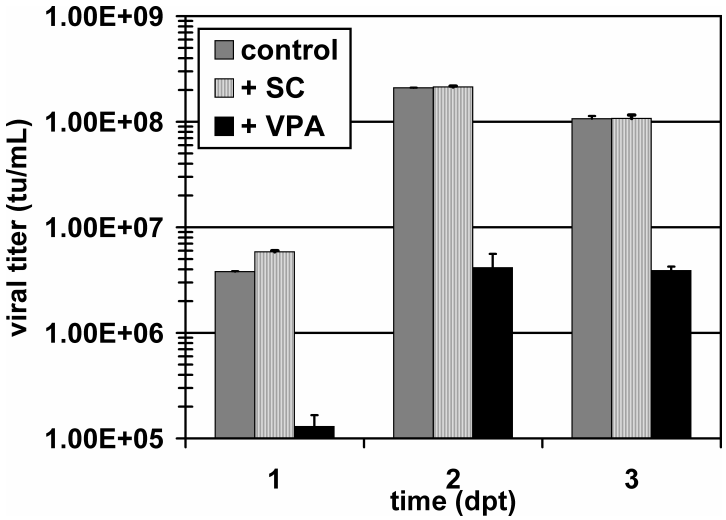


Figure AIV.7 : Addition of Synthechol (SC) or valproic acid (VPA) do not have positive effects on LV production

VPA (SIGMA P4543, prepared at a stoch concentration of 500 mM in MilliQ water) was added 16 hpt at 5 mM. VPA was described as a potent alternative histone deacetylase inhibitor (Backliwal, Hildinger et al. 2008); SC (SIGMA S5442; was added to HyQ medium at a concentration of 1:1000 which was determined to be the maximum concentration without negative impact on viability). Cholesterol was found in several studies to act as an enhancer or LV infectivitiy (Chen, Ott et al. 2009).

ADDITIONAL DATA FOR CHAPTER 5

Table AIV.2: Identification of process transition phases in bioreactor runs

Process transition points (PTP) were identified based on online monitoring data and used to define the process phases 1-4 (see chapter 6)

Culture	**Titer,** **GTA (max)[3],** **PCR (max)[3];** **Total yield[4] (GTA, PCR)**	**Operation** **(mode, perfusion rate, cell density at time of transfection,** **# of PTP; culture medium; Remarks)**	**PTP 1**		**PTP 2**		**PTP 3**		**PTP 4**	
			Identification Criteria; **Time (hpt); Titer (tu/mL, %);** **Process Phase**	**Change in $\Delta\varepsilon_{max}$ (rate pF/cm/h, %));** **Change in *fc* (kHz, %)**	**Identification Criteria;** **Time (hpt); Titer (tu/mL, %);** **Process Phase**	**Change in $\Delta\varepsilon_{max}$ (rate pF/cm/h, %));** **Change in *fc* (kHz, %)**	**Identification Criteria;** **Time (hpt); Titer (tu/mL, %);** **Process Phase**	**Change in $\Delta\varepsilon_{max}$ (rate pF/cm/h, %));** **Change in *fc* (kHz, %)**	**Identification Criteria;** **Time (hpt); Titer (tu/mL, %);** **Process Phase**	**Change in $\Delta\varepsilon_{max}$ (rate pF/cm/h, %));** **Change in *fc* (kHz, %)**
Run #1	none	Batch, -,-, 3, HyQ; no transfection batch culture	Decrease in *fc*, change in slope of $\Delta\varepsilon_{FC}$; 0-80 hinoc; n.A.; Exponential growth phase	12 pF/cm/80 h;780-700, 10% decrease over > 3 d	Increase $\Delta\varepsilon_{max}$, *fc*; 80-220h; n.A.; Linear growth at high viability, maximum in oxygen consumptino	12→34.5 /140 h; 700-630-730	Decrease $\Delta\varepsilon_{max}$, increase *fc*; 220-280 h; n.A.; Death phase, decrease in viability	34-26.1; 720-800		
Run #2	Low yield, $8x10^5$, $3.3x10^8$, $5.6x10^9$, $3.1x10^{12}$	Perfusion, 2 VVD, $1x10^6$, 3, LC-SFM L, results acquired with Aber B220	Increase $\Delta\varepsilon_{max}$, *fc* constant; -68-40 hpt; n.a./2-3%; Exponential growth phase/ pre-production	1-10; ~700[2]	Slope change in $\Delta\varepsilon_{max}$; 40-108; $7x10^5$; Production phase at maintained exponential growth, slope change in oxygen consumption.	10-40; 700 → 625, 11 %	Minimum in *fc*, slope change in $\Delta\varepsilon_{max}$; leveling of *fc*; 108-144; $3\text{-}4x10^5$; Decrease in viral titer/harvest time	40-50, +25 %; 625-700, 12 %		
Run #3	High yield, $7x10^7$, $5.6x10^9$, $3.6x10^{11}$, $2.3x10^{13}$	Perfusion, 2 VVD, $4.4x10^6$, 4, HyQ; results acquired with Aber B220	increase $\Delta\varepsilon_{max}$ after transfection, decrease in *fc*; 0-25; $10^{5\text{-}6}$, <10 %; Charging and induction, phase post transfection/pre-production	23.5-34 pF/25 h; 44.7 %; 480-425-515, 11% drop, 21 % increase	Increase in *fc*: decrease in $\Delta\varepsilon_{max}$; 25-70 hpt; $1x10^7$-~$7x10^7$, 10%-100 %; Viral release, start of production	34-28, -17.6 % decrease; 515-615-530, 19.4 %	Change in slope $\Delta\varepsilon_{max}$ and minimum in *fc*; 70-96 hpt; $3.5\text{-}1.6x10^7$, <20-40 %; Decrease in viral titer/harvest time	31-28.5, -8 %; 615-540, -12 %	Increase in *fc*, decrease $\Delta\varepsilon_{max}$; end of process ; 96 – process end, titer: < 20 %	$\Delta\varepsilon_{max}$ <20 *fc* >600

Run #4	**High yield,** **$4x10^7$,** **$3.8x10^9$,** **$4.25x10^{11}$,** **$5x10^{13}$**	**Perfusion, 2 VVD, $4.7x10^6$, 4, HyQ**	**increase $\Delta\varepsilon_{max}$; decrease in *fc*; maximum in $\Delta\varepsilon_{max}$, minimum in fc; 0-24;** **<$6x10^5$, <1.5 %; Charging/induction, phase post transfection/pre-production**	**35→ 64/24 h; 83 %,** **(aber: 30-48/24 h; 60 %);** **720→ 625, -13%; 1030-920, 10.6%)**	**decrease in $\Delta\varepsilon_{max}$, increase in *fc*; change in slope $\Delta\varepsilon_{max}$, maximum *fc*; 24-50 hpt;** **$3.8x10^7$-$3.5x10^7$; Viral release and peak titer, maximum in oxygen consumption**	**64-40; -37.5 %; (Aber: 48—33; -31.25 %;** **615-790, 28 % increase; (Aber: 920-1090, 18.5%**	**Slope change in $\Delta\varepsilon_{max}$ /slope change in *fc*; 50-75 (fc)-84 ($\Delta\varepsilon_{max}$); 2.8-1e7, 74-35 %;** **Decrease in viral titer/harvest time**	**40-28/31.5, 30 %; 770-715/73,-8 %**	**Decrease of $\Delta\varepsilon_{max}$ until; end of process; 75/84 hpt – process end,** **<30 %**	**$\Delta\varepsilon_{max}$ <30, *fc* = 730**
Run #5	**High yield,** **$3x10^7$,** **$4.6x10^9$,** **$3x10^{11}$,** **$8x10^{13}$**	**Perfusion, 2 VVD, $8x10^6$, 4; HyQ+**	**Increase $\Delta\varepsilon_{max}$ after tf, decrease in *fc*; maximum in $\Delta\varepsilon_{max}$; minimum in *fc*;** **0-24; <1e4, <1.5;** **Charging/induction, phase post transfection/pre-production; maximum in oxgen consumption**	**73→110/24h; 50 %;** **(Aber: 94-128/24 h, 36%);** **635→ 525, 17%;** **(Aber: 380-330, 13%)**	**1: decrease in $\Delta\varepsilon_{max}$, increase in *fc*; 2: change in slope $\Delta\varepsilon_{max}$, maximum *fc;*** **24-45/50;$3.1x10^7$ (max), 6.5-100-70 %;** **Viral release and peak titer, maximum in oxygen consumption**	**110 (24h)-55 ; 50 %; (Aber: 128-64, 50 %);** **525-825, 57 % increase; 330- 540; 63 %**	**Slope change in $\Delta\varepsilon_{max}$; 50-70 hpt, 172-192 h inoc; $2.2\text{-}1.4x10^7$, 70-45/40 %; Decrease in viral titer/harvest time**	**55-44pf/cm/26 h , 20 %; 825-780, 5.5 %**	**Decrease of $\Delta\varepsilon_{max}$ until, end of process; 70 hpt – process end,** **< 30 %**	**$\Delta\varepsilon_{max}$ <40,** ***fc* = 760-720**

PTP: process transition points

Remarks for Table AIV.2 :

1: LV titer GTA in bioreactor supernatant sample

2: high variation in fc at beginning of culture due to low biomass level

3: maximum titer in reactor supernatant

4: after 5 dpt

ADDITIONAL DATA FOR APPENDIX II:

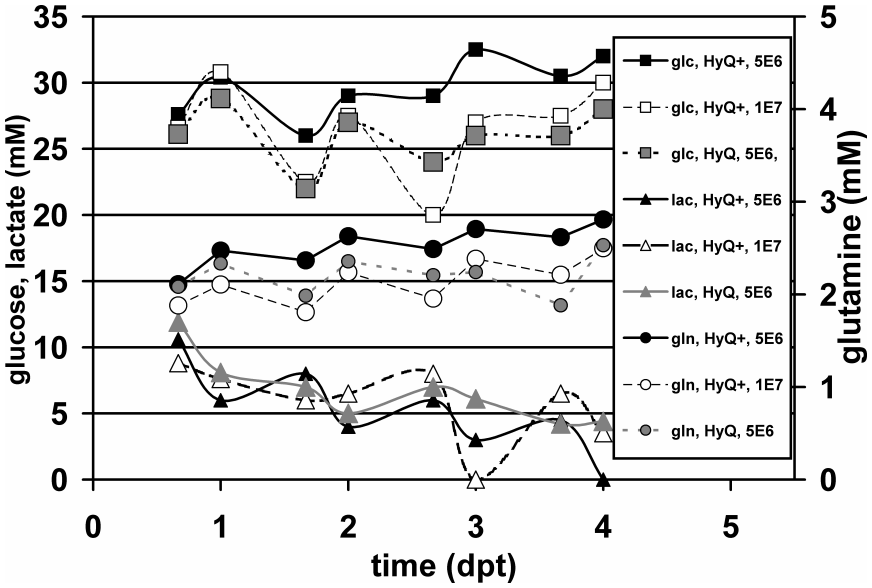


Figure AIV.8: Nutrient and byproduct concentrations (glucose, glutamine, lactate) in small scale LV production. Culture were transfected using improved production conditions at 2 DMD and two different cell densities ($5x10^6$ c/mL and $1x10^7$ c/mL). All concentrations remained below limiting or inhibiting values, except for lactate concentration before the first medium exchange

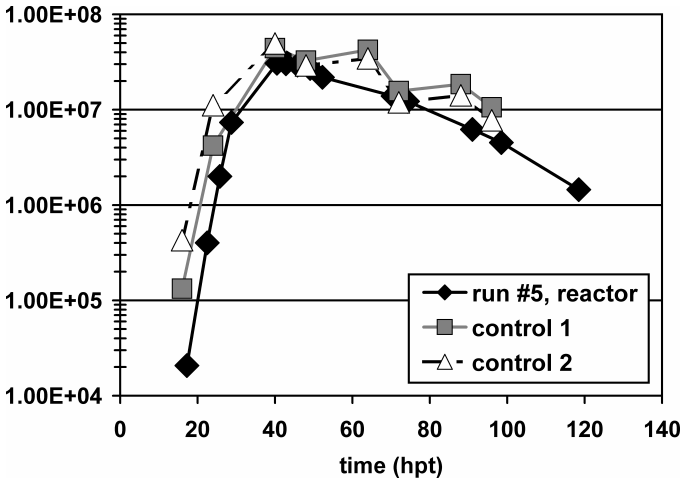


Figure AIV.9: Functional LV titer evolution is identical in bioreactor and shake flask control cultures; experiment shown corresponds to run # 5. Control 1 was taken from the bioreactor 3.5 h before transfection and transfected in the shake flask with the identical polyplex preparation than the mother culture; control 2 was taken from the bioreactor 1.5 h after transfection; both control cultures were subject to 2 DMD

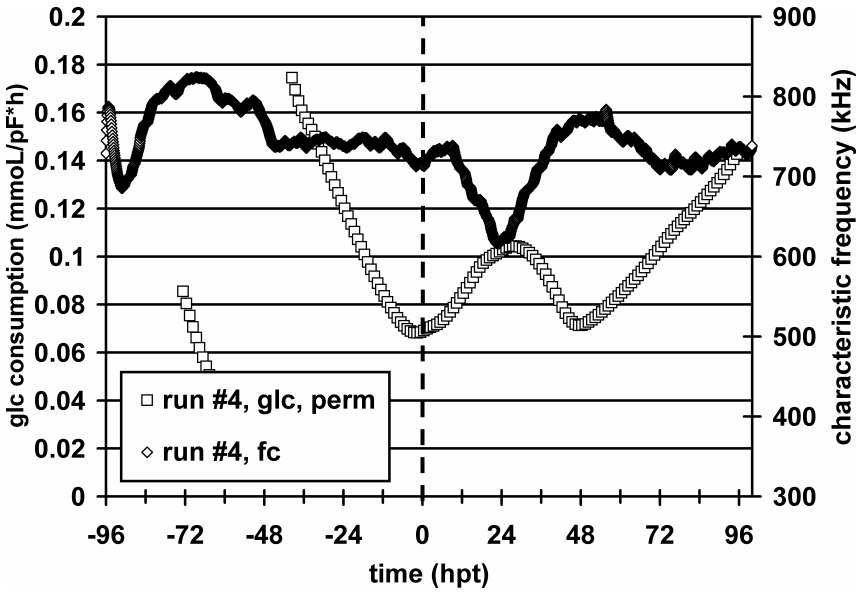


Figure AIV.10: Characteristic frequency and glucose consumption rate do not correlate during bioreactor LV production. Data correspond to run #4; dashed line indicates the time of transfection. The decrease in *fc* after transfection, paralleled an increase in glucose consumption rates indicates that the changes in *fc* is not linked with decreasing metabolic activity (as observed in chapter 5 for batch cultivation)

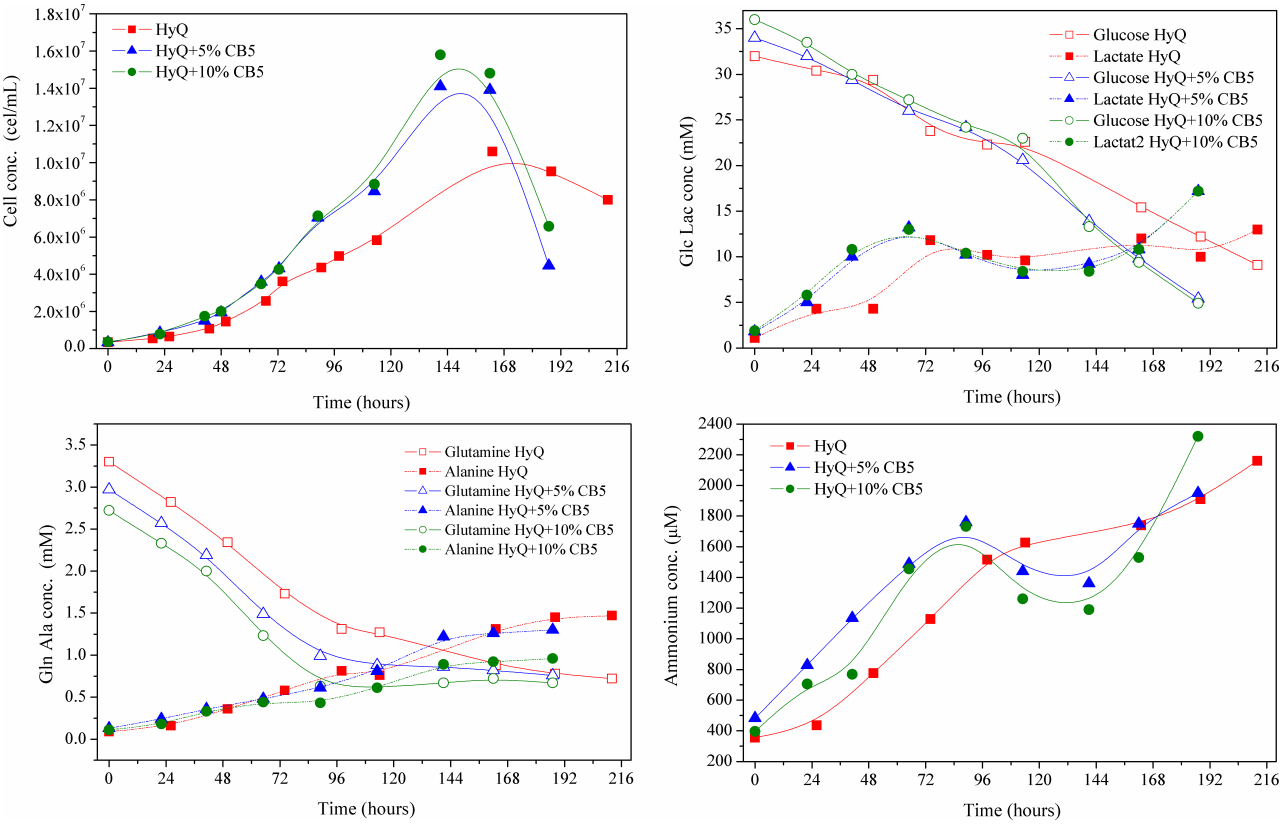
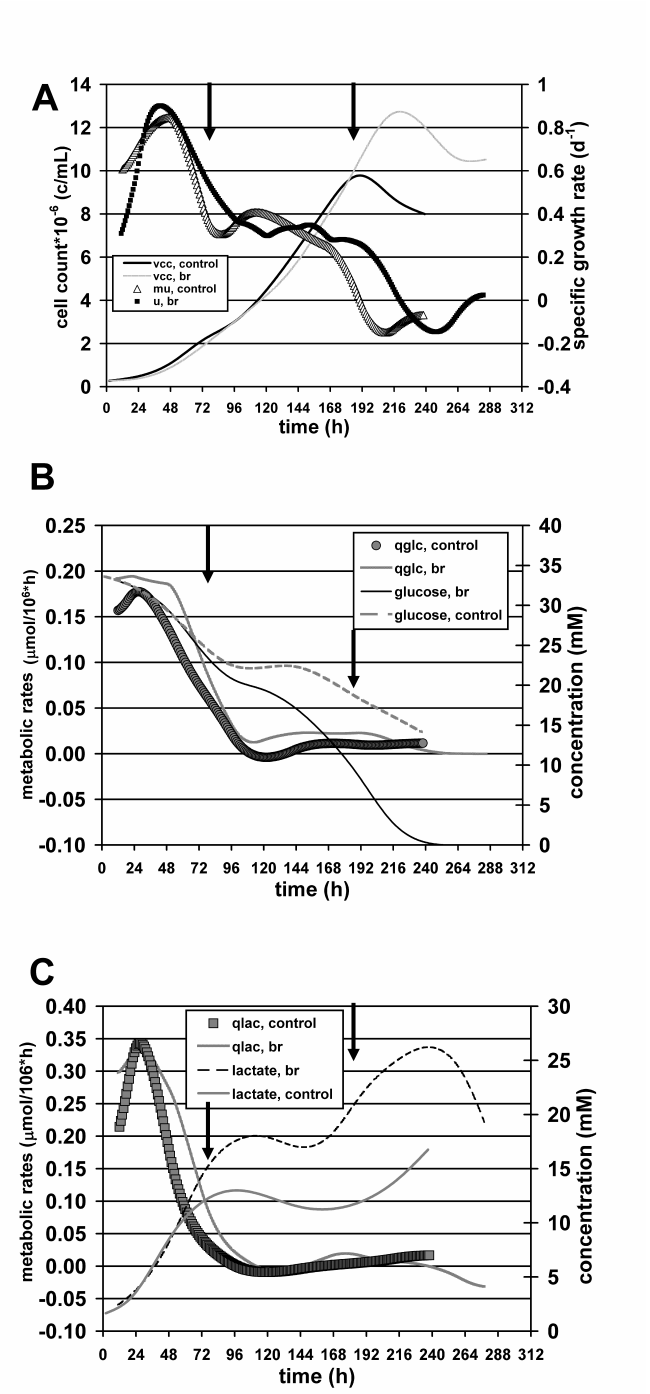


Figure AIV.11: Shake flask cultures in different medium compositions. Cultures were inoculated at cell density of ~3x10^5 c/mL and grown in regular medium (HyQ) and at supplement (CB5 reg) concentrations of 5 and 10 % v/v

ADDITIONAL DATA FOR APPENDIX III

Practical applications of Online Permittivity Signals: Characterization of HEK293 Batch Growth and Identification OF metabolic Shifts

Supplementary Figure AIII.1/Figure AIV.12:



Control shake flask cultures (sample taken from the bioreactor 1.5 h after inoculation) showed similar metabolic and growth profiles compared to the mother culture in the bioreactor; arrows mark the end of the exponential growth and beginning of death phase, respectively. The death phase began earlier in shake flask control cultures, most likely because of elevated lactate concentrations, causing a decrease in pH. At the end of the culture, (~250 h, the pH of control was determined at 6.3.

(A) *vcc* and growth rates

(B) glucose concentrations and consumption rates

(C) lactate concentrations and production rates

THE EFFECT OF SODIUM BUTYRATE ADDITION TIME POINT ON LV PRODUCTION

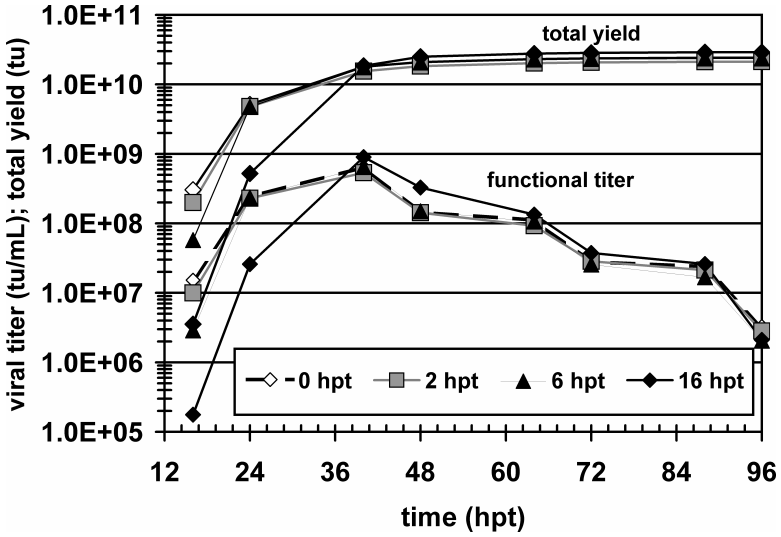


Figure AIV.13: The addition of sodium butyrate at different time points post-transfection (0, 2, 6, 16 hpt) was evaluated using transfection at $5x10^6$ c/mL in HyQ+ medium.

Impact of butyrate was previously observed (see chapter 3) to act as a strong inducer of LV production, resulting in yield improvement of ~15-fold at a concentration of 5 mM. When adding butyrate at different time points post-transfection (0-16 hpt), the LV release with titers $>1x10^7$ was shifted to earlier harvest points. The first harvest (16 hpt), which typically contained only small amounts of LV under improved production conditions, contained $>1x10^7$ tu/mL when butyrate was added as early as 0-2 hpt; simmilarly, the second harvest (24 hpt) also showed significantly increased titers). Yet, the overall yield was not improved in any of the tested alternative production conditions with the addition of butyrate at 16 hpt resulting in highest LV yields.

The addition of an even later addition of butyrate might therefore be evaluated. However, it needs to be kept in mind that this might further delay LV release/ i.e. prolong the overall process and, with regards to production in bioreactor, perfusion time.

The results also indicate that although the intial plasmid uptake is a fast process leading to high functional LV titers as early as 16 hpt, expression of all LV-encoding proteins at highest efficiency takes only places at later time points, i.e. 1-3 dpt. A further analysis of expression kinetics could this be useful to further optimize muli plasmid transfection systems.

ADDITIONAL OBSERVATIONS DURING LV PRODUCTION

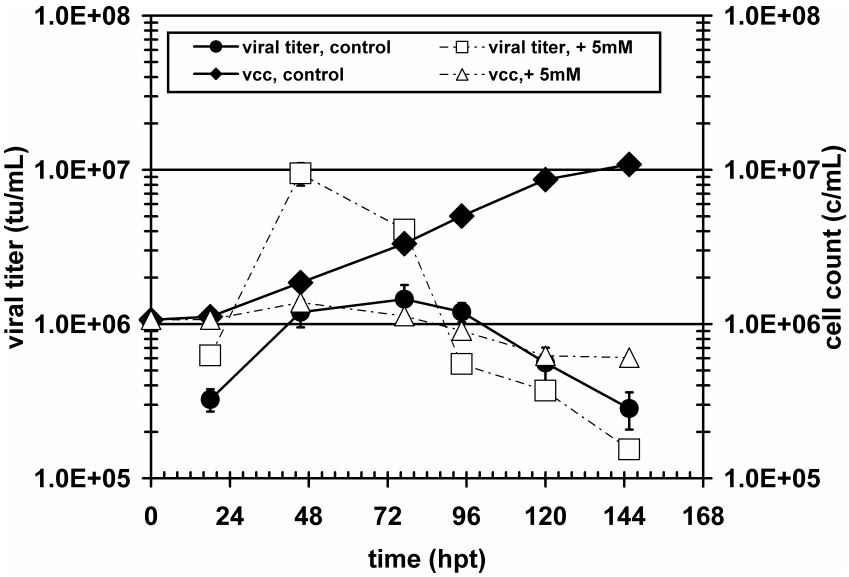


Figure AIV.14: LV production with and without the addition of sodium butyrate at 5 mM. In these initial experiments in LC-SFM L medium, the butyrate stock was not neutralized and pH <<7. This was corrected in later experiments (chapter 3).

Figure AIV.15: High total GFP expression at 4 dpt in bioreactor perfusion culture (picture taken during run #4)

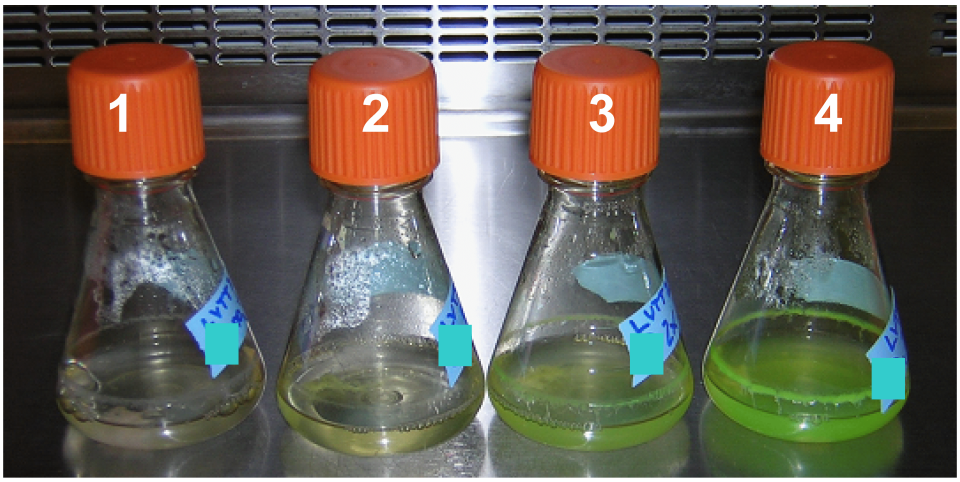


Figure AIV.16: Small scale LV production 4 dpt. Cultures were transfected using improved production conditions at different cell densities: (1): $2x10^6$; (2): $5x10^6$; (3): $1x10^7$; (4) $2x10^7$ c/mL. Note the dramatic and increasing cell ring formation at the inner shake flask surface

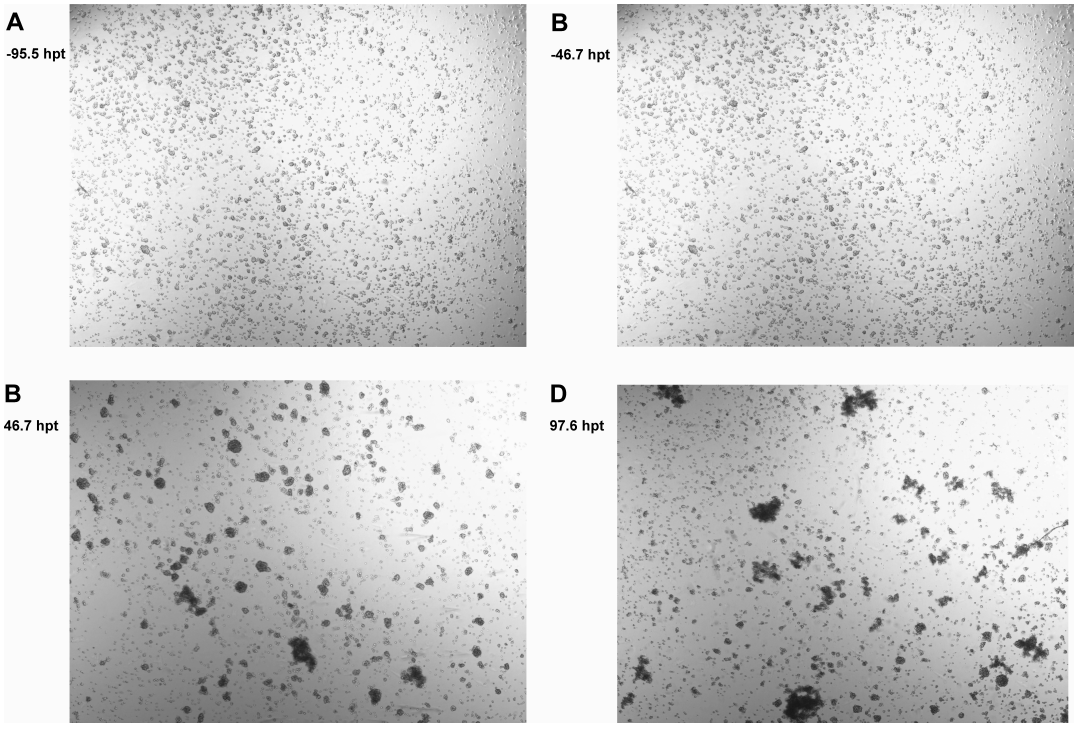


Figure AIV.17: Qualitative evolution of cellular aggregation during bioreactor perfusion culture (run #4). Figure legends indicate the time post-transfection. Immediately after inoculation (A), almost no cellular aggregates were observed. Aggregation then increased progressively after the perfusion start (B) and reached high levels at 2 (C) and 4 dpt (D)