Ghent University
Faculty of Pharmaceutical Sciences

EXPLORING NEW APPLICATIONS FOR PHOTOPHYSICALLY ENCODED MICROCARRIERS

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Ghent, September 2008

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تقدیم به، امین ونیلوفر

من گره خواهم زد، چشمان را با خورشید، دل ها را با عشق، سایه ها را با آب، شاخه ها را با باد.

هر چه دشنام از لب ها خواهم برچید.

هرچه دیوار، از جا خواهم بر کند.

آشتی خواهم داد.

آشنا خواهم کرد.

راه خواهم رفت.

نور خواهم خورد.

دوست خواهم داشت.

سهراب سپهری

I shall graft eyes to the sun, hearts to love, shadows to water and branches to the wind

I shall remove every obscenity from the lips

I shall remove every wall from the root

I shall reconcile

I shall familiarize

I shall eat light

I shall love

Sohrab Sepehri

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سپاس

Bedankt

Thank you

Farzaneh

September 2008

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AAV	Adeno-Associated Virus
AdV	Adenovirus
Ad-RFP	Adenovirus expressing Red Fluorescent Protein
APDN	Applied DNA Sciences
ATTC	American Type Culture Collection
A549	Carcinomic human alveolar basal epithelial cells
BSA	Bovine Serum Albumin
BPEI	Branched Poly(Ethyleneimine)
CHO	Chinese Hamster Ovary cells
CLSM	Confocal Laser Scanning Microscopy
COS	Monkey kidney fibroblasts
Cr	Chrome
CrO_2 NP	Ferromagnetic chromium dioxide nanoparticles
DEP	Dielectrophoresis
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Desoxyribonucleic Acid
DOPE	1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine
DOTAP	N-(1-(2,3-Dioleoyloxy)propyl)-N,N,N-Trimethylammoniumchloride
DS	Dextran Sulfate
dsDNA	Double Stranded DNA
dsRNA	Double Stranded RNA
EBL	Electron Beam Lithography
EDTA	Ethylenediaminetetraacetic Acid
eGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme-Linked Immuno Sorbent Assay
EtBr	Ethidium Bromide
EZ4U	'Easy for you' cytotoxicity assay
FBS	Fetal Bovine Serum
FCS	Fluorescence Correlation Spectroscopy
FDA	US food and Drug Administration

FITC	Fluorescein Isothiocyanate
GFP	Green Fluorescent Protein
GPCR	G-Protein-Coupled Receptor
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HEK	Human Embryonic Kidney cells
Hela	Carcinomic human cervical cells
HIV	Human Immunodeficiency Virus
HPMC	Hydroxypropylmethylcellulose
HSV	Herpes Simplex Virus
HuH-7	Human Hepatoma-7 cells
INF-7	Influenza virus - 7 derived peptide
IgG	Immunoglobulin G
kDa	Kilodalton
LbL	Layer-by-Layer
LD50	Lethal Oral Dose
L-Gln	L-Glutamine
LIF	Leukemia Inhibitory Factor
LPEI	Linear Poly(Ethylene imine)
LPS	Liposome
LPX	Lipoplex
MPa	Megapascal
4-MUP	4-Methylumbellifery Phosphate
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MW	Molecular Weight
N:P ratio	Nitrogen to Phosphate ratio
pAsp	Poly-L-Aspartic Acid
PAH	Poly(Allylamine hydrochloride)
PbAE	Poly(β-Amino Ester)
PBS	Phosphate Buffered Saline
PCS	Photon Correlation Spectroscopy
pDMAEMA	Poly(2-Dimethylaminoethyl Methacrylate)
pDNA	Plasmid DNA
PEG	Poly(ethylene Glycol)

PEs	Polyelectrolytes
PEI	Poly(ethylene imine)
PEM	Poly Electrolyte Multilayer
pLL	Poly-(L-Lysine)
PLGA	poly(lactic-co-glycolic acid)
PQ-4	Polyquarternium-4
PQ-10	Polyquarternium-10
PSS	Poly(Styrene sulfonate)
P/S	Penicilline-Streptomycine
Rcf	Relative Centrifugal Force
RFID	Radio Frequency Tags
RFP	Red Fluorescent Protein
RNA	Ribonucleic Acid
RTCM	Reversely Transfected Cell Microarray
SD	Standard Deviation
SEAP	Secreted Alkaline Phosphatase
siRNA	Small Interfering RNA
TCMs	Transfected-Cell Microarrays
TNF-α	Tumor Necrosis Factor-α
UV	Ultraviolet
Vero	African green monkey kidney epithelial cells
w/v	Weight/Volume
WHO	Word Health Organization

LIST OF SYMBOLS

λ_{ex}	Excitation Wavelenght
λ_{em}	Emission Wavelenght
ζ	Zeta Potential
η	Intrinsic viscosity in water at 25°C

General Introduction

Aim and Outline of this Thesis

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The need to carry out many assays simultaneously in biomedical research has promoted the development of multiplexing technologies. The main goal of “multiplexing” is the rapid and simultaneous screening of (a) biological samples (in the clinical biology) and/or (b) large libraries of chemical compounds for their binding to target molecules (in drug discovery programs). As explained in Chapter 1 of this thesis, multiplex assays can be performed on "positional arrays" or on "non positional arrays". In non positional cellular arrays cells are grown on encoded micron sized carriers; the biological activity of the compounds are screened in the cells that are growing at the surface of the carriers; the code of the microcarrier allows one to know which cell type is present at its surface.

Recently our group has introduced photophysically encoded microparticles named “Memobeads”. The major aim of this thesis is to investigate the applications of these microparticles. First we investigate to which extent Memobeads could be of use in non positional cellular array technologies. Later they were evaluated for a totally different purpose, namely as a tool to protect pharmaceutical products from counterfeiting.

In **Chapter 1** the main nucleic acid delivery systems are summarized and an overview is given on multiplexing technologies that allow screening of different compounds against a large numbers of targets. Additionally, “reverse transfection” and its use in cell microarrays are discussed. Finally, digitally encoded microparticles are introduced.

In **Chapter 2** the physicochemical and transfection properties of cationic hydroxyethylcellulose/plasmid DNA (pDNA) nanoparticles are investigated and compared with the properties of DNA nanoparticles based on polyethylene imine (PEI) which is widely investigated as gene carrier.

Chapter 3 shows how digitally encoded microparticles can be loaded with different kinds of cells, keeping the digital code in the microcarriers readable. Also the possibility to perform ELISA's on cells that grow on the encoded microcarriers is shown.

It is further shown in **Chapter 4** that, after optimizing the surface, encoded beads are suitable for immobilizing viral vectors or nucleic acids, either in naked form or complexed. We showed that cells growing at the surface of the beads could become transduced with adenoviral particles.

Finally, **Chapter 5** shows that Memobeads may become a major tool in combating counterfeiters of pharmaceuticals, by incorporating the encoded microcarriers in drug tablets.

Chapter 1

Introduction

ABSTRACT

The technology of gene transfection has become more and more important because of its application in therapeutic and basic research. Subsequently, this has led to the development of many different transfection techniques, all aiming to efficiently introduce genetic materials into host cells. Generally, two systems can be distinguished, viral and non-viral. Both of these methods have advantages and disadvantages. This chapter gives an overview of these methods.

The genetic materials, either in free form or complexed with chemical reagents, can be delivered to the cells by direct administration or after immobilization on a solid surface. Immobilizing nucleic acids onto the surface of a culture substrate prior to cell seeding, termed reverse transfection, has different applications than the traditional gene delivery approach such as transfected cell microarrays and tissue engineering.

Several approaches have been employed for nucleic acid immobilization on solid surface. Multilayered polyelectrolyte films are one of the possibilities not only to immobilize nucleic acid on solid surface but also to control its delivery. We introduced encoded microcarriers to use as a non-positional microarray for reverse transfection and also other molecular analysis.

Chapter 1

Introduction

INTRODUCTION

Gene Delivery

Gene delivery is a promising technology that has numerous applications in both basic and applied science such as gene therapy, tissue engineering, and transfected cell arrays. This method with high potential for treating chronic diseases and genetic disorders has drawn a lot of attention. Gene therapy involves the delivery of (a) DNA that encodes for an absent protein, to cells and/or (b) siRNA or antisense oligonucleotides to knock down unwanted or deregulated proteins [1-4].

Due to rapid degradation by the nucleases, its large size and its high negative charge, naked DNA is not really suitable for gene delivery though it has been shown that muscle cells can become transfected with naked DNA [5]. Especially, the membrane of cells is negatively charged and naked DNA can not interact with the cell membrane. Because of these limitations, to enhance the internalization of DNA into the cell, it is necessary to use DNA-carrier complexes. Development of efficient and safe delivery systems is really critical for successful gene transfer.

An efficient gene delivery system should overcome different extracellular and intracellular (Fig 1) barriers. These barriers include: (a) polynucleotide degradation in extracellular space, (b) internalization of the carrier, (c) intracellular trafficking from endosome to the lysosome, (d) dissociation of the polynucleotides from the carrier and (e) entry of the polynucleotides into the nucleus. For *in vivo* applications, many other factors, including the physicochemical properties of the carrier in the blood and its immunological properties should be considered as well [6].

Gene delivery systems can be categorized in two types: viral [7-10] and non-viral transfection [11-15] systems. Viruses are powerful gene transfer agents but there still remain concerns about insertional mutagenesis, immunogenicity and cytotoxicity of this system [16-21]. In contrast, non viral systems are much safer and additionally have less limitation in the molecular size of the DNA they have to carry [22]. However, low transfection efficiency seriously limits this method. To improve this low efficiency, several cationic liposome [23-25] and cationic polymers [26-32] have been investigated in different research programmes. The ideal vector should (1) be non-immunogenic (2) show high transfection/transduction; (3) allow controlled and targeted transgene expression and (4) be cheap.

Gene delivery must be selected according to the specific therapeutic aim. But the perfect vector covering all therapeutic and safety requirements does not exist and much work is needed in this important research field.

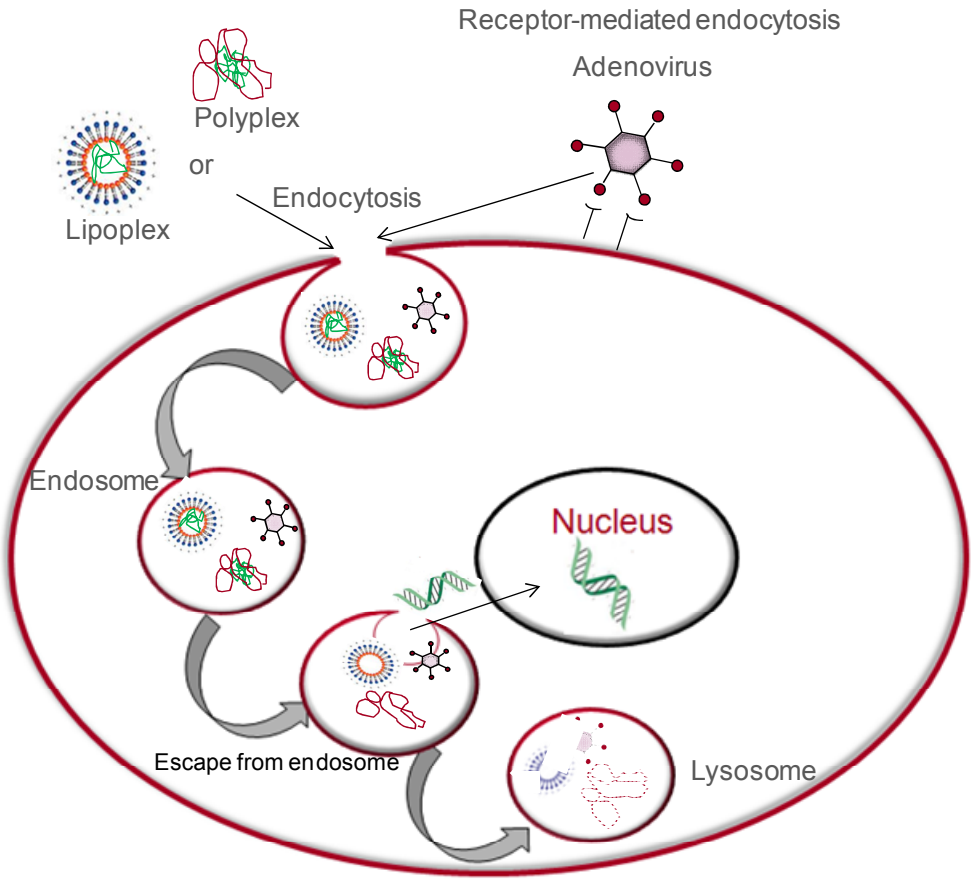

Figure 1. Schematic representation of the cellular steps of gene delivery using lipoplexes, polyplexes and viral vectors.

Viral Gene Delivery Systems

Viral vectors still provide the most efficient means of gene delivery. Particular viruses have been selected as gene delivery vehicles because of their capacity to carry foreign genes and their ability to efficiently deliver these genes associated with efficient gene expression. These are the major reasons why viral vectors derived from retroviruses, adenoviruses, adeno-associated viruses and herpes viruses are employed in more than 70% of the clinical gene therapy trials worldwide. Because these vector systems have unique advantages and limitations, each type of virus has specific applications for which it is best suited [33].

Novel developments of viral carrier systems mainly aim to reduce their side effects such as immunogenicity and cytotoxicity [34] and to improve infectivity, viral targeting, cell type specific expression and the duration of expression [35]. One of the major obstacles in the use of viral vectors as therapeutic tools is the inability to control vector targeting. Different strategies were developed for localized delivery of viral vectors [36].

Retroviruses

Retroviruses are small RNA viruses that replicate through a DNA intermediate and their average diameter is ~100 nm. The retrovirus infects target cells through a specific interaction between the viral envelope protein and a cell surface “receptor” on the target cell. Retroviral vectors require mitotic cell division for transduction and can permanently integrate into the genome of the infected cell without expressing any immunogenic viral proteins. Their disadvantages include the facts that the MLV-based retroviral vectors require cell division for stable infection, and that their coding capacity prevents the delivery of large genes. The recent development of vectors based on lentiviruses (a subclass of the retroviruses) such as HIV 1, which can infect certain non dividing cells, should allow the *in vivo* use of retroviral vectors for gene therapeutic applications [37].

Adenviruses

Adenoviruses are non-enveloped linear double-stranded DNA viruses of ~70 nm diameter. 51 human AdV serotypes have been identified, grouped in six species (A–F), which mostly cause mild infection of the upper respiratory tract and eye infections [38]. They can carry about 8 kb foreign DNA. Adenoviral vectors can efficiently deliver genes to a wide

variety of dividing and non-dividing cell types [39]. Briefly, they have the ability to infect a wide variety of cell types, including dividing and non-dividing cells, high efficiency of gene transfer, no integration into the host genome, relatively large transgene capacity, easy manipulation and high titers. Their drawbacks include limited duration of transgene expression and immunogenicity *in vivo* [40].

Adeno-associated viruses

Adeno-associated viruses (AAV), a member of the parvovirus family, are single-stranded DNA viruses that require a helper virus such as adenovirus or HSV for replication. They are icosahedral, ~20-25 nm diameter and contain a capsid protein. No pathology appears to be associated with infection by AAV. Wild-type AAV is able to infect non-dividing and dividing human cells and to stably integrate into a specific locus on chromosome 19 [41,42], but has a limited DNA loading capacity. AAV may be highly suitable for the delivery of genes to specific target cells *in vivo*, without inducing an immune response to the infected cells [43].

Herpes simplex viruses

Herpes simplex viruses (HSV) are also being developed for gene therapy applications [44-48]. HSV is a dsDNA virus which has the advantage of being able to infect non-dividing cells, establishing latency in some cell types, and having the capacity to carry large regions of exogenous DNA [49]. Although this virus can deliver large amounts of exogenous DNA, cytotoxicity and maintenance of transgene expression remain as obstacles. The ability to establish latency in neuronal cells makes HSV an attractive vector for treating neurological disorders such as Parkinson's and Alzheimer's [37].

Non-Viral Gene Delivery Systems

Although viral systems are still the most efficient vectors for gene delivery several problems are inherent to this system [50-53]. Besides the clinically dangers of viral gene delivery system, several non-viral systems have been proposed. They have advantages due to their low immunogenicity, their capacity to deliver large genes and the fact that large scale production is possible at low cost [54]. Therefore, there is increasing interest in the use of non-viral delivery system, including physical and chemical approaches.

Physical methods

Physical methods, which are carrier-free, enhance nucleic acid transfer into cells by employing a physical force and temporarily disrupting the cell membrane. Several physical methods have been investigated such as electroporation [55-59], hydrodynamic injection [60-62], sonoporation [63-65], or ballistic pressure (gene gun) [66,67].

Chemical methods

Chemical methods make mostly use of positively charged synthetic or natural carriesr to deliver the nucleic acids into the target cells. This delivery system is broadly subdivided in polymer- and lipid-based carriers. Because of the permanent cationic charge, these carriers interact electrostatically with the negatively charged nucleic acids to form "polyplexes" (being nucleic acid/cationic polymer complexes) or "lipoplexes" (being nucleic acid/cationic lipid complexes), respectively. This complexation protects the nucleic acids against degradation by nucleases, creates a less negative surface charge, compact the size of the nucleic acids which promotes internalization, and can aid the intracellular trafficking of the nucleic acids. Many nucleic acid-carrier complexes aggregate in the presence of salt or serum, which mostly needs the incorporation of poly(ethylene glycol) (PEG) in the complexes to stabilize them [68,69]. The molecular design of non-viral vectors is currently further advancing with the aim to overcome a multitude of barriers, both extracellular and intracellular, that presently limit gene transfer [70-72].

Polymer-based gene delivery

Cationic polymers are a leading class of molecular gene delivery systems [73-78]. In recent years, biodegradable polymers have been designed as nucleic acid delivery systems. Their reduced toxicity and the absence of accumulation of the polymer in the cells after repeated administration are the advantages of these polymers [73]. Commonly used polycations include: poly(ethylene imine) (PEI) [79], cationic dendrimers [80] , poly-(L-lysine) (pLL) [81], poly(ester)s and cationic polysaccharides (dextran spermine) [82] (Fig. 2). Among different polycations used for gene delivery, cationic polysaccharides are attractive candidates. They are natural, with low toxicity, biodegradable and generally biocompatible [54]. Chitosan is one of the most reported non-viral naturally derived polymeric gene carrier from this group [83,84].

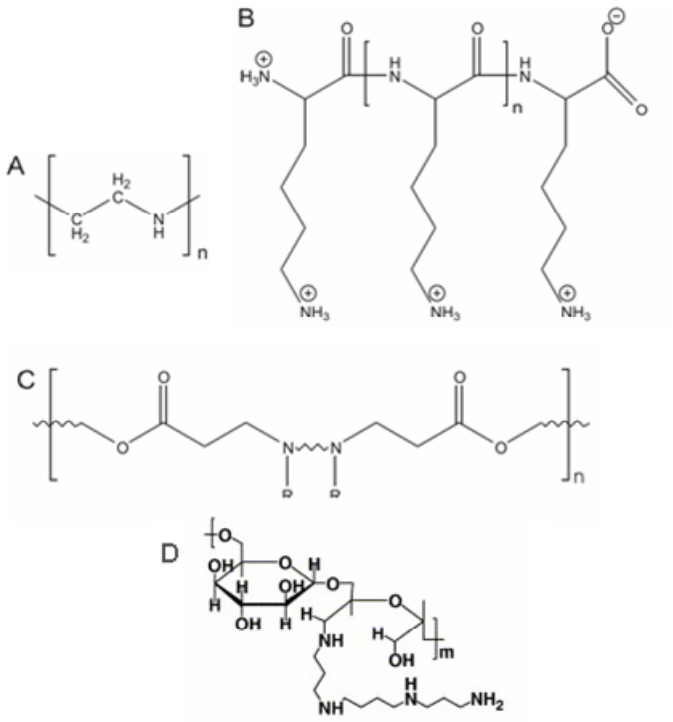

Figure 2. Structure of some cationic polymers commonly used for nucleid acid delivery: A) linear PEI, B) pLL, C) poly(β-amino ester), D) cationic polysaccharides.

Liposome-based gene delivery

Cationic liposomes are spherical, nano-sized artificial vesicles, composed of cationic and other (helper) lipids that form one or more (phospho) lipid bilayers enclosing aqueous compartments [85,86]. Complexation of nucleic acids to cationic liposomes results in positively charged lipoplexes (LPX) which facilitates the interaction with the negatively charged cell membrane. The cationic LPX structure is still under discussion and depends on many factors: an "external" model, in which the nucleic acids are adsorbed onto the surface of cationic liposomes, or an "internal" model, in which the nucleic acids are surrounded or "coated" by a lipid envelope [87]. Cationic liposomes have been investigated extensively as non-viral gene carrier for efficient gene delivery [88-91]. However, the liposomes are often toxic and transfection results can vary significantly between different cell lines. The commonly used cationic lipid N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammoniumchloride (DOTAP), the neutral lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), together with the general structure of LPXs are depicted in Figure 3.

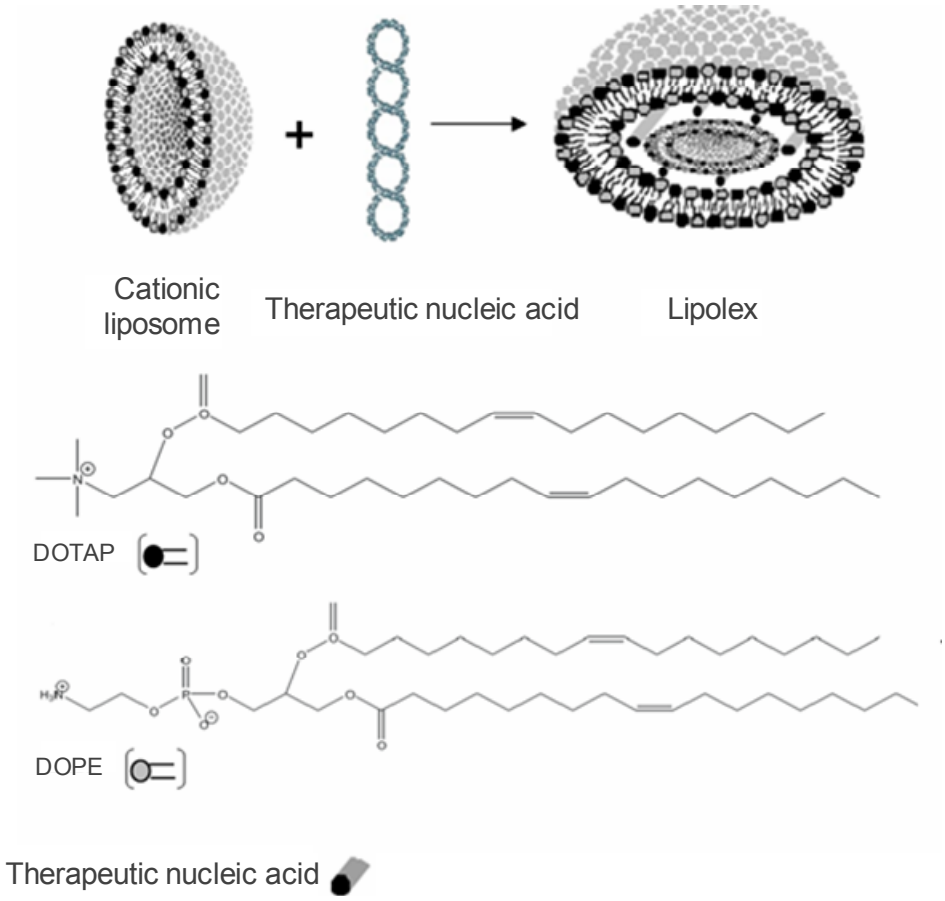

Figure 3. Representation of a cationic liposome, composed of DOTAP and DOPE, and the formation of a lipoplex

Reverse Transfection

Typically, in transfection experiments (*in vitro*) the nucleic acids, either in free form or complexed with chemical reagents, are delivered into target cells by "direct administration"; the nucleic acid solution/dispersion is applied on top of the cells which are typically cultured at the surface of wells.

Recently, immobilizing nucleic acids or viral vectors onto the surface of a culture substrate prior to cell seeding has been proposed. This alternative delivery system was termed "reverse transfection" (in case naked or complexed DNA or siRNA is used) or "reverse transduction" (in case viral vectors are used), because in contrast to conventional transfection protocols, first the DNA is "seeded" followed by addition of the cells (Fig. 4). Reverse transfection, also termed substrate-mediated delivery or solid-phase delivery, may improve transfection/infection efficiency and may be especially useful in the creation of cell

microarrays. The immobilization of nucleic acids may improve the transfection as the nucleic acids are in constant and close contact with the cells. Additionally, transfection is observed only in the location at which nucleic acids are immobilized [92].

Solid supports are being developed that optimally bind the viruses [93,94] or the non-viral DNA complexes [95,96]. Clearly, maximal transfection efficiency requires good balance between a sufficiently strong binding of the DNA material to the support on the one hand and a sufficient release of the DNA from the support into the cells on the other hand [97].

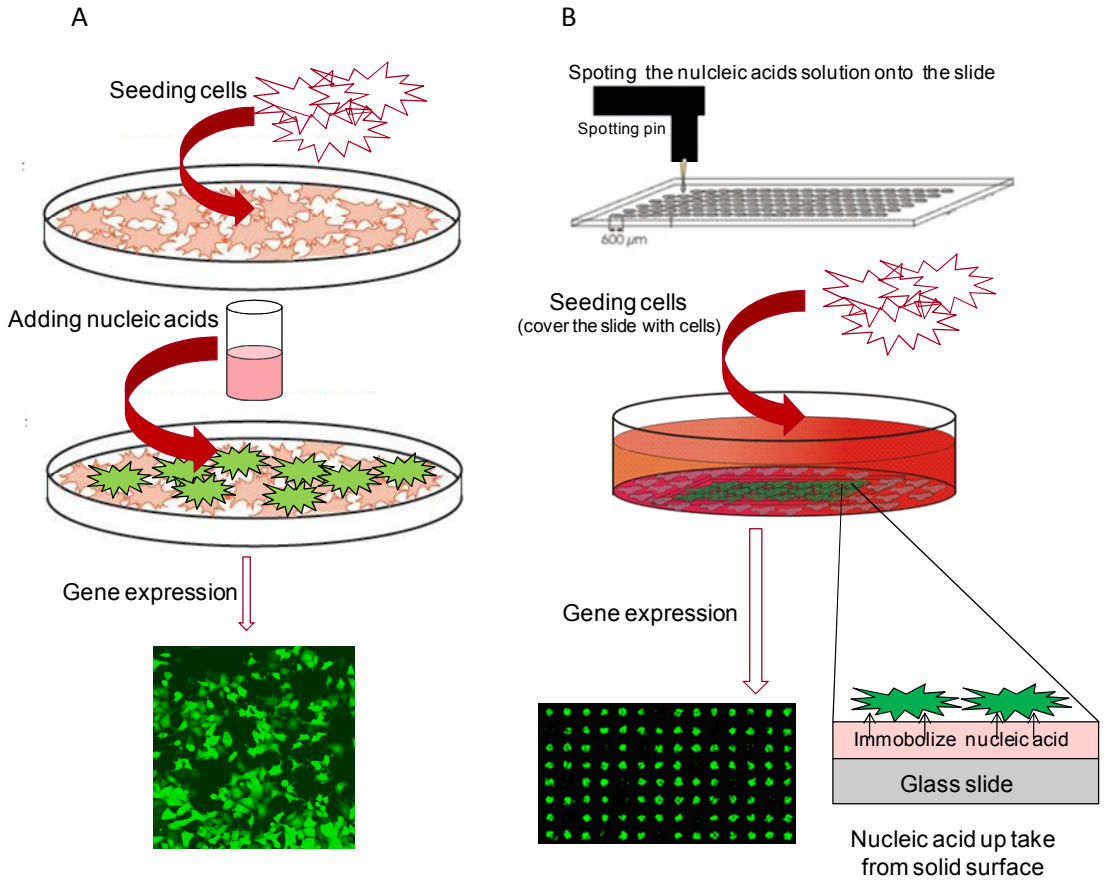

Figure 4. Schematic illustration of *in vitro* cell transfection. A) Bolus addition of nucleic acids to the media after cell seeding. B) Reverse transfection which immobilizes nucleic acid to a solid surface prior to cell seeding.

Several strategies have been developed to immobilize nucleic acids on solid surfaces. They can be immobilized through specific or non-specific interactions or even by multilayered assemblies. Specific interactions can be introduced through complementary functional groups on the vector and surface, such as antigen–antibody or biotin–avidin. Segura et al. have shown that PLL and PEI, modified with biotin residues could complex with DNA and bind to a neutravidin substrate [95,96]. Transfection was maximal when complexes

contained biotin residues attached to a small fraction of the cationic polymers [96] and it was observed only at the location where complexes were bound.

In non-specific interaction, non-covalent mechanisms [98-102], including hydrophobic, electrostatic, and van der Waals interactions are involved. Bielinska et al. [98] were the first to report the use of solid support membranes for DNA delivery mediated by dendrimer based nucleic acid complexes which were non-specifically bound to the support. Ziauddin and Sabatini further developed this challenge by miniaturizing and parallelizing the reverse transfection of cells [103].

A better understanding of the physicochemical properties of the nucleic acid complexes and the substrates may improve the extent of transgene expression and cell viability in reverse tranefection setups [101]. Delehanty et al. [104] quantified the transfection efficiency of mammalian cells on different microscope slide substrates**.** They found that the size and concentration of the pDNA spots on the slides were dependent on the hydrophobicity of the substrate.

Uchimura et al. reported a different method to improve the efficiency of reverse transfection. They proposed to conjugate gold nanoparticles to the DNA/reagent complex which can regulate the size and the density of the complex printed on the solid surface [108]. They also found that an antibody-coated surface allows the most effective delivery of genes by reverse transfection [106].

Multilayered polyelectrolyte films are another possibility to immobilize nucleic acids on solid surfaces. Polyelectrolyte multilayers (PEM) can be/are generated by sequential adsorption of oppositely charged polymers (i.e. polyelectrolytes; so called layer-by-layer (LbL) adsorption) on a charged planar substrate [107]**.** Figure 5 is a schematic illustration of the LbL process. A charged surface (being a flat surface or the surface of e.g. a microcarrier), is immersed into an aqueous solution of an oppositely charged polyelectrolyte. After a certain adsorption time (15 min) the substrate is removed and washed with water in order to remove the excess of polyelectrolyte. In the next step the substrate is immersed into the second polyelectrolyte solution which has a charge opposite to the first polyelectrolyte. This second polyelectrolyte adsorbs onto the layer of the first polyelectrolyte which reverses the surface charge. Again a washing step is performed and the whole procedure can be repeated as many times as one desires. In this way one can easily prepare multilayered films with

tunable physicochemical properties as both the number of layers as well as their composition can easily be varied.

Layer-by-Layer coating technique has become a new and general way to modify and functionalize surfaces and provided the opportunity to develop platforms for delivery of biological materials such as proteins and drugs to the cells [108-114]. Recently, different studies have demonstrated the efficient release of pDNA from multilayered assemblies made of naked pDNA and a degradable polyamine [102,115], or from pre-complexed DNA, with PEI, into multilayer architectures made of neutral degradable and synthetic polyelectrolytes [116,117]. Jessel et al have shown that gene delivery can be enhanced from multilayers by the presence of charged cyclodextrins [118,119].

In general, glass and plastics are used as solid surface in reverse transfection but other materials, such as metals, could contribute to the development of this approach. Many research groups have reported on the developments of stents, which permit gene delivery to endothelial cells by reverse transfection. In these studies, plasmid and adenovirus vectors are loaded onto the stent surface together with various polymers [120-121]. Yamauchi et al [122] made use of an LbL process to deposit cationic lipids and plasmid DNA on gold surfaces to efficiently transfer pDNA to cells. Lynn et al. reported on the fabrication of ultra thin polyelectrolyte films that permit immobilization and controlled release of pDNA from the surfaces of stainless steel intravascular stents [123].

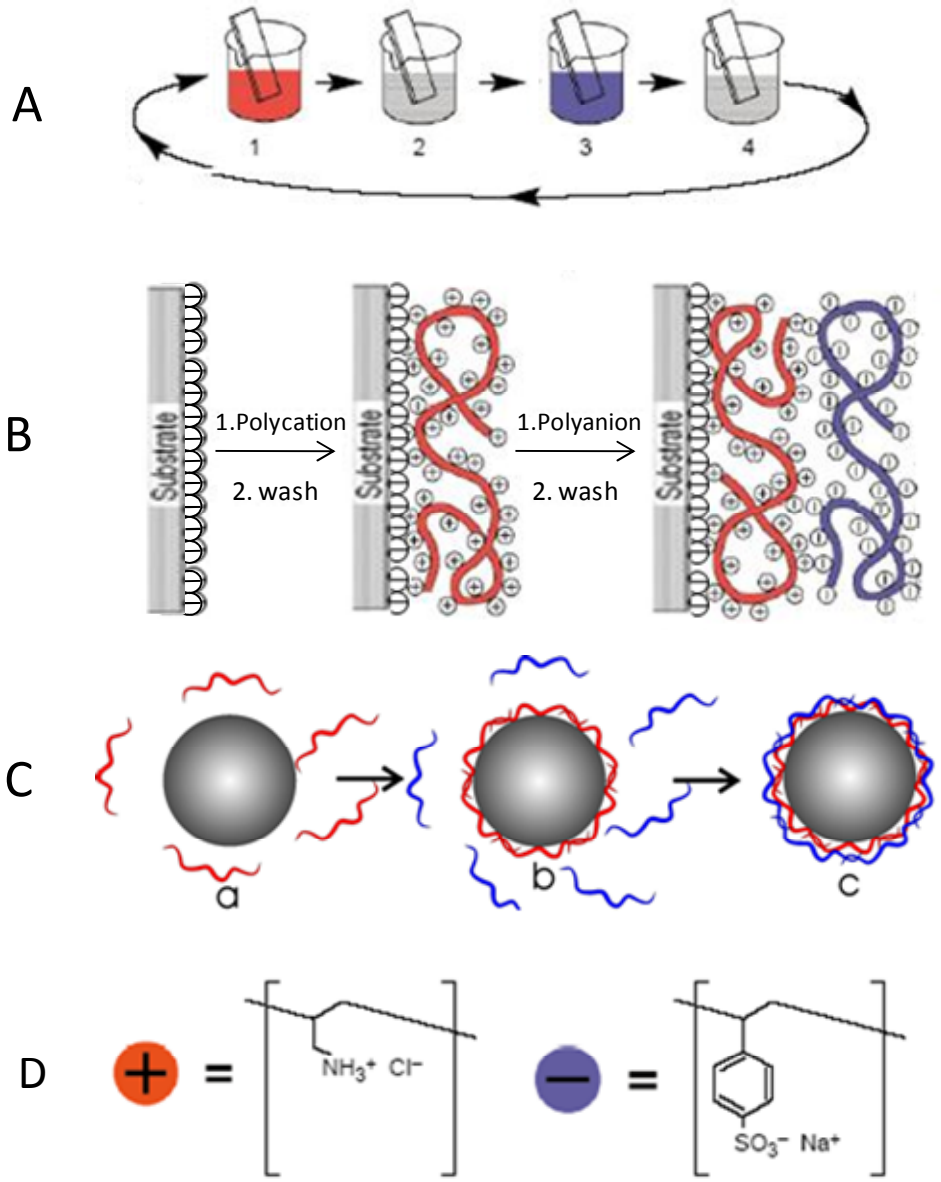

Figure 5. (A) Schematic representation of the deposition of a polyelectrolyte film on a slide. Steps 1 and 3 represent the adsorption of respectively a polycation and polyanion, steps 2 and 4 are washing steps. (B) Simplified molecular picture of the first two adsorption steps, depicting film deposition starting with a negatively charged substrate. (C) Schematic illustration showing the preparation of LbL coated microcarrier(D) Chemical structure of poly(styrene sulfonate) (PSS) (right) and poly(allylamine hydrochloride) (PAH) (left), often used polyions to build LbL films.

Multiplex Assay Technologies

The desire for screening more compounds against a large numbers of targets, for both research and diagnosis applications, has led to multiplex technologies.

A monoplex assay aim to analyse a single compound in e.g. a biological sample while multiplex assays aim to carry out multiple analysis simultaneously in the same sample.

Multiplex assays aim to save both time and reagents by parallelized and miniaturized analysis.

A main challenge in multiplexing technologies is to be able to identify quickly and accurately each reaction. There are two main strategies for doing this, resulting in positional and non-positional arrays [124-127]. One strategy relies on the exact location (x,y coordinate) of the molecules on the solid surface (microtiter plate or microarray) as illustrated in Figure 6A. This method of identification is known as positional encoding. In a second strategy (Fig. 6B), the molecules are bound to microcarriers and the reaction is carried out on their surface, therefore the identification is possible by the code on microcarriers.

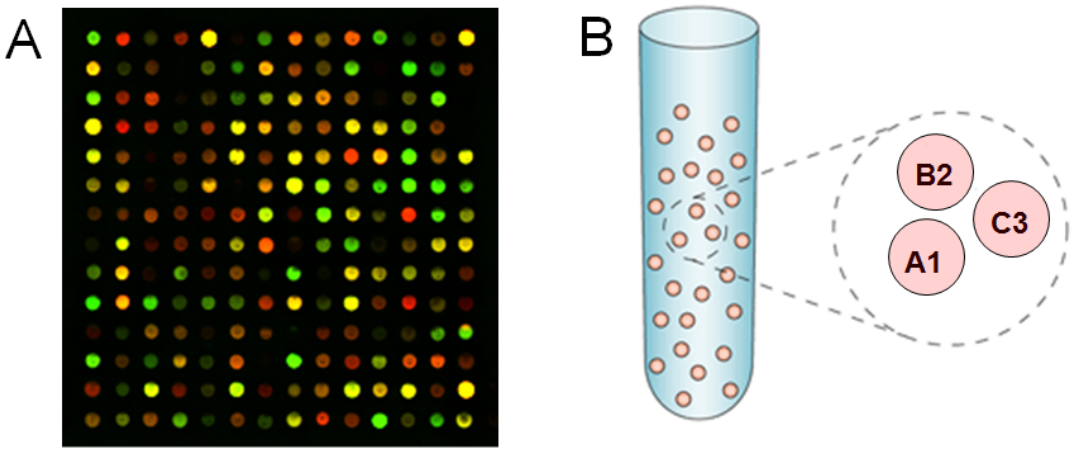

Figure 6. (A) In a positional array (flat surface array) the identity of each probe (being a nucleic acid, a protein or a cell) is known from its location on the array. (B) In a non- positional array (a suspension array) each probe at the surface of the microcarrier is identified by the code written in the microcarrier.

Microarrays

Over the last years, microarray technology has evolved as a propitious platform for the multiplex analysis [128-130]. This technology is a powerful method for life science researchers and is also beginning to be used in diagnostic and treatment application [131-133]. Microarrays consist of immobilized biomolecules (nucleic acids, proteins) on a planer or non-planer surface. The immobilized biomolecules (a) must retain their activity, (2) must remain stable, and (3) may not desorb during reaction or washing steps [134]. Several materials could be used as solid surface for microarrays such as glass and plastics. Though microarray technology was initially developed for DNA mapping [135] and sequencing by hybridization [136-

[139] and also as a tool for transcript-level analyses [140], it could be adapted for the analysis of other molecular entities, such as proteins [141-144], tissue samples and even living cells [145-147]. In fact this technology has meanwhile spread into many areas and became a device for diverse purposes [148-149].

Positional microarrays

In a positional or planer array (Fig. 6A), which is a common format to perform multiplexed assays, molecules such as antibodies or nucleic acids, are immobilized on a two-dimensional grid. The identification of each molecule relies on its position in the grid.

The main advantage of positional arrays is that they allow thousands of individual tests to be performed in parallel but they have also some limitations [150]. Positional arrays are often inflexible because they impose a predetermined panel of tests on the user. The rate of binding on positional arrays and also their sensitivity are important limiting factors. Positional arrays have had major impact on high throughput screenings, but because of their limitations recently the possibility of using suspension arrays (see below) has emerged as an alternative.

Non positional microarrays

Recently, non-positional microarrays (Fig. 6B), also known as suspension arrays, have proven to be a very promising alternative to positional arrays due to their inherent and unique characteristics, such as a higher flexibility in array implementation and no requirement for a sophisticated high-precision spotting device [151]. The use of microspheres also can facilitate the separation and washing steps and they are inexpensive to produce in large number [150].

In this technique, each of the microcarriers needs to be encoded to allow the identification of molecule bound to its surface. This method allows uniquely encoded microcarriers to be mixed and subjected to an assay simultaneously [152].

Meanwhile a large number of techniques for the encoding of microparticles have been reported: spectrometric [153-157], graphical [158-163], electronic [164-165] and physical [166-167].Recently, our group introduced a way for encoding microcarriers (being fluorescent polystyrene microspheres) that holds much promise for use in biomedical research and diagnostics. These microspheres, called Memobeads, were encoded by spatial selective

photobleaching. As shown in Figure 7 they can carry a digital code (like a dotcode or a barcode) in their middle plane. This technique provides a virtually unlimited number of unique codes, hence overcoming the (sometimes) limitations of other encoding strategies [162].

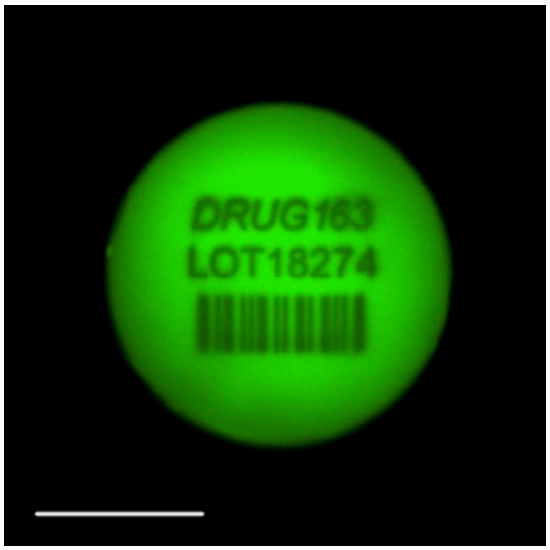

Figure 7. A 40 µm diameter fluorescent microsphere with a bleached barcode and alphanumeric code.

Our group showed recently that a LbL coating, composed of polyanions (like polystyrene sulfonate; PSS), polycations (like polyallyamine hydrochloride; PAH) and ferromagnetic chromium dioxide nanoparticles (CrO_2 NP), can be easily applied (Fig. 8) [168]. The CrO_2 NP allows an optimal positioning of the Memobeads in a magnetic field, which is necessary to read the code at the time of decoding (Fig 8). On top the LbL coating is ideally suitable for binding probes (like antibodies, nucleic acids) at the surface of the beads.

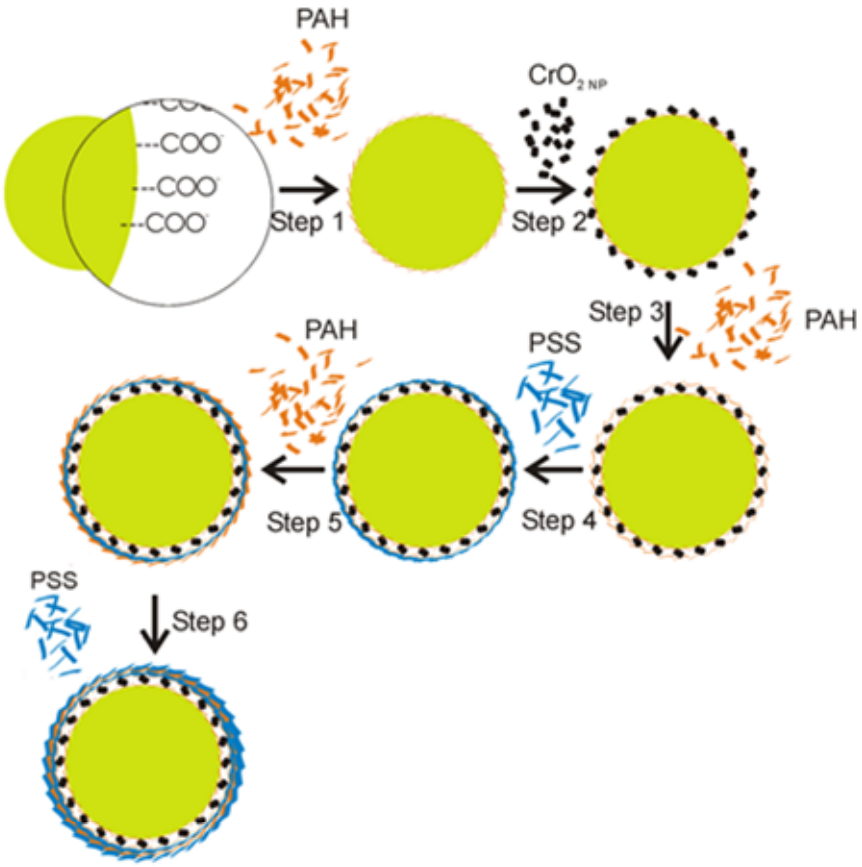

Figure 8. Schematic illustration showing LbL coating of green fluorescent polystyrene microspheres. The polycation PAH and the polyanion PSS are alternately adsorbed to the surface of the microcarrier. Magnetic CrO_2 nanoparticles are immobilized in the LbL coating [168].

Cell microarrays

In cell microarrays cells are grown respectively on the flat surface of a positional array or on the surface of encoded carriers in non positional arrays. Cell microarrays, in which the cells are analyzed for various types of responses, show many advantages as they allow using very small volumes of expensive reagents and small number of rare cells. The applications of cell microarrays could be very diverse. Though, challenges/limitations such as cell destruction, low efficiency of transfection, difficulties in detecting signals in the cells currently remain. The most obvious application for cell microarrays is the biological screening of compounds. An even more promising avenue would be the analysis of gene expression and/or gene knock down due to the uptake of compounds (or plasmid DNA or siRNA) by cells.

Cell microarray can be divided into two main categories depending on whether cells themselves are microarrayed (genuine cell arrays) or cells are bound to microarrayed biomolecules (substrate based cell array)

Genuine cell microarrays

The different types of cells are arrayed on a flat surface (and thus identified by the array coordinates; positional array) or on encoded carriers, and are probed with one or multiple reagents like e.g. antibodies or pharmaceutical compounds.

In a positional cell array, immobilization of cells into a precise position is challenging. One way to generate cell microarrays is by miniaturizing microliter plates: plates containing 96 wells (with a volume between 100 nl and 1 µl) which are addressable by a piezonozzle have been developed [169]. Stephan et al. developed an array containing several types of frozen cells that can be labelled with specific reagents, including antibodies, DNA or RNA probes.[170]. Another way to generate a cell array is by printing cells on a glass slide. Such slides are covered with cell-repulsive tri-ethoxy amino propylsilane (which inhibits adhesion of cells to the slide) except at certain spots. This results in a matrix of circular spots on which cells are growing [171,172].

Ink-jet printers have been adapted to print cells without major loss in viability [173]. It is also possible to print fixed cells using a pin ring arrayer, in which cells are captured within the ring and pushed onto the surface by the pin [174]. The force of dielectrophoresis (DEP) is another way to pattern cells in a microarray. As cells are polarizable bodies, when they flow over an array of electrodes they trap above the electrode [175,176].

However, direct cell arraying remains particularly challenging because most devices used for this purpose (such as piezonozzle and ink-jet devices) can have a deleterious effect on cells. Although cells can stay alive, it is possible that their physiology is affected by the arraying process. So far, this issue has not been extensively evaluated.

As explained above, in a non positional cell array cells are identified based on codes in the carriers. The nature of this code can be diverse but so far only two types of coding approaches have been demonstrated for carriers loaded with cells. The first technology describes a platform in which cells grow on small cards that can be manufactured with different materials, which are encoded colored bars. The array is decoded by imaging with an automated microscope and appropriate software. Using this system, up to nine cell lines

have been assayed in parallel [177]. Another technology uses nanometer-sized semiconductor crystals, quantum dots, which are incorporated into the cells (without affecting cell physiology) to encode the cells [178,179]. Matteakis et al. showed that different sizes of quantum dots emit their fluorescence at different wavelengths when excited by light. They were able to generate up to 1023 codes with combinations of five colours [180]. Such nanocrystals can be internalized by different cell types and the resulting encoded cells are analyzed by optical methods. Tagging of cells by the quantum dot-coding method can be applied to both adherent and suspension cells.

Substrate cell microarrays

For this type of array cells are seeded on biomaterials microarrayed on solid surface. Different substrates such as polymers, carbohydrates, antibodies, proteins, pDNA or siRNA are arrayed and interact with the attached cells. Therefore this technology allows exploration into the effects of a large variety of biomolecules on cell behaviour. They are promising tools for the large scale screening of such substrates. Here we further focused on transfected cell microarray technique which considered a substrate-cell microarray.

Transfected cell microarrays

Recently, several reports have suggested new screening approaches in which living-cell microarrays are used for reverse transfection with plasmid DNA or siRNA; such technologies should allow the simultaneous high throughput analysis of the function of many different genes in many different cell types [103, 181-184]. These new tools appear to be very promising for the rapid identification of protein function [185,186].

Sabatini et al. were pioneering reverse transfection of cells grown on glass slides [103]. One nanoliter of pDNA in an aqueous gelatin solution was printed at a number of locations on a glass slide, using a robotic arrayer. After drying, the DNA spots were exposed to a lipid transfection reagent and subsequently the slide was placed in a culture dish to become covered with adherent mammalian cells. Cells growing on the printed areas took up the DNA, creating spots of transfected cells. Figure 8 shows an example of the microarrays that can be created with this approach.

The employment of siRNA to knockdown expression of selected genes by Mousses et al. significantly extended the use of transfected microarray [187].

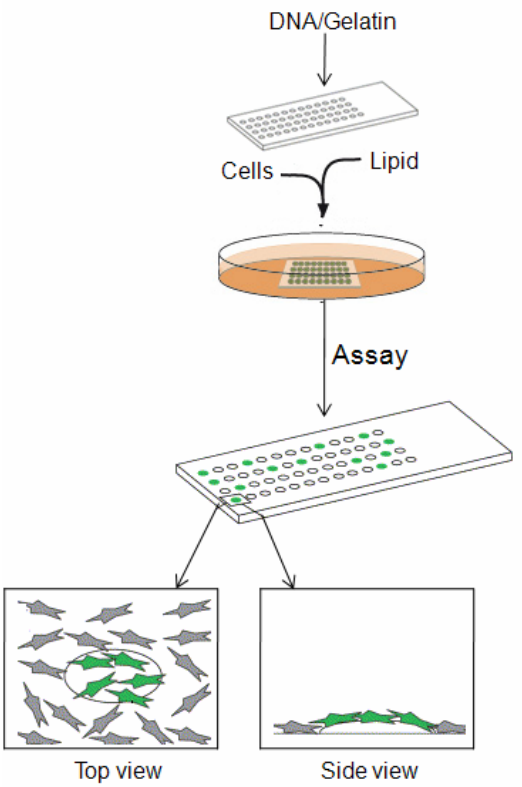

Figure 9. Schematic illustration of the procedure used to obtain a cell microarray by reverse transfection [110]. The array consists of cell clusters expressing green fluorescent protein, each cluster about 120 µm in diameter and containing about 50-80 transfected cells.

Later, Sabatini et al. also described lentivirus-infected cell microarrays for high-throughput screenings [188]. Carbone et al. [189] have presented a cell microarray for phenotype screening on primary cells and cancer cells based on the reverse transduction of the cells by retroviruses. In this work the viral vectors were immobilized on a glass slide coated with a titanium dioxide layer, cells were subsequently plated on the slide in the absence of reagents such as lipofectamine and polycations.

It has been recently demonstrated how transfected cell microarrays can be successfully used in drug discovery [190]. However, despite several attempts to improve this technology, it remains a challenge to obtain transfected cell microarrays for screening.

REFERENCES

1. Gilmore I.R., Fox S.P., Hollins A.J., Akhtar S., Delivery strategies for siRNA-mediated gene silencing, Curr. Drug Deliv. **2006**, 3, 145–47.

2. Li C.X., Parker A., Menocal E., S. Xiang, L. Borodyansky and J.H. Fruehauf, Delivery of RNA interference, Cell Cycle **2006**, 5, 2103–109.

3. Tan F.L., Yin J.Q., Application of RNAi to cancer research and therapy, Front. Biosci. **2005,** 10, 1946–60.

4. Tuschl T., Borkhardt A., Small interfering RNAs: a revolutionary tool for the analysis of gene function and gene therapy, Mol. Interv. **2002**, 2, 158–67.

5. Giannoukakis N., Thomson A.W., Gene therapy in transplantation, Gene Ther. **1999**, 6, 1499–1511.

6. Li S.D., Hung L., Gene therapy progress and prospects: non-viral gene therapy by systemic delivery, Gene therapy, **2006**, 13, 1313-19.

7. Robbins P.D., Ghivizzani S.C., Viral vectors for gene therapy, Pharmacol. Ther. **1998**, 80, 35–47.

8. Duisit G., Salvetti A., Moullier P., Cosset F.L., Functional characterization of adenoviral/retroviral chimeric vectors and their use for efficient screening of retroviral producer cell lines, Hum. Gene Ther. **1999**, 10,189–200.

9. Kim Y.S., Lim H.K., Kim K.J., Production of high-titer retroviral vectors and detection of replication-competent retroviruses, Mol. Cells **1998**, 8, 36–42.

10. Peng K.W., Russell S.J., Viral vector targeting, Curr. Opin. Biotechnol. **1999**, 10, 454–57.

11. Felgner P.L., Ringold G.M., Cationic liposome-mediated transfection, Nature 337 **1989**, 337, 387–88.

12. Nishi T., Yoshizato K., Yamashiro S., Takeshima H., Sato K., Hamada, Kitamura I., Yoshimora T., Saya H., Kuratsu J., Ushio Y., High-efficiency in vivo gene transfer using intraarterial plasmid DNA injection following in vivo electroporation, Cancer Res. **1996**, 56, 1050–55.

13. Toncheva V., Wolfert M.A., Dash P.R., Oupicky D., Ulbrich K., Seymour L.W., et al., Novel vectors for gene delivery formed by self-assembly of DNA with poly(L-lysine) grafted with hydrophilic polymers, Biochim. Biophys. Acta **1998**, 1380. 354–68.

14. Oupicky D., Konak C., Ulbrich K., Wolfert M.A., Seymour L.W., DNA delivery systems based on complexes of DNA with synthetic polycations and their copolymers, J. Control. Release **2000**, 65, 149–71.

15. Nishikawa M., Huang L., Nonviral vectors in the new millennium: delivery barriers in gene transfer, Hum. Gene Ther. **2001**, 12, 861–70.

16. Zhang X., Godbey W.T., Viral vectors for gene delivery in tissue engineering, Adv. Drug Deliv. Rev. **2006**, 58, 515–34.

17. McTaggart S., Al-Rubeai M., Retroviral vectors for human gene delivery, Biotechnol. Adv. **2002**, 20, 1–31.

18. Gorecki D.C., Prospects and problems of gene therapy: an update, Expert Opin. Emerging Drugs **2001**, 6, 187–98.

19. Liu Q., Muruve D.A., Molecular basis of the inflammatory response to adenovirus vectors, Gene Ther. **2003**, 10, 935–40.

20. Sun J.Y., Anand-Jawa V., Chatterjee S., Wong K.K., Immune responses to adeno-associated virus and its recombinant vectors, Gene Ther. **2003**, 10, 964–76.

21. Zaiss A.K. & Muruve D.A. Immune responses to adeno-associated virus vectors. Curr. Gene Ther.**2005** 5(3) 323-331.

22. Jo J., Tabata Y., Non-viral gene transfection technologies for genetic engineering of stem cells. Eur. J. Pharm. Biopharm. **2008**, 68, 90-104.

23. Audouy S.A., de Leij L.F., Hoekstra D. Molema G., In vivo characteristics of cationic liposomes as delivery vectors for gene therapy, Pharm. Res. **2002**, 19, 1599–1605.

24. Voinea M., Simionescu M., Designing of 'intelligent' liposomes for efficient delivery of drugs, J. Cell Mol. Med. **2002**, 6, 465–74.

25. Pedroso de Lima M.C., Neves S., Filipe A., Duzgunes N., Simoes S., Cationic liposomes for gene delivery: from biophysics to biological applications, Curr. Med. Chem. **2003**, 10, 1221–31.

26. Nah J.W., Yu L., Han S.O., Ahn C.H., Kim S.W., Artery wall binding peptide-poly(ethylene glycol)-grafted-poly(l-lysine)-based gene delivery to artery wall cells, J. Control. Release **2002**, 78, 273–84.

27. Molas M., Bartrons R., Perales J.C., Single-stranded DNA condensed with poly-l-lysine results in nanometric particles that are significantly smaller, more stable in physiological ionic strength fluids and afford higher efficiency of gene delivery than their double-stranded counterparts, Biochim. Biophys. Acta **2002**, 1572, 37–44.

28. Fischer D., Bieber T., Li Y., Elsasser H.P., Kissel T., A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity, Pharm. Res. **1999**, 16, 1273–79.

29. Boussif O., Lezoualc'h F., Zanta M.A., Mergny M.D., Scherman D., Demeneix B. , A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylene imine, Proc. Natl. Acad. Sci. USA **1995**, 92, 7297–301

30. Gosselin M.A., Guo W., Lee R.J., Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine, Bioconjug. Chem. **2001**, 12,. 989–94.

31. Wightman L., Kircheis R., Rossler V., Carotta S., Ruzicka R., Kursa., Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo, J. Gene Med. **2001**, 3, 362–72.

32. Brunner S., Furtbauer E., Sauer T., Kursa M., Wagner E., Overcoming the nuclear barrier: cell cycle independent nonviral gene transfer with linear polyethylenimine or electroporation, Mol. Ther. **2002**, 5, 80–86
33. Mancheño-Corvo P., Martín-Duque P., Viral gene therapy. Clin Transl Oncol. **2006**, 12, 858-67.

34. Young L.S., Searle P.F., Onion D., Mautner V., Viral gene therapy strategies: from basic science to clinical application.J Pathol. **2006**, 208(2), 299-318.

35. Walther W., Stein U., Viral vectors for gene transfer: a review of their use in the treatment of human diseases. Drugs. **2000**, 60(2), 249-71.

36. Pandori M.W., Hobson D.A., Sano T., Adenovirus-microbead conjugates possess enhanced infectivity: A new strategy for localized gene delivery, Virology, **2002**, 299, 204-12.

37. Robbins P.D., Ghivizzani S.C., Viral vectors for gene therapy. Pharmacol Ther. **1998**, 80(1), 35-47.

38. Horwitz M.S. Adenovirus immunoregulatory genes and their cellular targets. Virology, **2001,** 279(1), 1-8.

39. Hitt M.M., Graham F.L., Adenovirus vectors for human gene therapy. Adv. Virus Res. **2000**, 55479-505.

40. Stone D., David A., Bolognani F., Lowenstein P.R., Castro M.G., Viral vectors for gene delivery and gene therapy within the endocrine system. Journal of Endocrinology, **2000**, 164, 103-18

41. Surosky R.T., Urabe M., Godwin S.G., McQuiston S.A,. Kurtzman G.J., Ozawa K., Natsoulis G.. Adeno-associated virus Rep proteins target DNA sequences to a unique locus in the human genome. J. Virol. **1997**, 71(10), 7951-59.

42. Samulski R.J., Adeno-associated virus: integration at a specific chromosomal locus. Curr. Opin. Genet Dev, **1993**, 1, 74-80.

43. Robbins P.D., Tahara H., Ghivizzani S.C., Viral vectors for gene therapy. Trends Biotechnol. **1998,** 16(1), 35-40.

44. Fink D.J., Glorioso J.C., Engineering herpes simplex virus vectors for gene transfer to neurons. Nat. Med. **1997**, 3, 357–59.

45. Fink D.J., Glorioso J.C., Herpes simplex virus-based vectors: problems and some solutions. Adv. Neurol. **1997**, 72,149–156.

46. Geller A.I., Herpes simplex virus-1 plasmid vectors for gene transfer into neurons. Adv. Neurol. **1997**, 72, 143–48.

47. Marconi P., Krisky D., Oligino T., Poliani P.L., Ramakrishnan R., Goins W.F., Fink D.J., Glorioso, J.C., Replication-defective herpes simplex virus vectors for gene transfer in vivo. Proc. Natl. Acad. Sci. USA, **1996**, 93, 11319-320.

48. Neve R.L., Geller A.I., A defective herpes simplex virus vector system for gene delivery into the brain: comparison with alternative gene delivery systems and usefulness for gene therapy. Clin Neurosci. **1995**, 3, 262–67.

49. Cuchet D., Potel C., Thomas J., Epstein A.L., HSV-1 amplicon vectors: a promising and versatile tool for gene delivery. Expert. Opin. Biol. Ther. **2007,** 7(7), 975-95.

50. Baum C., Kustikova O., Modlich U., Li Z., Fehse B., Mutagenesis and oncogenesis by chromosomal insertion of gene transfer vectors, Hum Gene Ther. **2006,** 17:253-263.

51. Thomas C. E., Ehrhardt A., Kay M. A., Progress and problems with the use of viral vectors for gene therapy, Nat Rev Genet **2003***,* 4:346-358

52. Lehrman S., Virus treatment questioned after gene therapy death, Nature **1999**, 401, 517–18.

53. Roberts D.M., Nanda A., Havenga M.J. et al. Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. Nature **2006,** 441(7090), 239-243.

54. Azzam T.; Eliyahu H.; Makovitzki A.; Linial M.; Domb A.J., Hydrophobized dextran-spermine conjugate as potential vector for in vitro gene transfection J Control Release **2004**, 96, 309-23.

55. Isaka Y., Imai E., Electroporation-mediated gene therapy. Expert. Opin. Drug Deliv. **2007,** 4(5), 561-71.

56. Heller L.C., Ugen K., Heller R., Electroporation for targeted gene transfer. Expert. Opin. Drug Deliv., **2005**, 2(2), 255-68.

57. Favard C., Dean D.S., Rols M.P. Electrotransfer as a non viral method of gene delivery. Curr. Gene Ther. **2007**, 7(1) 67-77.

58. Mir L.M., Moller P.H., Andre F., Gehl J., Electric pulse-mediated gene delivery to various animal tissues, Adv. Genet. **2005**, 54, 83–114

59. Gehl J., Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research, Acta Physiol. Scand., **2003**, 177, 437–47.

60. Zhang G., Gao X., Song Y.K., Vollmer R., Stolz D.B., Gasiorowski JZ, Dean DA, Liu D., Hydroporation as the mechanism of hydrodynamic delivery, Gene Ther., **2004**, 11, 675–82.

61. Kobayashi N., Nishikawa M., Takakura Y., The hydrodynamics-based procedure for controlling the pharmacokinetics of gene medicines at whole body, organ and cellular levels, Adv. Drug Deliv. Rev., **2005**, 57, 713–731

62. Herweijer H., Wolff J.A. Gene therapy progress and prospects: hydrodynamic gene delivery. Gene Ther**. 2007,** 14(2), 99-107.

63. Lu Q.L., Liang H.D., Partridge T., Blomley M.J., Microbubble ultrasound improves the efficiency of gene transduction in skeletal muscle in vivo with reduced tissue damage, Gene Ther., **2003**, 10, 396–405.

64. Hosseinkhani H., Tabata Y., Ultrasound enhances in vivo tumor expression of plasmid DNA by PEG-introduced cationized dextran, J. Control. Release. **2005**, 108, 540–56.

65. Ferrara K., Pollard R., Borden M., Ultrasound microbubble contrast agents: fundamentals and application to gene and drug delivery. Annu. Rev. Biomed. Eng **2007,** 9415-47.

66. Yang N., Horn R., Evidence for voltage-dependent S4 movement in sodium channels, Neuron, **1995,** 15, 213–18

67. Cui Z., Mumper R.J., Microparticles and nanoparticles as delivery systems for DNA vaccines, Crit. Rev. Ther. Drug Carrier Syst., **2003**, 20, 103–137.

68. Zhang Y., Boado R.J., Pardridge W.M. Marked enhancement in gene expression by targeting the human insulin receptor, J. Gene Med., **2003**, 5, 157–63.

69. Kichler A., Gene transfer with modified polyethylenimines, J. Gene Med., **2004**, 6, 3–10.

70. Read M.L., Logan A., Seymour L.W., Barriers to gene delivery using synthetic vectors. Adv Genet., **2005**, 53, 19-46.

71. Shea L.D, Houchin T.L., Modular design of non-viral vectors with bioactive components.Trends Biotechnol. **2004**, 22(9), 429-31

72. Hirko A., Tang F., Hughes J.A., Cationic lipid vectors for plasmid DNA delivery.
Curr Med Chem. **2003**, 10(14), 1185-93.

73. Luten J., van Nostrum C.F., De Smedt S.C., Henninck W.E., Biodegradable polymers as non-viral carriers for plasmid DNA delivery. J. Control Release **2008,** 126(2), 97-110

74. Pietersz G.A., Tang C.K., Apostolopoulos V., Structure and design of polycationic carriers for gene delivery. Mini. Rev. Med. Chem. **2006**, 6(12), 1285-98.

75. Dubruel P., Schacht E., Vinyl polymers as non-viral gene delivery carriers: current status and prospects. Macromol. Biosci., **2006**, 6(10), 789-810.

76. Brown M.D., Schatzlein A.G., Uchegbu I.F., Gene delivery with synthetic (non viral) carriers. Int. J. Pharm. **2001,** 229(1-2), 1-21.

77. Merdan T., Kopecek J., Kissel T. Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. Adv. Drug Deliv. Rev. **2002,** 54(5), 715-58.

78. De Smedt S.C., Demeester J., Hennink W.E. Cationic polymer based gene delivery systems. Pharm. Res., **2000,** 17(2), 113-26.

79. Wagner E., Orgis M., Zauner W., Polylysine-based transfection systems utilizing receptor-mediated delivery, Adv. Drug delivery Rev.,**1998**, 30, 97-113

80. Dennig J., Duncan E., Gene transfer into eukaryotic cells using activated polyamidoamine dendrimers. J Biotechnol. **2002**, 90(3-4),339-47

81. Godbey W.T., Wu K.K., MikosA.G, Poly(ethylenimine)and its role in gene delivery, J. Control. Rel.**1999**, 60,149-160.

82. Yudovin-Farber I., Domb A.J., Cationic polysaccharides for gene delivery , Mat Sci Eng **2007**, 27, 595-98.

83. Mansouri S., Lavigne P., Corsi K., Benderdour M., Beaumont E., Fernandes J.C., Chitosan-DNA nanoparticles as non-viral vectors in gene therapy: strategies to improve transfection efficacy. Eur J Pharm Biopharm. **2004**, 57(1),1-8.

84. Dang J.M., Leong K.W., Natural polymers for gene delivery and tissue engineering, Advanced Drug Delivery Reviews, **2006**, 58(4), 487-99

85. Liu D., Ren T., Gao X. Cationic transfection lipids. Curr. Med. Chem. **2003,** 10(14) 1307-15.

86. Ma B., Zhang S., Jiang H., Zhao B., Lv H., Lipoplex morphologies and their influences on transfection efficiency in gene delivery. J. Control Release **2007,** 123(3) 184-94.

87. Eastman S.J., Siegel C., Tousignant J., Smith A.E., Cheng S.H., Scheule R.K. Biophysical characterization of cationic lipid: DNA complexes. Biochim. Biophys. Acta **1997,** 1325(1) 41-62.

88. Torchilin V.P., Recent advances with liposomes as pharmaceutical carriers, Nat. Rev. Drug Discov. **2005**, 4,145–60.

89. Simoes S., Filipe A., Faneca H., Mano M., Penacho N., Duzgunes N., de Lima MP, Cationic liposomes for gene delivery, Expert Opin. Drug Deliv. **2005**, 2, 237–54.

90. Dass C.R., Lipoplex-mediated delivery of nucleic acids: factors affecting in vivo transfection, J. Mol. Med.**2004** 82, 579–91

91. Felgner P.L., Gadek T.R., Holm M., Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M.Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. U. S. A **1987,** 84(21) 7413-17

92. Jang J.H., Houchin T.L., Shea L.D. Gene delivery from polymer scaffolds for tissue engineering. Expert Rev Med Devices. **2004**,1(1), 127-38.

93. Levy R.J., Song C., Tallapragada S., DeFelice S., Hinson J.T., Vyavahare N., Connolly J., Ryan K., Li Q., Localized adenovirus gene delivery using antiviral IgG complexation. Gene Ther. **2001**, 9, 659-67.

94. Honma K., Ochiya T., Nagahara S., Sano A., Yamamoto H, Hirai K, Aso Y, Terada M.Atelocollagen-based gene transfer in cells allows high-throughput screening of gene functions. Biochem Biophys Res Commun **2001**, 289, 1075–81.

95. Segura T., Shea L.D., Surface-tethered DNA complexes for enhanced gene delivery. Bioconjug Chem **2002**, 13, 621–29

96. Segura T., Volk M.J., Shea L.D., Substrate-mediated DNA delivery: role of the cationic polymer structure and extent of modification. J Control Release **2003**, 93, 69–84.

97. Segura T., Chung P.H., Shea L.D., DNA delivery from hyaluronic acid-collagen hydrogels via a substrate-mediated approach. Biomaterials. **2005**, 26(13), 1575-84.

98. Bielinska A.U., Yen A., Wu H.L., Zahos K.M., Sun R., Weiner N.D., Baker J.R., Roessler B.J., Application of membrane-based dendrimer/DNA complexes for solid phase transfection in vitro and in vivo. Biomaterials. **2000**, 21(9), 877-87.

99. Kneuer C., Sameti M., Bakowsky U., Schiestel T., Schirra H., Schmidt H., Lehr CM., A nonviral DNA delivery system based on surface modified silica-nanoparticles can efficiently transfect cells in vitro, Bioconjug Chem **2000**, 11, 926–32.

100. Manuel W.S., Zheng J.I., Hornsby P.J., Transfection by polyethyleneimine-coated microspheres, J Drug Target **2001**, 9, 15–22.

101. Bengali Z., Pannier A.K., Segura T., Anderson B.C., Jang J.H. , Mustoe T.A, Shea L.D., Gene delivery through cell culture substrate adsorbed DNA complexes, Biotech Bioeng **2005**, 90, 290–302.

102. Zhang J.T., Chua L.S., Lynn D.M., Multilayered thin films that sustain the release of functional DNA under physiological conditions, Langmuir **2004**, 20, 8015–21.

103. Ziauddin J., Sabatini D.M., Microarrays of cells expressing defined cDNAs. Nature. **2001**, 411(6833), 107-10.

104. Delehanty J.B., Shaffer K.M., Lin B., A comparison of microscope slide substrates for use in transfected cell microarrays. Biosensors and Bioelectronics **2004**, 20(4), 773-779

105. Uchimura E., Yamada S., Uebersax L., Fujita S., Miyake M., Miyake J., Method for reverse transfection using gold colloid as a nano-scaffold. J Biosci Bioeng. **2007,** 103(1),101-3.

106. Uchimura E., Yamada S., Nomura T., Matsumoto K., Fujita S., Miyake M., Miyake J. Reverse transfection using antibodies against a cell surface antigen in mammalian adherent cell lines. J Biosci Bioeng. **2007**, 104(2),152-5.

107. Decher G., Fuzzy nanoassemblies: Toward layered polymeric multicomposites Science, **1997**, 227, 1232-37

108. Chluba J., Voegel J.C., Decher G., Erbacher P., Schaaf P., Ogier J., Peptide hormone covalently bound to polyelectrolytes and embedded into multilayer architectures conserving full biological activity, Biomacromolecules **2001**, **2** (3),.800–805

109. Jessel N., Atalar F., Lavalle P., Mutterer J., Decher G., Schaaf P. Voegel JC, Ogier J., Bioactive coatings based on polyelectrolyte multilayer architecture functionalised by embedded proteins, Adv Mater **2003**, 15, 692–95.

110. Thierry B., Winnik F.M., Merhi Y., Tabrizian M., Nanocoatings onto arteries via layer-by-layer deposition: Toward the in vivo repair of damaged blood vessels, J Am Chem Soc **2003**, 125, 7494–95

111. Benkirane Jessel N., Lavalle P., Meyer F., Audouin F., Frisch B., Schaaf P., Ogier J., Decher G., Voegel J.C., Control of monocyte morphology and response to model surfaces for implants equipped with anti-inflammatory agents, Adv Mater **2004**, 16, 1507–11.

112. Picart C., Elkaim R., Richert L., Audoin T., Arntz Y., Cardoso M.D., Schaaf P., Voegel J.C., Frisch B. Primary cell adhesion on RGD-functionalized and covalently crosslinked thin polyelectrolyte multilayer films, Adv Funct Mater **2005**, 15, 83–94.

113. Thierry B., Kujawa P., Tkaczyk C., Winnik F.M., Bilodeau L., Tabrizian M., Delivery platform for hydrophobic drugs: prodrug approach combined with self-assembled multilayers, J Am Chem Soc **2005**, 127, 1626–27

114. Benkirane-Jessel N., Schwinté P., Falvey P., Darcy R., Haïkel Y., Schaaf P., Voegel J.C., Ogier J., Build-up of polypeptide multilayer coatings with anti-inflammatory properties based on the embedding of Piroxicam-cyclodextrin complexes, Adv Funct Mater **2004**,14(2), 174–182.

115. Jewell C.M., Zhang J., Fredin N.J., Lynn D.M, Multilayered polyelectrolyte films promote the direct and localized delivery of DNA to cells, J Control Release **2005**, 106 (1–2), 214–23.

116. Meyer F., Ball V., Schaaf P., Voegel J.C., Ogier J., Polyplex-embedding in polyelectrolyte multilayers for gene delivery, Biochim Biophys Acta **2006**, 1758 (3), 419–422

117. Meyer F., Dimitrova M., Jedrzejenska J., Arntz Y., Schaaf P., Frisch B., Voegel J.C., Ogier J., Relevance of bi-functionalized polyelectrolyte multilayers for cell transfection. Biomaterials. **2008**, 29(5), 618-24.

118. Jessel N., Oulad-Abdelghani M., Meyer F., Lavalle P., Haikel Y., Schaaf P., Voegel J.C., Multiple and time scheduled in situ DNA delivery mediated by β-cyclodextrin embedded in a polyelectrolyte multilayer, Proc Natl Acad Sci USA **2006**, 103 (23), 8618–21.

119. Zhang X, Sharma KK, Boeglin M , Ogier J., Mainard D., Voegel JC., Mely Y., Benkirane-Jessel N., Transfection ability and intracellular DNA pathway of nanostructured gene-delivery systems**:** NANO LETT **2008,** 8**(**8**),** 2432-2436

120. Takahashi A., Palmer-Opolski M., Smith R.C., Walsh K., Transgene delivery of plasmid DNA to smooth muscle cells and macrophages from a biostable polymer-coated stent, Gene Ther **2003**, 10, 1471–78

121. Nakayama Y., Ji-Youn K., Nishi S., Ueno H., Matsuda T., Development of high-performance stent: gelatinous photogel-coated stent that permits drug delivery and gene transfer, J Biomed Mater Res **2001**, **57**, 559–566.

122. Yamauchi F., Koyamatsu Y., Kato K., Iwata H., Layer-by-layer assembly of cationic lipid and plasmid DNA onto gold surface for stent-assisted gene transfer. Biomaterials. **2006**, 27(18), 3497-504.

123. Jewell C.M., Zhang J., Fredin N.J., Wolff M.R., Hacker T.A., Lynn D.M., Release of plasmid DNA from intravascular stents coated with ultrathin multilayered polyelectrolyte films. Biomacromolecules. **2006**, 7(9), 2483-91.

124. Nolan J.P., Mandy F.F. Suspension array technology: new tools for gene and protein analysis, Cell Mol Biol **2001**, 47, 1241-56.

125. Zhou, H., Roy, S., Schulman, H. & Natan, M.J., Solution and chip arrays in protein profiling. Trends Biotechnol **2001, 19**, S34-39.

126. Armstrong B., Stewart M., Mazumder A., Suspension arrays for high throughput, multiplexed single nucleotide polymorphism genotyping. Cytometry **2000**, 40, 102-108.

127. 131. Martins T.B. Development of internal controls for the Luminex instrument as part of a multiplex seven-analyte viral respiratory antibody profile, Clin Diagn Lab Immunol **2002**, 9, 41-45.

128. Howbrook D.N, van der Valk A.M., O'Shaughnessy M.C., Sarker D.K., Baker S.C., Lloyd A.W. Developments in microarray technologies. Drug Discov Today. **2003**, 8(14), 642-51.

129. Sobek J., Bartscherer K., Jacob A., Hoheisel J.D., Angenendt P., Microarray technology as a universal tool for high-throughput analysis of biological systems. Comb Chem High Throughput Screen. **2006**, 9(5), 365-80.

130. Hoheisel J.D., Microarray technology: beyond transcript profiling and genotype analysis. Nat Rev Genet. **2006,** 7(3), 200-10.

131. Steller S., Angenendt P., Cahill D.J., Heuberger S., Lehrach H., Kreutzberger J., Bacterial protein microarrays for identification of new potential diagnostic markers for Neisseria meningitides infections, Proteomics **2005**, 5, 2048–55.

132. Groathouse N.A., Amin A., Marques M.A., Spencer J.S., Gelber R., Knudson D.L., Belisle J.T., Brennan P.J., Slayden R.A., Use of protein microarrays to define the humoral immune response in leprosy patients and identification of disease-state-specific antigenic profiles, Infect Immun **2006**, 74, 6458–66

133. Gomase V.S, Tagore S., Kale K.V., Microarray: an approach for current drug targets. Curr Drug Metab. **2008,** 9(3), 221-31.

134. Venkatasubbarao S., Microarrays--status and prospects. Trends Biotechnol. **2004**, 22(12):630-7.

135. Poustka, A., Barlow D.P., Zehetner G., Craig A., Michiels F., Ehrich E., Frischauf A.M., Lehrach H. Molecular approaches to mammalian genetics. Cold Spring Harb. Symp. Quant. Biol. **1986**, 51, 131–39.
The first paper to describe the potential of array technology in genomics.

136. Bains, W., Smith, G., A novel method for nucleic acid sequence determination. J. Theor. Biol. **1988**, 135, 303–307.
The first publication on sequencing by hybridization.

137. 1Cantor C. R., Mirzabekov, A., Southern E., Report on the sequencing by hybridisation workshop. Genomics **1992**, 13, 1378–83.

138. Drmanac, R., Labat, I., Brukner, I., Crkvenjakov, R., Sequencing of megabase plus DNA by hybridisation: theory of the method. Genomics **1989**, 4, 114–28.

139. Khrapko K., Lysov Y.P., Khorlyn A.A., Shick V.V., Florentiev V.L., Mirzabekov A.D., An oligonucleotide hybridization approach to DNA sequencing. FEBS Lett. **256**, **1989**, 118–22.

140. 144. Schena, M., Shalon, D., Davis, R. W. & Brown, P. O., Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science **1995**, 270,467–70.
A publication that made the wider scientific community aware of the potential of arraytechnology.

141. Mitchell P., A perspective on protein microarrays. Nat Biotechnol. **2002**, 20(3):225-29.

142. 146. Tao S.C., Chen C.S., Zhu H., Applications of protein microarray technology. Comb Chem High Throughput Screen. **2007**, 10(8), 706-18.

143. Wingren C.; Borrebaeck C.A.K., Progress in miniaturization of protein arrays--a step closer to high-density nanoarrays. Drug Discov Today. **2007**, 12(19-20), 813-9.

144. Hall D.A.; Ptacek J., Snyder M., Protein microarray technology. Mech Ageing Dev. **2007**, 128(1):161-7.

145. Starkuviene V., Pepperkok R., Erfle H., Transfected cell microarrays: an efficient tool for high-throughput functional analysis. Expert Rev Proteomics. **2007,** 4(4):479-89.

146. Hook A.L., Thissen H., Voelcker N.H., Surface manipulation of biomolecules for cell microarray applications. Trends Biotechnol. **2006**, 24(10):471-7.

147. Wu R.Z., Bailey S.N., Sabatini D.M., Cell-biological applications of transfected-cell microarrays. Trends Cell Biol. **2002**, 12(10):485-8.

148. Bubendorf L., Nocito A., Moch H., Sauter G., Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies. J Pathol. **2001**, 195(1), 72-9.

149. Li B., Jiang L., Song Q., Yang J., Chen Z., Guo Z., Zhou D., Du Z., Song Y., Wang J., Wang H, Yu S, Wang J, Yang R., Protein microarray for profiling antibody responses to Yersinia pestis live vaccine, Infect Immun **2005**, 73, 3734–39.

150. Wilson R., Cossins A.R., Spiller D.G., Encoded microcarriers for high-throughput multiplexed detection. Angew Chem Int Ed Engl. **2006**, 45(37), 6104-17.

151. Zhi Z.L., Morita Y., Hasan Q., Tamiya E., Micromachining microcarrier-based biomolecular encoding for miniaturized and multiplexed immunoassay. Anal Chem **2003**, 75(16), 4125-31.

152. Braeckmans K., De Smedt S.C., Leblans M., Pauwels R., Demeester J., Encoding microcarriers: present and future technologies. Nat Rev Drug Discov. **2002**, 1(6), 447-56.

153. Battersby B.J., Bryant D., Meutermans W., Matthews D., Smythe M.L., Trau M., Toward larger chemical libraries: Encoding with fluorescent colloids in combinatorial chemistryJ. Am. Chem. Soc. **2000**, 122, 2138-39.

154. Han M.Y., Gao X.H., Su J.Z., Nie S., Nie, Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. Nat. Biotechnol. **2001**, 19, 631-35.

155. Zhao W., Liu Z.B., Yang H., Nagai K., Zhao Y.H., Gu Z.Z. , Uniformly colorized beads for multiplex immunoassay Chem. Mater**.2006**, 18, 2443-49.

156. Cunin F., Schmedake T.A., Link J.R., Li Y.Y., Koh J., Bhatia S.N., Sailor M.J., Biomolecular screening with encoded porous-silicon photonic crystals. Nat. Mater. **2002**, 1, 39-41.

157. Su X., Zhang J., Sun L., Koo T.W., Chan S., Sundararajan N., Yamakawa M., Berlin A.A., Composite organic-inorganic nanoparticles (COINs) with chemically encoded optical signatures Nano Lett **2005** 5 (1), 49-54.

158. Nicewarner-Pena S.R., Freeman R.G., Reiss B.D., He L., Pena D.J., Walton I.D., Cromer R., Keating C.D., Natan M.J. Submicrometer metallic barcodes Science**2001**, 294, 137-41.

159. Sha M.Y., Walton I.D., Norton S.M., Taylor M., Yamanaka M., Natan M.J., Xu C.J., Drmanac S., Huang S., Borcherding A., Drmanac R., Penn S.G.,Multiplexed SNP genotyping using nanobarcode particle technology ., Anal. Bioanal. Chem **2006**, 384(3), 658-66

160. Evans M.; Sewter C.; Hill E., An encoded particle array tool for multiplex bioassaysAssay Drug Dev. Technol **2003**, 1, 199-207.

161. Zhi Z.L., Morita Y., Hasan Q., Tamiya E., Micromachining microcarrier-based biomolecular encoding for miniaturized and multiplexed immunoassay Anal. Chem **2003**, 75, 4125
162. Braeckmans K., De Smedt S.C., Roelant C., Leblans M., Pauwels R., Demeester J., Encoding microcarriers by spatial selective photobleaching Nat. Mater **2003**, 2(3), 169.

163. Pregibon D.C.; Toner M., Doyle P.S., Multifunctional encoded particles for high-throughput biomolecule analysis, , Science **2007**, 315, 1393-1396.

164. Moran E.J., Sarshar S., Cargill J.F., Shahbaz M.M., Lio A., Mjalli A.M.M., Armstrong R.W., Radio-frequency tag encoded combinatorial library method for the discovery of tripeptide-substitued cinnamic acid inhibitors of the protein-tyrosine phosphatise PTP1B. J. Am. Chem. Soc **1995**, 117 (43), 10787-88.

165. Nicolaou K. C., Xiao X. Y., Parandoosh Z., Senyei A., Nova M. P., Radiofrequency encoded combinatorial chemistry, Angew Chem(int Ed Engl), **1995**, 34(20), 2289-91**.**

166. McHugh T. M., Miner R. C., Logan L. H., Stites D. P., Simultaneous detection of antibodies to cytomegalo-virus and herpes-simplex virus by using flow-cyometery and microsphere-based fluorescence immunoassay, J. Clin. Microbiol. **1998**, 26, 1957-61.

167. Vaino A. R., Janda K. D., Euclidean shape-encoded combinatorial chemical libraries,Proc. Natl. Acad. Sci. U.S.A. **2000**, 97(14), 7692-96.

168. Derveaux S., De Geest B.G., Roelant C. , Braeckmans K., Demeester J., De Smedt S.C., Langmuir. Multifunctional layer-by-layer coating of digitally encoded microparticles, **2007**, 23, 10272-79.

169. Angenendt P., Nyarsik L., Szaflarski W., Glokler J., Nierhaus K.H., Lehrach H., Cahill D.J., Lueking A., Cell-free protein expression and functional assay in nanowell chip format, Anal. Chem. **2004**, 76(7), 1844–49.

170. Stephan J.P., Schanz S., Wong A., Schow P, Wong W.L.T., Development of a frozen cell array as a high-throughput approach for cell-based analysis, Am. J. Pathol. **2002**, 161, 787–797

171. Oode K., Furuya T., Harada K, Kawauchi S., Yamamoto K., Hirano T., Sasaki K., The development of a cell array and its combination with laser-scanning cytometry allows a high-throughput analysis of nuclear DNA content, Am. J. Pathol. **2000**, 157, 723–28.

172. Takita M., Furuya T., Sugita T., Kawauchi S., Oga A., Hirano T., Tsunoda S., Sasaki K., An analysis of changes in the expression of cyclins A and B1 by the cell array system during the cell cycle: comparison between cell synchronization methods, Cytometry A **2003**, 55, 24–29.

173. Xu T., Jin J, Gregory C, Hickman JJ, Boland T Inkjet printing of viable mammalian cells, Biomaterials **2005**, 26, 93–99

174. SchwenkJ.M., Stoll D., Templin M.F., Joos T.O., Cell microarrays: an emerging technology for the characterization of antibodies, Biotechniques **2002**, S, 54–6

175. Huang Y., Joo S., Duhon M., Heller M., Wallace B., Xu X., Dielectrophoretic cell separation and gene expression profiling on microelectronic chip arrays, Anal. Chem. **2002,** 74(14), 3362–71.

176. Gray D.S., Tan J.L., Voldman J., Chen C.S., Dielectrophoretic registration of living cells to a microelectrode array, Biosens. Bioelectron. **2004**, 19, 1765–1774.

177. Beske O., Guo J.J., Li J.R., Bassoni D., Bland K., Marciniak H., Zarowitz M., Temov V., Ravkin I., Goldbard S., A novel encoded particle technology that enables simultaneous interrogation of multiple cell types, J. Biomol. Screen. **2004**, 9, 173–185.

178. A. Watson, Wu X.Y., Bruchez M., Lighting up cells with quantum dots, Biotechniques **2003**, 34, 296–303.

179. Smith A.M., Gao X.H., Nie S.M., Quantum dot nanocrystals for in vivo molecular and cellular imaging, Photochem. Photobiol. **2004**, 80, 377–385.

180. Mattheakis L.C., Dias J.M., Choi Y.J., Gong J., Bruchez M.P., Liu J.Q., Wang E., Optical coding of mammalian cells using semiconductor quantum dots, Anal. Biochem. **2004**, 327, 200–208.

181. Silva J.M., Mizuno H., Brady A., Lucito R., Hannon G.J., RNA interference microarrays: high-throughput loss-of-function genetics in mammalian cells. Proc Natl Acad Sci U S A **2004**, 101, 6548-52.

182. Wheele D.B., Carpenter A.E., Sabatini D.M., Cell microarrays and RNA interference chip away at gene function, Nat Genet **2005**, 37, S25–S30.

183. Wheeler D.B., Bailey S.N., Guertin D.A., Carpenter A.E., Higgins C.O., Sabatini D.M., RNAi living-cell microarrays for loss-of-function screens in Drosophila melanogaster cells, Nat Methods **2004**, 1(2), 127–132.

184. Baghdoyan S., Roupioz Y, Pitaval A, Castel D, Khomyakova E, Papine A, Soussaline F, Gidrol X., Quantitative analysis of highly parallel transfection in cell microarrayse: Nucleic Acids Res **2004**;32:e77.

185. Wu R.Z., Bailey S.N., Sabatini D.M., Cell-biological applications of transfected-cell microarrays, Trends Cell Biol **2002**, 12(10), 485–88.

186. Bailey S.N., Wu R.Z., Sabatini D.M., Applications of transfected cell microarrays in high-throughput drug discovery, Drug Discov Today **2002**, 7(18), S113–18

187. Mousses S, Caplen NJ, Cornelison R, Weaver D, Basik M, Hautaniemi S, Elkahloun AG, Lotufo RA, Choudary A, Dougherty ER, Suh E, Kallioniemi O., RNAi microarray analysis in cultured mammalian cells Genom Res **2003**, 13(10), 2341-47

188. Bailey S.N., Ali S.M., Carpenter A.E., Higgins C.O., Sabatini D.M. , Microarrays of lentiviruses for gene function screens in immortalized and primary cells Nat Methods **2006**, 3(2), 117-22.

189. Carbone R., Giorgetti L., Zanardi A., Marangi I., Chierici E., Bongiorno G., Fiorentini F., Faretta M., Piseri P., Pelicci P.G., Milani P., Retroviral microarray-based platform on nanostructured TiO_2 for functional genomics and drug discovery. Biomaterials **2007**, 28(13), 2244-53

190. Mishina Y.M., Wilson C.J., Bruett L., Smith J.J., Stoop-Myer C., Jong S., Amaral L.P., Pedersen R., Lyman S.K., Myer V.E., Kreider B.L., Thompson C.M., Multiplex GPCR assay in reverse transfection cell microarrays, J. Biomol. Screen. **2004**, 9, 196–207**.**

Chapter 2

Physicochemical and Transfection Properties of Cationic Hydroxycellulose/DNA Nanoparticles

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ABSTRACT

In this study the physicochemical and transfection properties of cationic hydroxyethylcellulose/plasmid DNA (pDNA) nanoparticles were investigated and compared with the properties of DNA nanoparticles based on poly(ethylene imine) (PEI) which is widely investigated as gene carrier. The two types of cationic hydroxyethylcelluloses studied, polyquarternium-4 (PQ-4) and polyquarternium-10 (PQ-10), are already commonly used in cosmetic and in topical drug delivery devices. Both PQ-4 and PQ-10 spontaneously interact with pDNA with the formation of nanoparticles of approximately 200 nm in size. Gelelectrophoresis and fluorescence dequenching experiments indicated stronger interactions between pDNA and the cationic celluloses than between pDNA and PEI. The cationic cellulose/pDNA nanoparticles transfected cells to a much lesser extent than the PEI based pDNA nanoparticles. The low transfection property of the PQ-4 /pDNA nanoparticles was attributed to their neutrally charged surface which does not allow an optimal binding of PQ-4/pDNA nanoparticles to cellular membranes. Although the PQ-10/pDNA nanoparticles were positively charged, and thus expected to be taken up by cells, they were also much less efficient in transfecting cells than PEI/pDNA nanoparticles. Agents known to enhance the endosomal escape were not able to improve the transfection properties of PQ-10/pDNA nanoparticles indicating that a poor endosomal escape is, highly likely, not the major reason for the low transfection activity of PQ-10/pDNA nanoparticles. We hypothesized that the strong binding of pDNA to PQ-10 prohibits the release of pDNA from PQ-10 once the PQ-10/pDNA nanoparticles arrive in the cytosol of the cells. Tailoring of the nature and extent of cationic side chains on this type of cationic hydroxyethylcellulose may be promising to further enhance their DNA delivery properties.

Chapter 2

Physicochemical and Transfection Properties of Cationic Hydroxycellulose/DNA Nanoparticles

INTRODUCTION

The major progress in the field of genomics has encouraged numerous scientists to work on suitable carriers for delivering genes to target cells. Indeed, the biological efficacy (i.e. the production of a certain protein) of administered "naked" DNA is restrained as it cannot efficiently overcome cellular barriers on its way to the nucleus. E.g. the cellular uptake of naked DNA is poor due to its large size, hydrophilicity, and negatively charged backbone. In addition, naked DNA is very susceptible to degradation by nucleases in the extracellular matrix and in the cells. Consequently, efficient intracellular delivery of DNA is highly dependent on suitable carriers which protect the DNA against degradation, allow the DNA to cross cellular membranes and to escape from endosomes, and guide the DNA into the nuclei of the cells.

Worldwide many laboratories are searching for appropriate pharmaceutical carriers for DNA. Different types of cationic lipids and cationic polymers are under investigation as non-viral DNA carriers [1,2]. Cationic lipids, as well as cationic polymers, spontaneously form interpolyelectrolyte complexes with negatively charged nucleic acids, called respectively

lipoplexes and polyplexes. The physicochemical features that govern the biological activity of lipo- and polyplexes are, however, not well understood, partly due to the complexity of the association and dissociation behavior of such complexes.

Cationic polysaccharides (like e.g. cationic dextrans, cationic celluloses, cationic guar, chitosan) are, for various reasons, widely used in both cosmetics and topical drug delivery systems[3]. Recently, cationic polysaccharides were also investigated for DNA delivery purposes. In particular, Azzam et al.[4] tested over 300 different types of cationic polysaccharides for gene transfection, starting from various polysaccharides (of various molecular weight) and oligoamines. Although most of the cationic polysaccharides formed stable complexes with various plasmids, only certain types of the dextran-spermine based polyplexes transfected cells *in vitro* [4,5].

A major obstacle for *in vivo* gene delivery is the interaction of the DNA complexes with (extra) cellular fluids [6,7,8]. Indeed, binding of (extra)cellular proteins can induce aggregation, (partial) dissociation and opsonization of the charged DNA-complexes. It is well known that pharmaceutical carriers bearing neutral, hydrophilic segments (such as poly(ethylene glycol) (PEG)) at their surface are attractive to avoid opsonization. Pegylated derivatives of dextran-spermine were reported to give efficient transfection in serum rich media and to induce gene expression in the liver of mice after IV administration[9]. Transfection efficiency could be also increased by grafting fatty acids on the dextran-spermine polymers. It was believed that hereby the surface of the particles becomes more hydrophobic which would favour the hydrophobic interactions with the cell membranes [10]. Cationic polysaccharides containing quaternary ammonium oligoamines were developed to make DNA particles with a highly positive surface which would promote binding of the particles to the negatively charged cell membranes and would thus improve cellular uptake. However, quaternary ammonium polysaccharides as synthesized by Yudovin-Farber et al. were less efficient in gene transfer than dextran-spermine based carriers [11].

A first aim of this study is to evaluate the DNA complexation properties of two types of cationic hydroxyethylcellulose. Polyquaternium-4 cellulose (PQ-4; Fig. 1) and polyquaternium-10 cellulose (PQ-10; Fig. 1) were previously characterized [3,12] ,and already have applications in cosmetics and topic drug delivery devices [13]. In both PQ-4 and PQ-10 the sugar monomers are substituted with PEG. However, in PQ-4 the quaternary ammonium groups are directly attached to the cellulose backbone, while in PQ-10 the quaternary

ammonium groups are present at the end of the PEG chains [14]. Also, as Table 1 shows, compared to PQ-10, PQ-4 is less substituted with ammonium groups. The second aim of this study is to investigate whether quaternized cationic celluloses are suitable for gene delivery. The transfection properties of quaternized cationic cellulose/DNA nanoparticles were compared with the transfection properties of DNA nanoparticles based on respectively branched and linear poly(ethylene imine) (PEI) which are widely investigated cationic polymers for DNA delivery purposes.

Table 1

Polymer	MW (kDa)	[η]dl/g	X_{PEG}	n	X_N
PQ-4	1400	14.59	1.33	1.04	0.24
PQ-10	1700	70.09	0.51	1.52	0.51

Table 1. Molecular weight (MW), intrinsic viscosity in water at 25°C ([η]), the number of PEG-chains per glucose residue (X_{PEG}), the average number of repeating units of the hydroxyethyl chains (n) and the number of nitrogen atoms per glucose residue (X_N) of the cationic celluloses.

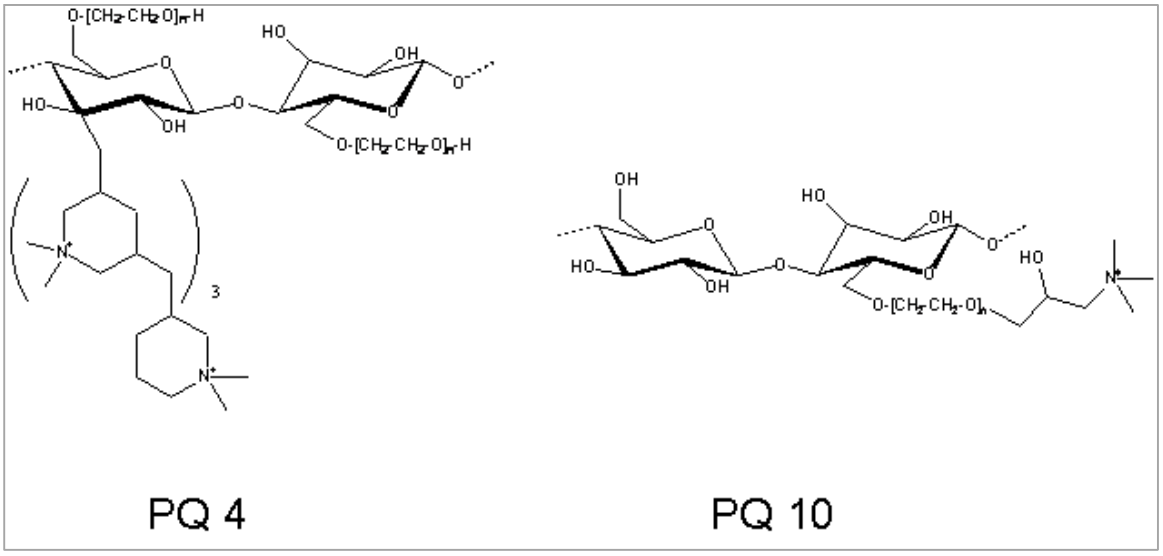


Figure1. Schematic molecular structures of PQ-4 and PQ-10.

MATERIALS & METHODS

Materials

PQ-4 (Celquat H-100) and PQ-10 (Celquat SC-230M) were obtained from National Starch and Chemical Ltd. The physicochemical properties of PQ-4 and PQ-10 as provided by the supplier are shown in Table 1. Branched poly(ethylene imine) (BPEI, MW 25 KDa) was purchased from Sigma. Linear poly(ethylene imine) (LPEI, MW 22 KDa) was a kind gift from Professor Ernst Wagner (Ludwig Maximilian University, Munich, Germany). Secretory alkaline phosphatase (SEAP) plasmid DNA (pMet7-hβc-SEAP) was a kind gift from Professor Tavernier (Ghent University, Belgium). pMet7-hβc-SEAP was amplified in *Escherichia Coli* and purified by the Qiagen Plasmid Mega Kit. The plasmid DNA was fluorescently labeled using the Mirus *Label IT* Nucleic Acid Labeling Kit (Panvera Corporation, USA). Picogreen was purchased from Molecular Probes. Poly-L-aspartic acid (pAsp), dextran sulfate (DS), chloroquine and ethidium bromide were purchased from Sigma. The cellulase used in this study was the FIP (Féderation Internationale Pharmaceutique) standard (0.147U/mg). The fusogenic peptide INF-7, a 24 amino acid containing peptide with fusogenic activity derived from the influenza virus, was a kind gift from Professor Hennink (Utrecht University, The Netherlands).

Preparation of Polyplexes

Stock solutions of the cationic polymers (PQ-4(3.0 mg/ml), PQ-10(3.0 mg/ml), BPEI (12.98 mg/ml) and LPEI (7.6 mg/ml)) were diluted in 20mM HEPES buffer at pH 7.4. In general polyplexes (varying in N/P ratio, see below) were prepared by adding (in one step) different volumes of the polymer stock solution (depending on ratio) to a fixed amount (1µg) of the DNA stock solution (1mg/ml in HEPES buffer) and adding HEPES buffer to reach final volume, 40 µl. The mixture was vortexed for 10 sec and the polyplexes were allowed to equilibrate for 30 min at room temperature prior to use.

In this study the N/P ratio is the ratio of the number of nitrogen atoms on the cationic cellulose to the number of phosphor atoms on the DNA. It was assumed that 1 µg of DNA contains 3.43 nmol phosphor and that 1 µg of PQ-4, PQ-10 and PEI contains respectively 2.0 nmol, 3.2 nmol and 23.3 nmol nitrogen. Polyplex were also prepared from

'low molecular weight PQ-10'. The low molecular weight PQ-10 was obtained by incubating PQ-10 solutions (3 mg/ml in 20mM HEPES buffer at pH 7.4) with cellulase at 37°C. The reaction was terminated by heating the sample for 10 min at 95°C.

Particle Size and Zeta Potential Measurements on the Polyplexes

Dynamic light scattering measurements (DLS) on the polyplexes were carried out on a Malvern 4700 instrument at 25°C and at an angle of 90 degrees. The incident beam was a HeNe laser beam (633 nm). To avoid dust particles, the HEPES buffer used in the preparation of the polyplexes was filtered through a 0.45 μm membrane (Schleicher & Schuell). Polystyrene nanospheres (220 ± 6 nm; Duke Scientific) were used to verify the performance of the instrument. The particle size of each polyplex sample was measured three times.

Zeta potential measurements on the polyplexes were performed by electrophoretic light scattering at 25 °C on a Malvern Zetasizer 2000. Polystyrene nanospheres (-50 mV; Duke Scientific) were used to verify the performance of the instrument. The zeta potential of each polyplex sample was measured three times.

Gelelectrophoresis on the Polyplexes

Agarose gelelectrophoresis experiments were performed to monitor the complexation of pDNA to the cationic polymers. The slots of the agarose gel (1% agarose in Tris-borate EDTA buffer) were loaded with 40 μl of sample. Electrophoresis was carried out at 100 V for 60 min. The bands containing pDNA were visualized by staining the gels with an ethidium bromide solution for 30 min at room temperature, followed by UV illumination.

Fluorescence Measurements on the Polyplexes in the Presence of Picogreen

The complexation of pDNA to the cationic polymers was further investigated by measuring the extent of the quenching of picogreen. Picogreen is only fluorescent when it can intercalate with pDNA, thus when free (uncomplexed) pDNA is present. Briefly, 1μg of pDNA was mixed with increasing amounts of cationic polymer in HEPES buffer (at pH 7.4) and incubated for 30 min at room temperature to equilibrate. The fluorescence of the polyplex dispersions was measured 10 min after adding the picogreen to the dispersions. A SLM-Aminco Bowman spectrofluorimeter was used (λ_{ex} 480 nm, λ_{em} 520 nm).

Cell Viability and Cell Transfection Measurements

For evaluating the cytotoxicity and transfection properties of the polyplexes COS-7 cells were cultured in DMEM (Dulbecco's modified eagle medium) containing glutamine (Gibco) supplemented with 10% FBS (fetal bovine serum) (Gibco) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin, Sigma). The cells were seeded in 24-well culture plates at a density of about 25 000 cells/cm^2. After 24-48h incubation at 37°C in humidified air containing 5% CO_2, the cells were rinsed with PBS (phosphate buffer saline). Subsequently 500µl of culture medium (without serum), containing 40µl polyplexes, was added to each well and incubated at 37°C in a humidified air (5% CO_2) atmosphere for 2h. Subsequently, the transfection medium was removed, 500 µl DMEM (containing 10% FBS) was added to each well. Two days later, SEAP activity was determined in the medium using 4-MUP (4-methylumbellifery phosphate) [15]. Then the fluorescence due to conversion of 4-MUP to 4-methylumbelliferone was measured with a Wallac Victor 2 plate reader (Perkin Elmer; λ_{ex} 360 nm, λ_{em} 449 nm). To take into account the number of cells per well, the measured SEAP activity was normalized; therefore we divided the SEAP activity by the total protein content in the corresponding well which was measured using the DC protein assay (BioRad). The cytotoxicity of the polyplexes was measured by the EZ4U assay (Lucron Bioproducts; Biomedica). COS-7 cells were seeded in 96-well culture plates at a density of about 7000 cells/well. The cells were incubated with polyplexes at different ratio. Consequently the polyplex dispersions were removed from the cells in the wells. To each well 20µL EZ4U substrate in 200µl culture medium was added and incubated with the cells for 2-5 h. The absorbance of each well was measured with a Wallac Victor 2 plate reader (Perkin Elmer; λ_{ex} 450 nm, λ_{em} 495).

Dissociation of the Polyplexes

To study the dissociation of the polyplexes by polyanions, an excess of dextran sulfate (DS) or poly-L-aspartic acid (p(Asp)) was added to the polyplex dispersions (at a ratio of respectively 40:1 for DS/pDNA and 10:1 for p(Asp)/pDNA). The resulting mixtures were incubated for 30 min at room temperature. Gelelectrophoresis and the picogreen fluorescence assay (see above) were used to determine the extent of dissociation of the polyplexes.

Confocal Laser Scanning Microscopy (CLSM)

CSLM (MRC1024 Bio-Rad) was performed on COS-7 cells which were transfected by polyplexes containing fluorescently labeled pDNA (see below). The transfection of the cells with polyplexes occurred as described above. The cells were seeded in a glass culture dish to allow optical imaging.

Plasmid DNA Labeling

The plasmid DNA labelling reagents were obtained from Mirus Corporation. DNA (pMet7 hβ_c SEAP) was modified using *Label* IT reagents according to the recommendations of the manufacturer. Briefly, *Label* IT reagent was added to a DNA solution (100 µg DNA/ml (20 mM) HEPES buffer at pH 7.4) at 1:1 ratio (weight label/weight DNA) and incubated at 37°C for 1 h. To remove unbound fluorescein the pDNA was precipitated by adding 0.1 volumes of 5M NaCl and 2 volume of ice cold 100% ethanol to the pDNA. After centrifugation, the pellet was washed with 70% ethanol until the supernatant contained no fluorescein. Consequently the pellet was dissolved in HEPES buffer and the concentration was determined by absorption at 260 nm.

RESULTS & DISCUSSION

pDNA Binds to Cationic Cellulose

As outlined above, the binding of pDNA to the cationic polymers was studied by gelelectrophoresis. The gelelectrophoresis measurements in Figure 2 clearly show that at N/P ratios of 1 and higher both the cationic celluloses and poly(ethylene imine)s bind all the pDNA. The polymer/pDNA complexes remain in the slots as they are unable to migrate through the gel. Clearly, a difference between PQ-4 and PQ-10 is that at N/P ratio 0.1 PQ-4 binds all the pDNA whereas PQ-10 binds only a part of the pDNA.

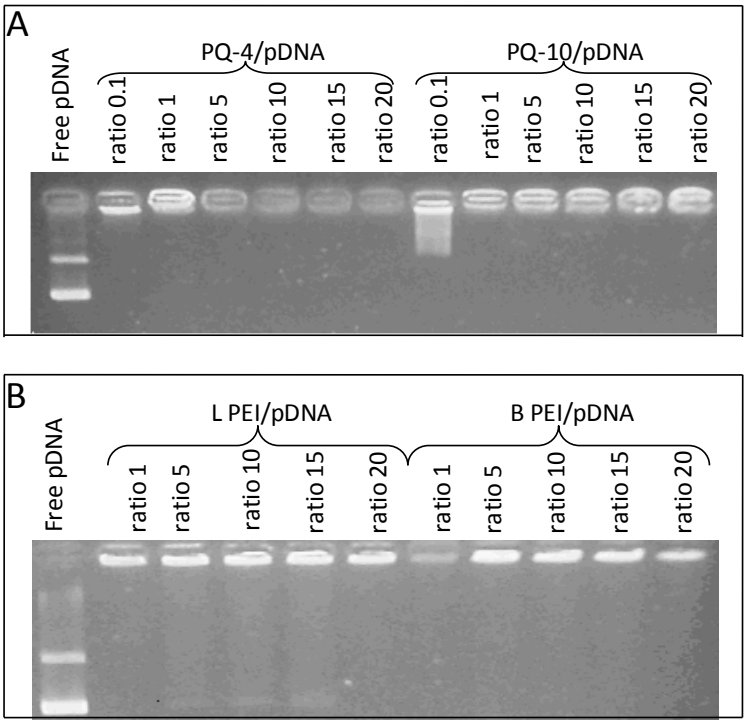


Figure 2. Gelelectrophoresis on dispersions of (A) PQ-4/pDNA and PQ-10/pDNA complexes and (B) BPEI/pDNA and LPEI/pDNA complexes. The N/P ratio was varied.

Figure 3 shows the complexation between the cationic celluloses and pDNA as revealed from the fluorescence quenching assay. As explained in the materials and method section, picrogreen was added to the dispersions to detect free pDNA. As expected, the fluorescence decreases at higher concentrations of the cationic polymers: it indicates that the pDNA molecules not only bind to the cationic polymer chains but that pDNA molecules also condense to such an extent that picrogreen has less access to/ less intercalates in the double stranded DNA yielding less fluorescence. In case of PQ-4, LPEI and BPEI the fluorescence is almost completely disappeared at an N/P higher than 1. This confirms the gelelectrophoresis data in Figure 2 as free pDNA was not detected in the dispersions. In case of PQ-10, however, even at higher N/P ratio's the fluorescence seemed to be only partially quenched when compared to the fluorescence of the corresponding free pDNA solution. Although, the gelelectrophoresis data showed that at these N/P ratios PQ-10 binds all the pDNA. The partial quenching in Figure 3 probably indicates that PQ-10 does not condense pDNA as tightly as the other cationic polymers, still allowing picrogreen to intercalate to a certain extent in the pDNA.

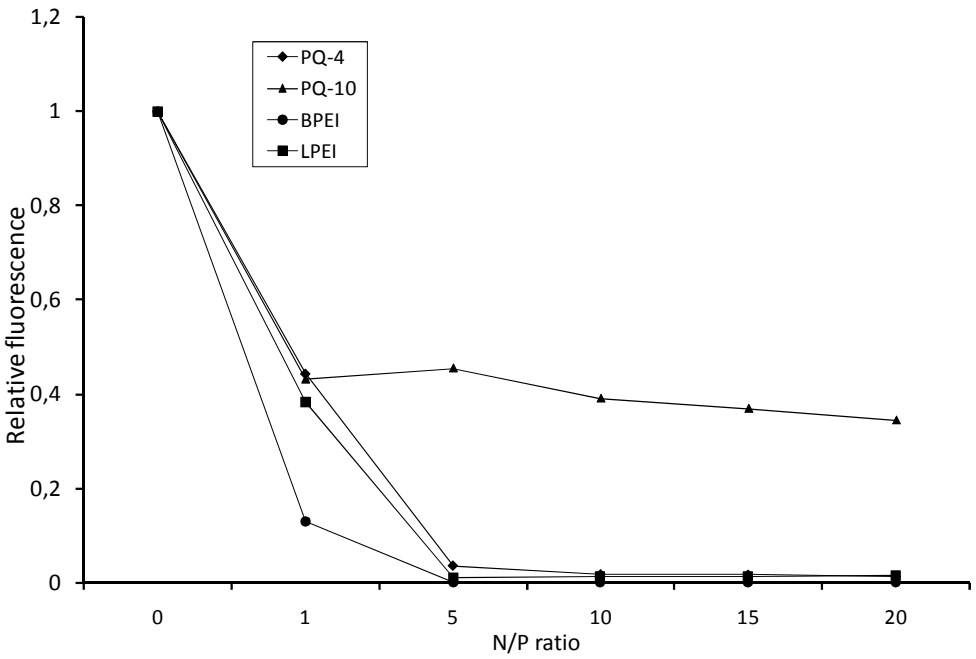
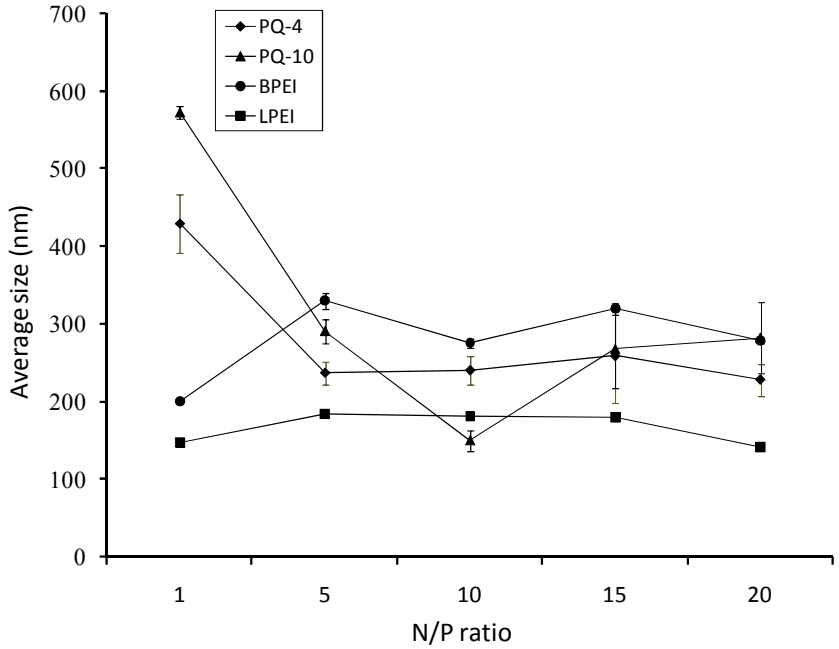


Figure 3. Relative fluorescence of cationic cellulose/pDNA and PEI/pDNA dispersions (as a function of the N/P ratio of the complexes) containing picogreen. The amount of pDNA was kept constant (1µg) and the concentration of cationic polymers was increased. The fluorescence of a (free) pDNA solution was set to 1. The fluorescence of three samples was measured. The error bars were smaller than the size of the symbol.

Size and Zeta Potential of pDNA /Cationic Cellulose Complexes

Figure 4A shows that at N/P ratio of 1 the cationic cellulose/pDNA complexes are substantially larger than the PEI/pDNA complexes; at higher N/P ratio's all types of pDNA complexes are of similar size (between 150 and 300 nm). Figure 4B reveals that at high N/P ratio's the PQ-10 based complexes are clearly positively charged while the zeta-potential of the PQ-4 based complexes is significantly lower. This can be explained by the structural differences between PQ-4 and PQ-10 (Fig. 1). The sugar backbone of PQ-4 is substituted with both polycation chains (which bind the DNA) as well as with neutrally charged PEG chains (which are not involved in the binding of pDNA). We assume that the PEG chains form a neutral shell around the inner core of the polyelectrolyte complexes. The resulting complexes might resemble the core-shell polyplexes formed between graft-polyelectrolytes and oppositely charged polymers or biomacromolecules[16]. In contrast, in PQ-10 the cationic groups are attached to the free end of the PEG side chains. Therefore we assume that, upon binding the pDNA, the PEG strands are (sterically) not allowed to create a neutralizing PEG-shell around the complexes.

A



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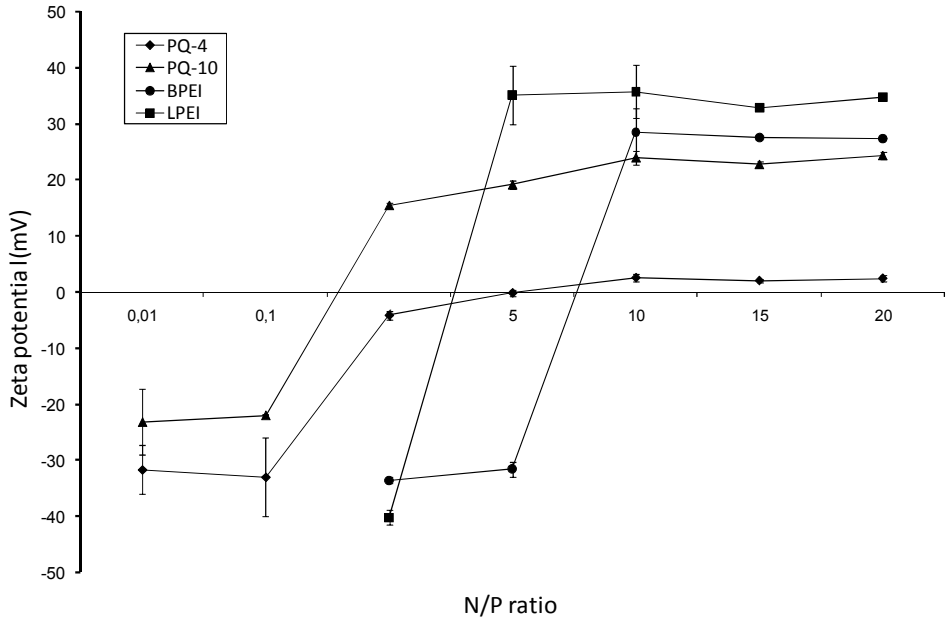

Figure 4. Hydrodynamic diameter (A) and zeta potential (B) of cationic cellulose and PEI based pDNA nanoparticles. Data represent the mean values obtained on 5 (independently prepared) samples.

Cytotoxicity of pDNA/Cationic Cellulose Complexes

As outlined in the introduction, we are interested in how cationic cellulose based DNA nanoparticles behave in a cellular environment. Clearly, an absolute requirement is that

the nanoparticles should be not toxic for cells. Therefore the cytotoxicity of the cellulose based polyplexes was studied using COS-7 cells. Figure 5 shows the results of the cell viability assay. PQ-4 and PQ-10 based pDNA complexes show low toxicity as more than 90% of the cells remains alive. At higher N/P ratio the cationic cellulose/ pDNA dispersions seemed to be slightly more toxic which may be due to the presence of a higher amount of (free) cationic cellulose damaging the cellular membranes [17]. The PEI based complexes demonstrated low cytotoxicity as well. Indeed, COS-7 cells seemed to tolerate well the PEI based complexes used in this study as the cell viability was around 80% compared to untreated cells. Although it has been reported that low molecular weight PEI shows low membrane damaging [18] and that cell damaging is more severe for free PEI compared to PEI/DNA complexes [19], this low cytotoxity of the PEI polyplexes was rather unexpected as some studies reported significant cytotoxicity [20,21]

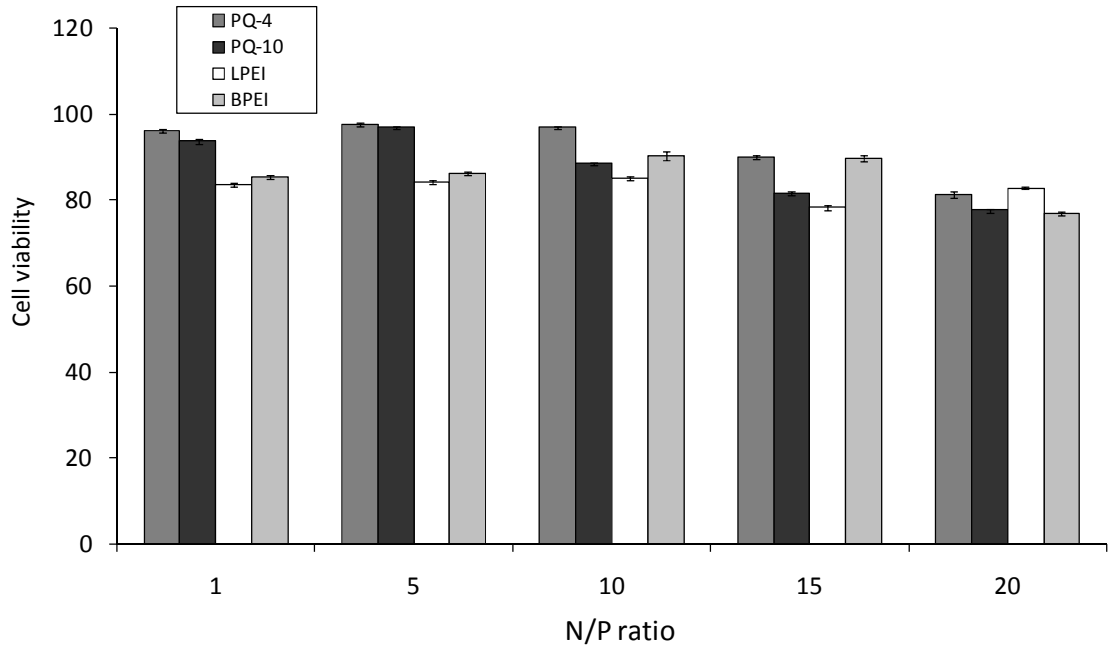

Figure 5. Viability of COS-7 cells after incubation with cationic cellulose and PEI based pDNA nanoparticles. The data are the mean values obtained on three (independently prepared) samples.

Also, the group of Kissel [22,23] showed that the molecular weight and type of PEI influences the cytotoxicity. From MTT-assay's [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] they concluded that cytotoxicity of PEI is affected by polymer size and structure: high cationic charge densities, compact and highly branched structures, and high

molecular weights render PEI less biocompatible [18]. Anyhow, the fact that the cationic cellulose based polyplexes seemed to be well tolerated by cells was encouraging for further transfection experiments.

In Vitro Transfection of Cells by pDNA/Cationic Cellulose Complexes

Figure 6A shows the transfection efficiency of cationic cellulose and PEI based polyplexes in COS-7 cells as a function of the amount of pDNA per well. Note that the N/P ratio of all the complexes studied in Figure 6 equalled 10. Clearly, PEI based polyplexes transfect significantly better than cationic cellulose/pDNA complexes. LPEI proofs to be a better transfectant than BPEI, in agreement with earlier findings [24,25]. PQ-10/pDNA complexes show minor transfection efficiency while PQ-4 does not transfect the COS-7 cells at all. As there is evidence that a positive surface charge promotes cellular uptake due to binding of the complexes to the negatively charged cell membranes, we hypothesize that PQ-4/pDNA-complexes do not transfect due to their neutral surface charge (Fig. 4B). Indeed, confocal microscopy measurements did not evidence any binding or uptake of the PQ-4/pDNA nanoparticles (data not shown). We observed the same trend in transfection behaviour when we used Hela cells in stead of COS-7 cells (Fig. 6).

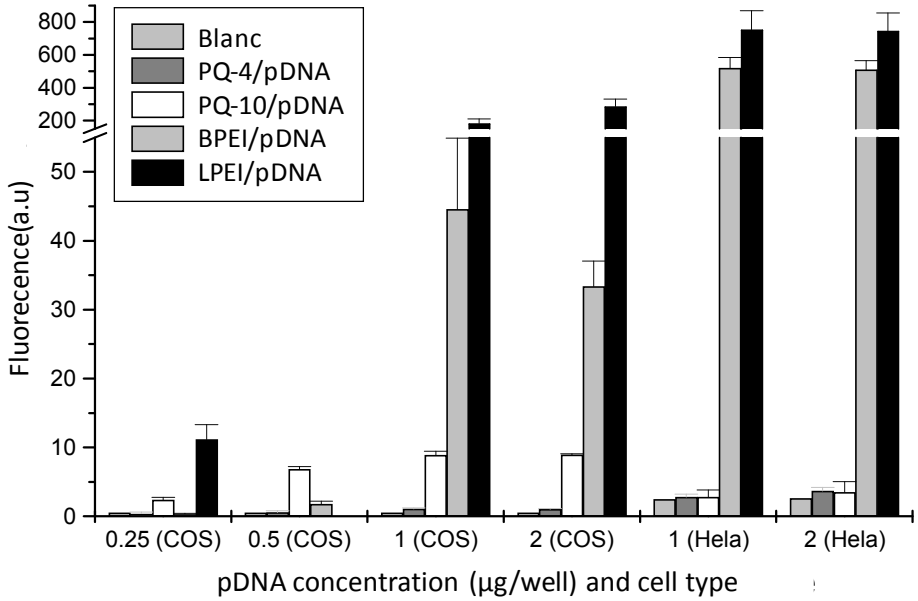

Figure 6. Transfection efficiency of cationic cellulose and PEI based pDNA nanoparticles in COS-7 cells and Hela cells as a function of the concentration of pDNA per well (n = 6). The N/P ratio of all the complexes equalled 10. (note: COS cells 0.5µg/well for LPEI/pDNA: not determined)

These results lead to the intriguing question why the positively charged PQ-10/pDNA complexes fail in transfecting COS-7 and Hela cells efficiently, while PEI/pDNA complexes of similar surface charge successfully deliver the same pDNA in the cells. The molecular weight of PQ-10 is 1700 kDa while the molecular weight of respectively LPEI and BPEI is 22 kDa and 25 kDa. To find out whether a lower molecular weight of PQ-10 would improve the transfection efficiency we degraded the sugar backbone of PQ-10 by treating it with endocellulase prior to complexation with pDNA. Capillar viscosity measurements on PQ-10 solutions incubated with cellulase showed a significant decrease (65%) in viscosity indicating that that PQ-10 indeed degraded. Figure 7 shows the transfection efficiency of the pretreated PQ-10: compared to high molecular weight PQ-10, lower molecular weight PQ-10 transfects better, however, still less efficient than BPEI.

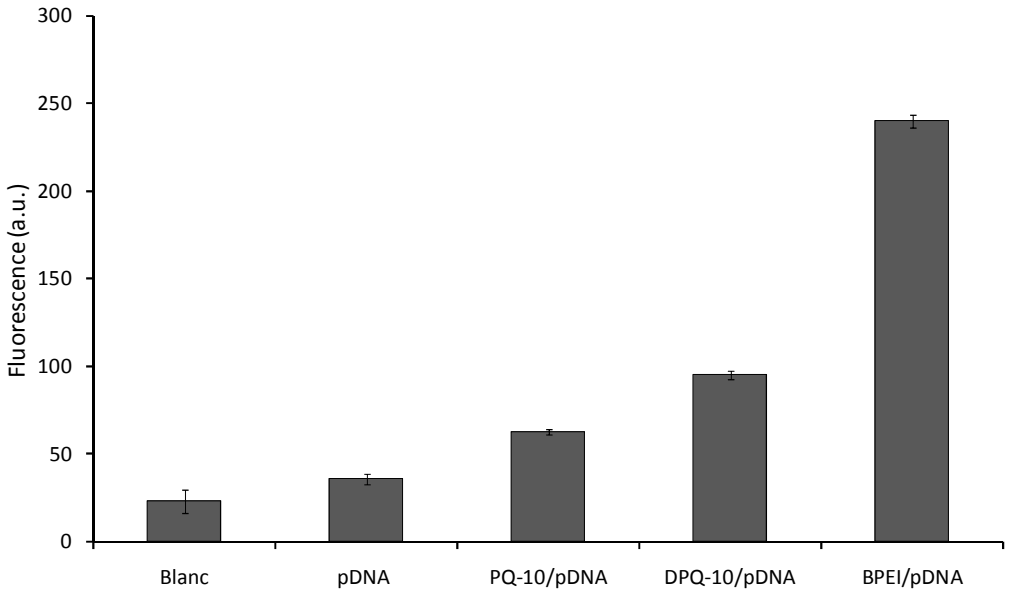

Figure 7. Transfection efficiency of cationic cellulose and PEI based pDNA nanoparticles in COS-7 cells. DPQ-10 denotes the complexes made from degraded PQ-10. The N/P ratio of the complexes is 10. The data are the mean values obtained on three (independently) prepared samples.

It has been proposed in many studies that the good transfection properties of PEI are partly due to the fact that it facilitates the escape of pDNA from the endosomes. This is usually explained by the fact that in the endosomes PEI works as a buffer ("proton-sponge") based on the uniqueness of its chemical structure [26] which eventually causes swelling and disruption of the endosomes. Consequently, PEI polyplexes in the endosomes can be efficiently delivered into the cytoplasm [27]. Because of quaternary ammonium, PQ-10 cannot buffer the endosomal pH. To investigate whether the low transfection efficiency of PQ-10

was in part attributed to a lack of endosomal escape of the polyplexes, we tested compounds known to improve the cytosolic delivery of macromolecules after their cellular uptake by the endosomes. Chloroquine is a lysosomotropic agent thought to have a buffering capacity preventing endosomal acidification[27]. In addition, it can lead to swelling and bursting of the endosomes and was shown to enhance DNA transfection [28,29,30]. However, co-incubation of the COS-7 cells with 100µM chloroquine did not improve the transfection activity by PQ-10/pDNA complexes (data not shown). In a second approach, a membrane disrupting peptide derived from the influenza virus (INF-7) was used, which is known to destabilize endosomal membranes (facilitating the escape of the viral particles from the endosomes) [31]. While other studies have shown that INF-7 significantly enhances the trancfection activity in COS-7 cells [31,32], addition of this peptide to PQ-10/pDNA polyplexes did not improve their transfection behaviour (data not shown). These data indicate that the lack of endosomal escape is probably not the major reason for the low transfection activity of PQ-10/DNA nanoparticles.

pDNA Release from Cationic Cellulose

Another critical step for obtaining successful transfection is the intracellular release of the pDNA from its carrier. While the pDNA has to remain associated with its carrier as long as it is outside the cells, intracellularly it has to release its carrier. In other words, a critical balance between being associated extracellularly and becoming dissociated intracellularly has to be maintained. To obtain information on 'the ease of disassembling' of the complexes we studied in buffer to which extent pDNA could be displaced from the complexes by the polyanions poly-L-aspartic acid and dextran sulfate. Their disassembling behavior was studied using agarose gel electrophoresis and a fluorescence dequenching assay.

After incubation of the polyplexes with an excess of p(Asp) gelelectrophoresis revealed that both linear and branched PEI almost completely released the pDNA (Fig. 8A). In contrast, PQ-4 only partially released the pDNA whereas PQ-10 did not release pDNA at all (Fig. 8B), suggesting a stronger binding of pDNA to cationic celluloses than to PEI. Similar results were obtained when dextran sulfate was added to the cationic cellulose/pDNA nanoparticles (data no shown). Fluorescence dequenching experiments (Fig. 9) show that upon adding p(Asp) all the pDNA is released from the PEI based complexes, in agreement

with the gelelectrophoresis results in Figure 8A. Also, p(Asp) (almost) completely releases pDNA from the PQ-4/pDNA complexes which does fully agree with the outcome of the gelelectrophoresis experiments in Figure 8B which suggested that the pDNA was only partially released at higher N/P ratios. Possibly p(Asp) only "partially" disassembles PQ-4/pDNA complexes in the sense that the pDNA molecules become fully accessible for picogreen without becoming really released from the cationic cellulose polymers. Importantly, the fluorescence dequenching experiments in Figure 9 indicate that PQ-10/pDNA complexes cannot be destabilized by p(Asp) in full agreement with the observations in the gelelectrophoresis experiments in Figure 8B.

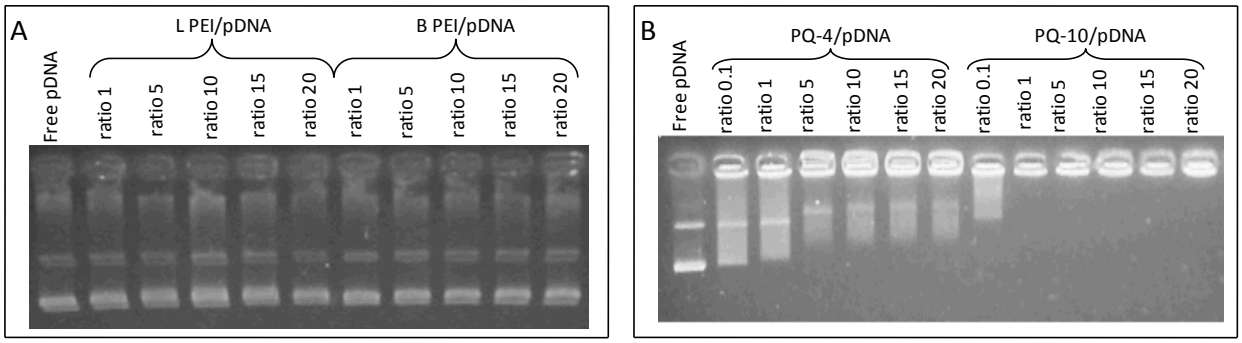

Figure 8. Gelelectrophoresis on dispersions of (A) BPEI/pDNA and LPEI/pDNA complexes and (B) PQ-4/pDNA and PQ-10/pDNA complexes. p(Asp) was added to the dispersions. The N/P ratio of the complexes was varied.

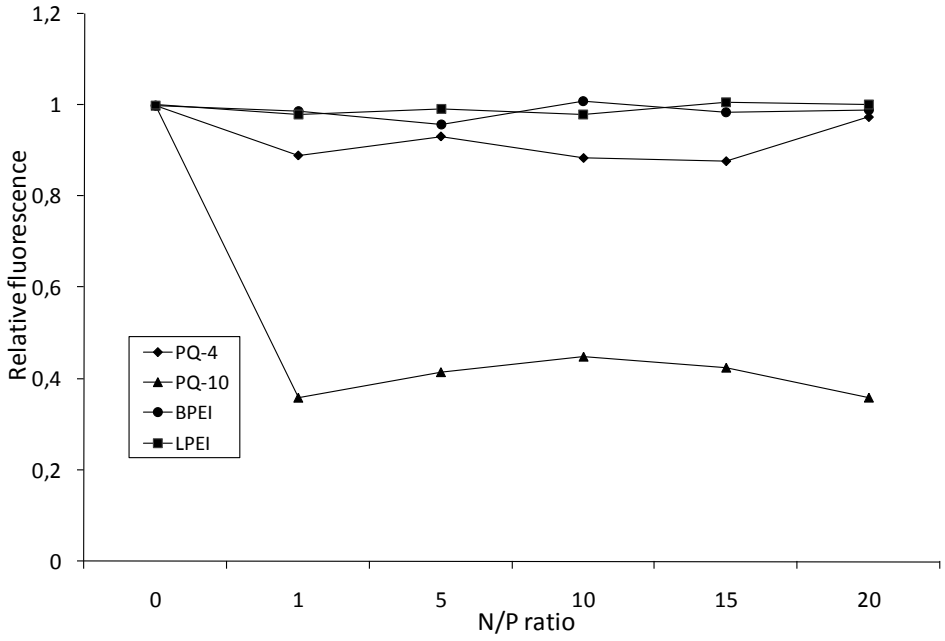

Figure 9. Fluorescence of (picogreen containing) cationic cellulose/pDNA and PEI/pDNA dispersions after adding p(Asp). The N/P ratio of the complexes was varied.

CONCLUSION

We have studied the physicochemical and transfection properties of PQ-4/pDNA and PQ-10/pDNA nanoparticles and compared them with DNA nanoparticles based on respectively branched and linear PEI. Gelelectrophoresis and fluorescence experiments indicated that PQ-4 can bind and condense pDNA, through stronger interactions than PEI. The resulting PQ-4/pDNA nanoparticles are approximately 200 nm in size and have a neutral zeta-potential which is explained by the fact that the sugar backbone of PQ-4 bears, besides polycation side chains, also PEG side chains. We observed that PQ-4/pDNA nanoparticles do not transfect COS-7 and Hela cells which we attribute to their neutrally charged surface which does not allow an optimal binding of PQ-4/pDNA nanoparticles to cellular membranes, thus preventing an efficient cellular uptake. Developing a PQ-4 like cationic cellulose with a lower degree of pegylation might improve the transfection properties of PQ-4/pDNA nanoparticles.

PQ-10/pDNA nanoparticles have a positively charged surface. Gelectrophoresis experiments indicated that PQ-10 binds pDNA stronger than PEI and PQ-4. However, upon binding to PQ-10 pDNA does not completely condense as picogreen can still, to a certain extent, intercalate in the pDNA. Although the PQ10/pDNA nanoparticles are positively charged, and thus expected to be taken up by cells, they are much less efficient in transfecting cells than PEI/pDNA nanoparticles. Agents known to enhance the endosomal escape (like chloroquine and the INF-7 peptide) were not able to improve the transfection properties of PQ-10/pDNA nanoparticles indicating that a poor endosomal escape is, highly likely, not the major reason for the low transfection activity of PQ-10/pDNA nanoparticles. Based on our findings we hypothesize that the strong binding of pDNA to PQ10 inhibits the release of pDNA from PQ-10 once the PQ-10/pDNA nanoparticles arrive in the cytosol of the cells. As the PQ-10/pDNA nanoparticles did transfect the cells to a certain extent, although less efficient than PEI/pDNA nanoparticles, tailoring of the nature and extent of cationic side chains on this type of cationic hydroxyethylcellulose may be promising to further enhance their DNA delivery properties.

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REFERENCES

1. Brown M.D., Schatzlein A.G., Uchegbu I.F., Gene delivery with synthetic (non viral) carriers. Int J Pharm **2001**, 23, 1-21.

2. Wagner E., Ogris M., Zauner W., Polylysine-based transfection systems utilizing receptor-mediated delivery. Adv Drug Delivery Rev **1998**, 30, 97-113.

3. Rodriguez R., Alvarez-Lorenzo C,. Concheiro A., Rheological evaluation of the interactions between cationic celluloses and Carbopol 974P in water. Biomacromolecules **2001**, 3, 886-93.

4. Azzam T., Eliyahu H., Shapira L., Linial M., Barenholz Y., Domb A.J., Polysaccharide-oligoamine based conjugates for gene delivery. J Med Chem **2002**, 45, 1817-24.

5. Azzam T., Raskin A., Makovitzki A., Brem H., Vierling P., Lineal M., Domb A.J., Cationic polysaccharides for gene delivery Macromolecules **2002**, 35, 9947-53.

6. Ruponen M., Honkakoski P., Ronkko S., Pelkonen J., Tammi M., Urtti A., Extracellular and intracellular barriers in non-viral gene delivery. J Control Release **2003**, 93, 213-7.

7. Wiethoff C.M., Middaugh C.R., Barriers to non-viral gene delivery. J Pharm Sci **2003**, 92:203-17.

8. Sanders N.N., De Smedt S.C., Demeester J., Mobility and stability of gene complexes in biogels. J Control Release **2003**, 87, 117-29.

9. Hosseinkhani H., Azzam T., Tabata Y., Domb A.J., Dextran-spermine polycation: an efficient nonviral vector for in vitro and in vivo gene transfection. Gene Therapy **2004**,11, 194-203.

10. Azzam T., Eliyahu H., Makovitzki A., Linial M., Domb A.J., Hydrophobized dextran-spermine conjugate as potential vector for in vitro gene transfection. J Control Release **2004**, 96, 309-23.

11. Yudovin-Farber I., Yanay C., Azzam T., Linial M., Domb A.J., Quaternary ammonium polysaccharides for gene delivery. Bioconjug Chem **2005**, 16, 1196-203.

12. Rodriguez R., Alvarez-Lorenzo C., Concheiro A., Influence of cationic cellulose structure on its interactions with sodium dodecylsulfate: implications on the properties of the aqueous dispersions and hydrogels. Eur J Pharm Biopharm **2003**, 56, 133-42.

13. Brode G.L., Goddard E.D., Harris W.C., Salensky G.A., In cosmetic and pharmaceutical applications of polymers; Gebelein, C. G., Cheng, T. C., Yang, V. C., Plenum press: New York, **1991**, 117-128.

14. Rodriguez R., Alvarez-Lorenzo C., Concheiro A., Cationic cellulose hydrogels: kinetics of the cross-linking process and characterization as pH-/ion-sensitive drug delivery systems. J Control Release **2003**, 86, 253-65.

15. Berger J., Hauber J., Hauber R., Geiger R., Cullen B.R., Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. Gene. **1988**, 66, 1-10.

16. Petersen H., Fechner P.M., Martin A.L., Kunath, K., Stolnik S., Roberts C.J., Fischer D., Davies M.C., Kissel T., Poly(ethylene imine)-graft-poly(ethylene glycol) copolymers: influence of copolymer block structure on DNA complexation and biological activities as gene delivery system , Bioconjug. Chem., **2002**, 13, 845-854.

17. Choksakulnimitr S., Masuda S., Tokuda H., Takakura Y., Hashida M., *In vitro* cytotoxicity of macromolecules in different cell culture systems. J. Control. Release **1995**, 34, 233–241.

18. Kunath K., Von Harpe A., Fischer D., Petersen H., Bickel U., Voigt K., Kissel T., Low-molecular-weight poly(ethylene imine) as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight poly(ethylene imine). J Control Release **2003**, 89, 113-25.

19. Godbey W.T., Wu K.K., Mikos A.G., Poly(ethylene imine) and its role in gene delivery. J Control Release. **1999**, 60, 149-60.

20. Godbey W.T., Wu K.K., Mikos A.G., Poly(ethylene imine)-mediated gene delivery affects endothelial cell function and viability. Biomaterials. **2001**, 22, 471-80.

21. Putnam D., Gentry C.A., Pack D.W., Langer R., Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. Proc Natl Acad Sci U S A **2001**, 98, 1200-5.

22. Fischer D., Bieber T., Li Y., Elsasser H.P., Kissel T., A novel non-viral vector for DNA delivery based on low molecular weight, branched poly(ethylene imine): effect of molecular weight on transfection efficiency and cytotoxicity. Pharm Res **1999**, 16, 1273–79.

23. Fischer D., Bieber T, Elsasser HP, Kissel T. Poly(ethylene imine); synthesis and *in vitro* cytotoxicity of a low molecular weight polycation for gene transfer. Eur J Cell Biol **1998**, 75, 108–116.

24. Wightman L., Kircheis R., Rossler V., Carotta S., Ruzicka R., Kursa M., Wagner E., Different behavior of branched and linear poly(ethylene imine) for gene delivery in vitro and in vivo. J Gene Med **2001**, 3, 362-72.

25. Itaka K., Harada A., Yamasaki Y., Nakamura K., Kawaguchi H., Kataoka K., *In situ* single cell observation by fluorescence resonance energy transfer reveals fast intra-cytoplasmic delivery and easy release of plasmid DNA complexed with linear poly(ethylene imine). J Gene Med **2004**, 6, 76-84.

26. Boussif O., Lezoualc'h F., Zanta M.A., Mergny M.D., Scherman D., Demeneix B., Behr J.P., A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: poly(ethylene imine). Proc Natl Acad Sci U S A **1995**, 92, 7297-301.

27. Funhoff A.M., Van Nostrum C.F., Koning G.A., Schuurmans-Nieuwenbroek N.M., Crommelin D.J., Hennink W.E., Endosomal escape of polymeric gene delivery complexes is not always enhanced by polymers buffering at low pH. Biomacromolecules **2004**, 5, 32-9.

28. Ciftci K., Levy R.J., Enhanced plasmid DNA transfection with lysosomotropic agents in cultured fibroblasts. Int J Pharm **2001**;218, 81-92.

29. Erbacher P., Roche A.C., Monsigny M., Midoux P., Putative role of chloroquine in gene transfer into a human hepatoma cell line by DNA/lactosylated polylysine complexes. Exp Cell Res **1996**, 225, 186–194.

30. Mellman I., Fuchs R., Helenius A., Acidification of the endocytic and exocytic pathways. Annu Rev Biochem **1986**, 55, 663-700.

31. Plank C., Oberhauser B., Mechtler K., Koch C., Wagner E.J., The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. J Biol Chem **1994**, 269, 12918-24.

32. Zuidam N.J., Posthuma G., de Vries E.T., Crommelin D.J., Hennink W.E., Storm G., Effects of physicochemical characteristics of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes on cellular association and internalization. J Drug Target. **2000**, 8, 51-66.

Chapter 3

Evaluation of Digitally Encoded Layer-by-Layer Coated Microparticles as Cell Carriers

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ABSTRACT

To obtain more biologically relevant data there is a growing interest in the use of living cells for assaying the biological activity of unknown chemical compounds. Density "multiplex" cell-based assays, where different cell types are mixed in one well and simultaneously investigated upon exposure to a certain compound are beginning to emerge. To be able to identify the cells they should be attached to microscopic carriers that are encoded. This paper investigates how digitally encoded microparticles can be loaded with cells keeping the digital code in the microcarriers readable. It turns out that coating the surface of the encoded microcarriers with polyelectrolytes using the Layer-by-Layer (LbL) approach provides the microcarriers with a "highly functional" surface. The polyelectrolyte layer (a) allows the growth of the cells (b) allows the orientation of the cell loaded microcarriers in a magnetic field and (c) does not hamper the reading of the code. We conclude that the digitally encoded microparticles are promising materials for use in biomedical and pharmaceutical *in vitro* research where cells are used as tools.

Chapter 3

Evaluation of Digitally Encoded Layer-by-Layer Coated Microparticles as Cell Carriers

INTRODUCTION

Due to significant progress in combinatorial chemistry the synthesis of huge libraries of drug candidates is no longer a bottleneck in the drug discovery process. However, screening the biological activity of these numerous compounds at high throughput remains very challenging. Historically, "biochemical assays" were often employed to screen the interaction between unknown compounds and targets in solution. To obtain more biologically relevant data from screening work, there is a growing interest in the use of living cells for assaying the biological activity of unknown compounds as this approach is better suited for the complex biology surrounding the target molecules [1]. For example, because a majority of today's drugs target G-protein-coupled receptors (GPCRs) in the cell membranes, major efforts have been made to develop cell-based, high throughput assays that screen compounds for their interaction with GPCRs [2,3]. Typically, in such cellular assays, the response of living cells (e.g. the calcium flux in the cells or the activation of a reporter gene) upon exposure of the cells to an unknown compound is measured to detect GPCR responses.

Initially, cell based assays were performed in the wells of microtiter plates. Typically, each well contained one type of cells that were exposed to a certain compound. In an attempt to increase data density and quality and to save time and reagents, "multiplex" cell based assays are beginning to emerge. In "multiplex cellular assays" different cell types are mixed in one well and simultaneously investigated upon exposure to a certain compound.

In general, multiplex technologies are divided in two categories; "positional arrays" (also named “flat surface arrays”) [4-9] and "non positional arrays" (also named “suspension arrays”) [10-16]. A positional cell array offers a format without wells for high-throughput cell based assaying; the identity of each cell is determined by its x,y-position on the array. As the name implies, non positional cell arrays do not rely on the location of a cell on an array to identify the cell type but on a "code" written in a (micro) particle which carries the cell. Each cell type is thus grown on an encoded microcarrier and therefore physically associated with the code of this microcarrier. It is believed that non positional arrays have greater flexibility and require smaller sample volumes compared to positional arrays [16]. Non positional arrays are not only of interest for multiplex cell based assaying but also for multiplex protein and nucleic acid assaying. Various strategies have been applied to encode microparticles: spectrometric encoding [17,18], electronic encoding [19,20], physical encoding [21,22] and graphical encoding [23-25].

Interesting information on the growth of (normal and genetically engineered) mammalian cells on the surface of non-encoded microcarriers is available [26] . Cell loaded microcarriers (based on e.g. cross-linked dextran or polystyrene) have been used for a long time, e.g. producing recombinant proteins (used as biopharmaceuticals in therapy and diagnostics) and viruses (used as vaccines) [26-28]. Clearly, the surface of the microcarrier at physiological pH should supply sites which favour the attachment, spreading and proliferation of the cells. Also, the density of the microcarriers should not be too high because a settling down of the beads could possibly prevent cell growth. Furthermore, the size-distribution of the microcarriers should be narrow so that cells on the different microcarriers attain confluency at approximately the same time and, last but not least, the microcarriers should be non-toxic to the cells.

To our knowledge there are only few reports which deal with the growth and the behaviour of cells on encoded microcarriers [29-32]. This paper studies whether photophysically encoded microparticles, recently introduced by our group for various applications [33-35] and named “Memobeads”, are suitable to grow cells on. As shown in (Fig. 1B, 1C), photophysically encoded microparticles carry a digital code (like a dotcode or a barcode) in their middle plane. The code in a particular bead reads out which”sensing molecule” or”probe” is bound to its surface. The probe can be an antibody (for screening antigens), a single stranded oligonucleotide (for single nucleotide polymorphism detection) or, in this

study, a cell. Before attaching the probe, we first applied at the surface of a Memobead a "Layer by Layer" (LbL) coating which is composed of polyelectrolytes (PEs) (Fig. 1A). 'LbL coating' is based on the alternate adsorption of oppositely charged PE's (or charged nanoparticles) onto a charged substrate [36-39] LbL technology has raised considerable interest [40,41]. A large variety of functionalized material such as DNA [42-46], drugs [47,48], proteins and peptides [49-51] can be incorporated into these assemblies. Therefore LbL films could play an important role in development of localized and controlled delivery of macromolecules.

We reported previously that the polystyrene core of a Memobead can be successfully LbL coated with polycations (PAH =poly(allylamine hydrochloride)), polyanions (PSS = poly(styrene sulfonate)) and ferromagnetic chromium dioxide nanoparticles (CrO_2 NP). As explained in detail by Derveaux *et al.* [33] the CrO_2 NP allow an optimal positioning of the Memobeads in a magnetic field, which is necessary to read the code at the time of decoding (Fig. 1D).

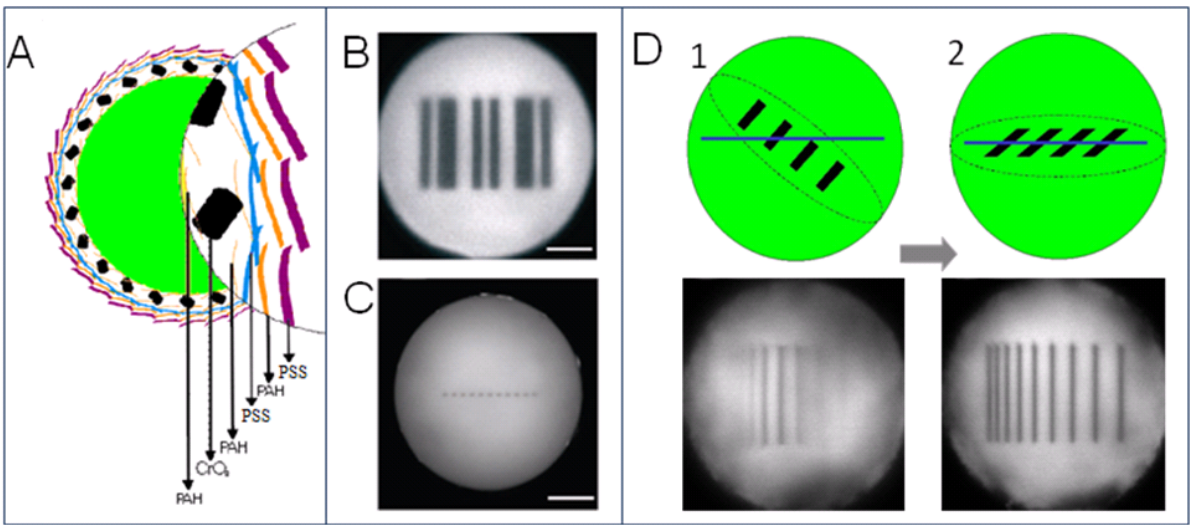

Figure 1. (A) Schematic representation of the LbL coating of green fluorescent polystyrene microspheres. The polycation PAH and the polyanion PSS are alternately adsorbed to the surface of the microcarrier. Magnetic CrO_2 nanoparticles are immobilized in the LbL coating. (B,C) Confocal images of the central plane of fluorescent microspheres encoded with respectively a bar code (B) and a dot code (C). The scale bar represents 10 µm. (D) A correct orientation and position of the microsphere is required at readout time. At position D1, the code is tilted with respect to the microscope focal plane and only the intersection of the code with the focal plane is visible. At position D2 the entire code is visible because it coincides with the focal plane.

In the present study we aim to evaluate (a) whether cells can be grown on the magnetic LbL surface of digitally encoded microcarriers and (b) whether the cell layer at the surface does not hamper the decoding of the beads. Indeed, the heavy cell load might

prevent the orientation of the beads in a magnetic field while the fluorescence in the cells (due to the expression of a fluorescent reporter protein) may interfere with the optical reading of the code. Further we aim to evaluate (c) whether we can perform an ELISA assay on the cells at the LbL surface. The possibility to perform ELISA's in the cells on the encoded microcarriers may be important tools for cell-based multiplexing with such encoded microcarriers.

MATERIALS & METHODS

Materials

Non-magnetic fluorescent carboxylated microspheres (CFP-40052-100, ø = 39 µm) were purchased from Spherotech (Libertyville, Illinois, USA). Poly (allylamine hydrochloride) (PAH; 70 kDa) and sodium poly (styrene sulfonate) (PSS; 70 kDa) were obtained from Sigma Aldrich (Steinheim, Germany). LysoTracker Red DND-99 was obtained from Molecular Probes. HEK-293 cells and Antibody against human IL5R-alpha were a kind gift of Prof. J. Tavernier (Ghent University). CHO-320 cells were a kind gift from Prof. Y.J. Schneider (Catholic University of Louvain). Cy5- goat anti- mouse IgG (H+L) conjugate was purchased from Zymed (San Francisco, CA). Leukemia Inhibitory Factor (LIF) was obtained from Sigma Aldrich (Steinheim, Germany). HEK-293, Vero-1, COS-7, Hela, HuH-7 and CHO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Merelbeke, Belgium) containing 2 mM L-glutamine (L-Gln), 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S).

Layer-by-Layer coating of the microcarriers

The polystyrene microspheres were coated with the polyelectrolytes PSS and PAH as reported previously [33]. The microspheres were LbL coated by suspending them in 1 ml (2 mg/ml) PAH solution (prepared in 0.5 M NaCl); the suspension was continuously vortexed (1000 rpm, 25°C) for 15 min. The non-adsorbed PAH was removed by repeated centrifugation and washing. Subsequently, the microspheres were dispersed in deionised water containing sub 500 nm CrO_2 NP. The CrO_2 NP are used to position the microspheres in a magnetic field to allow the reading of the code, as described in detail elsewhere [33]. The microsphere dispersion was continuously shaken for 15 min and the excess of CrO_2 NP was

removed by repeated centrifugation/washing steps. The third, fourth… polyelectrolyte layer was applied in a similar way as the first layer. Finally as illustrated in Figure 2(A) the microspheres were coated with 5 or 6 layers in the following order: PAH / sub 500 nm CrO_2 NP / PAH / PSS / PAH / PSS. Then resulting LbL coated microspheres were resuspended in 1 ml of ultrapure demiwater (± 400 000 microspheres/ml) and subsequently encoded (see below).

Growing cells on the microcarriers

The LbL coated (encoded) beads were dispersed in cell culture medium (DMEM containing about 50 % bovine serum). An appropriate volume of the microsphere dispersion (approximately 100 µl) was applied in polycarbonate Erlenmeyer shake-flasks (Corning) that were treated with a silicone solution (Sigmacoat®; Sigma) following the manufacturer's protocol. The silicone treatment should prevent the attachment of cells to the surface of the flasks. Subsequently, an appropriate amount of cell suspension (in DMEM supplemented with 2% P/S, 1% L-GLn and 10% FBS) was added to the flasks for 3 hours at 37°C and 5% CO_2 to allow the attachment of cells to the surface of the beads. During this 3 hours period the flasks were not shaken to allow the initial attachment of the cells. Subsequently we began to agitate the flasks on an orbital shaker under appropriate conditions to prevent the precipitation of the microcarriers, which prevents an optimal cell growth. Typically after one night the cell loaded microcarriers were used for further examination.

Encoding of the microcarriers

The LbL coated microspheres were encoded by spatial selective photobleaching as previously described [23]. An in-house-developed encoding device was used, being a microscopy platform equipped with an Aerotech ALS3600 scanning stage, a SpectraPhysics 2060 Argon laser and an Acousto-Optic-Modulator (AA.MQ/A0.5-VIS, A.A-Opto-Electronique, Orsay Cedex, France). The encoding process consists of two steps, a writing step (i.e. the photobleaching process) and a magnetizing step, during which the CrO_2 loaded microspheres are exposed to an external magnetic field sufficient to provide them with a magnetic memory. The microspheres were fixed on a grid during the encoding process to prevent rotation between the two steps.

Decoding of the cell loaded microcarriers

The cell loaded microspheres were decoded using a confocal laser scanning microscope (CLSM using a Bio-Rad MRC1024) equipped with a 60x water immersion objective lens. To visualize the beads and to read their code upon magnetic orientation an excitation light of 488 nm was used. To visualize the fluorescence in the cells at the surface of the microcarriers, excitation wavelengths of 567 nm and 647 nm were used.

Autoclaving of the microcarriers

A sample of encoded microcarriers was autoclaved for 20 min. These encoded microcarriers were decoded by the use of a confocal microscope as described above.

Cell toxicity of the microcarriers

The cell toxicity of the Memobeads was evaluated by the EZ4U assay (Biomedica GmbH, Vienna, Austria) [52]. A large amount of LbL coated microcarriers (> 10^6) was dispersed for 48, 72 and 140 hours in cell culture medium. In this way, compounds that are possibly present in the LbL coating and the polystyrene core of the microcarriers, and which may be toxic to cells, were "extracted" in the cell culture medium. Cells were subsequently grown in the cell culture medium obtained in this manner. An EZ4U test was performed in the exponential phase of growth. Briefly, 50 µl of EZ4U substrate, together with 450 µl culture medium, was added to each well and incubated for 2-5 hours. The absorbance was measured with a Wallac Victor^2 absorbance plate reader (Perkin Elmer; Waltham, MA) set at 450 nm with a reference wavelength of 620 nm. Negative and positive control experiments were included by using cell culture medium that had not been exposed to Memobeads and by using cell culture medium that was supplemented with 10 mg/ml of phenol, respectively.

Detecting ligand responsive cells on encoded microcarriers

HEK-293 FlpIn cells (Invitrogen) stably expressing a STAT3-responsive rPAP1-hIL5Ralpha deltacyt reporter [53] were cultured in DMEM containing glutamine supplemented with 10% FBS and 2% P/S. The HEK-293 cells were stimulated with 10 ng/ml Leukemia Inhibition Factor (LIF) for 24 hours. Upon confluency, the stimulated cells were trypsinized and were grown on the beads as described above. The cell loaded microcarriers were

incubated at 4°C in a 1 µg/ml of primary antibody solution (against human IL5R-alpha.) [54]. After 20 min incubation the antibody was removed by washing with culture medium. Subsequently the cell loaded microcarriers were incubated at 4°C with fluorescently labeled secondary antibody (Cy5- goat anti- mouse IgG (H+L) conjugate). After 20 min incubation the antibody was removed by washing with culture medium and the cells on the microcarriers were checked by fluorescence microscopy.

RESULTS & DISCUSSION

Autoclaving of encoded microcarriers

To allow the growth of cells on the surface of the encoded microcarriers, it is necessary to use sterile samples of encoded microcarriers. Therefore, a dispersion of encoded microcarriers was autoclaved and we ascertained that autoclaving neither destroys the code inside, nor the LbL surface of the microcarriers. As Figure 2 shows, we could still observe the code in the autoclaved microcarriers, indicating that autoclaving does not destroy the polystyrene matrix of the microcarriers. It also indicates that autoclaving did not destroy the LbL coating and the magnetic orientation of the chromium dioxide nanoparticles. Remember, changes in the LbL coating or displacement of the magnetic nanoparticles would have no longer allowed a good positioning of the beads in the magnetic field, thereby making the read-out of the code impossible.

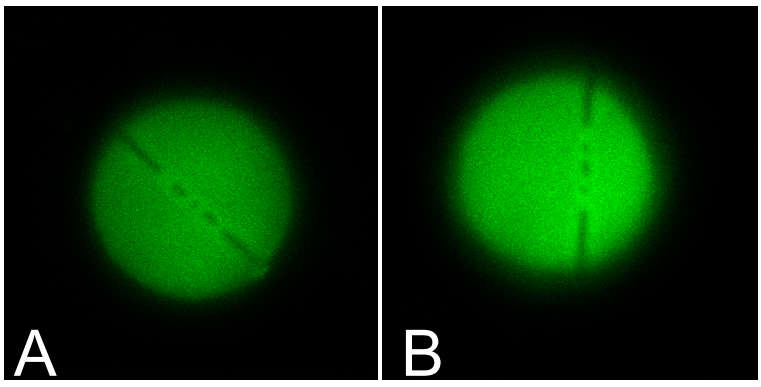

Figure 2. Confocal image of the central plane of (dot) encoded microcarriers respectively (A) before and (B) after autoclaving. The code is written in the green fluorescently dyed microspheres (about 40 µm in diameter) by local photobleaching of the dye.

Cell toxicity of the microcarriers

As described in the materials and methods section, a monolayer of cells was cultured in an extract of the microcarriers and the cytotoxicity of this extract was evaluated. The cells survived well (Fig. 3) indicating that the LbL encoded microcarriers do not release toxic compounds in sufficient amounts to kill the cells.

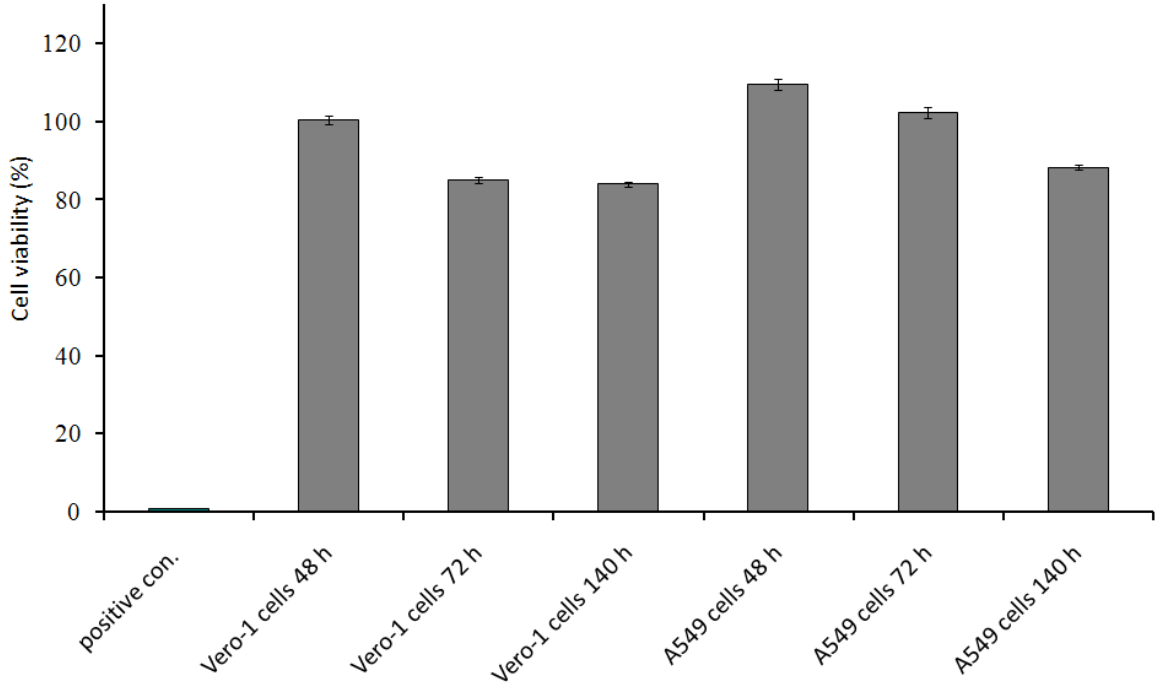

Figure 3. Toxicity of a "Memobead extract" for Vero-1 and A549 cells. Data represent the mean values obtained on three independently prepared samples ± SD compared to the appropriate control.

Growing cells on encoded microspheres

First we investigated to what extent CHO-320, Vero-1, Hela, COS-7 and HuH-7 cells can be cultured on the surface of LbL coated encoded microcarriers. As Figures 4 and 5 show, all these mammalian cells grew on the encoded microcarriers, though differences in the growth of the cells could be observed. Once attached to the microcarriers, HuH- 7 and CHO-320 cells grew quickly while Vero-1 cells needed a longer time to completely cover the surface of a microcarrier with a single cell layer. When the encoded microcarriers were incubated for longer times with the cells, the surface of the microcarriers became covered with multiple cell layers (Fig. 4, bottom row).

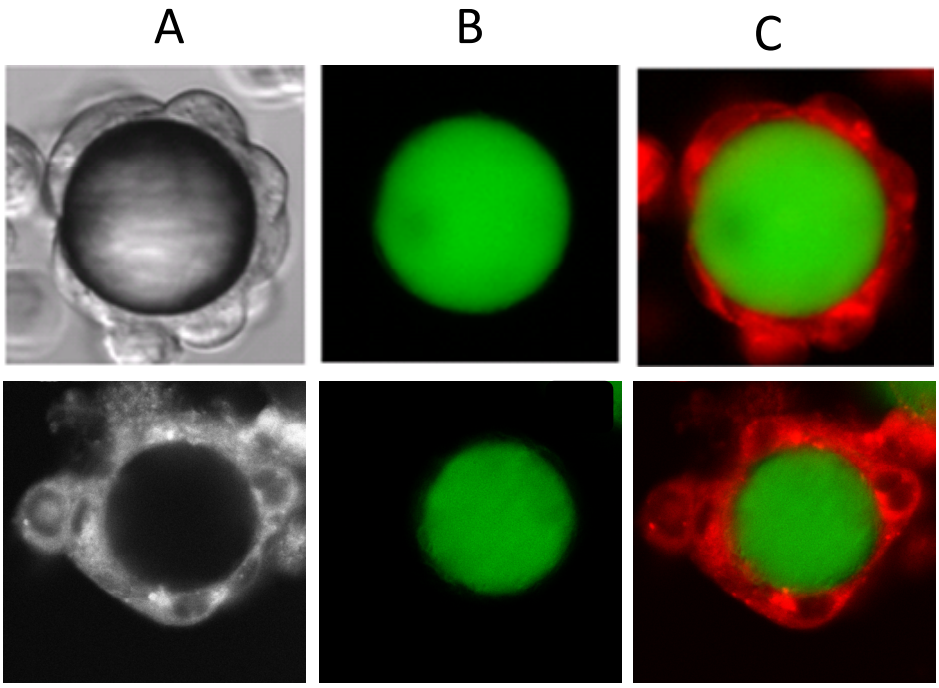

Figure 4. Microscopy images of Vero-1 cells grown on the surface of LbL coated microcarriers. (A) Transmission image, (B) green fluorescence image (λ_{ex} 488 nm) and (C) merged green/red fluorescence image (λ_{ex} 488 nm and 567nm). The cytosol of the Vero-1 cells was colored red using LysoTracker Red. Note that non-encoded beads (about 40 µm in diameter) were used. Bottom row shows the overgrowing of cell after longer incubation.

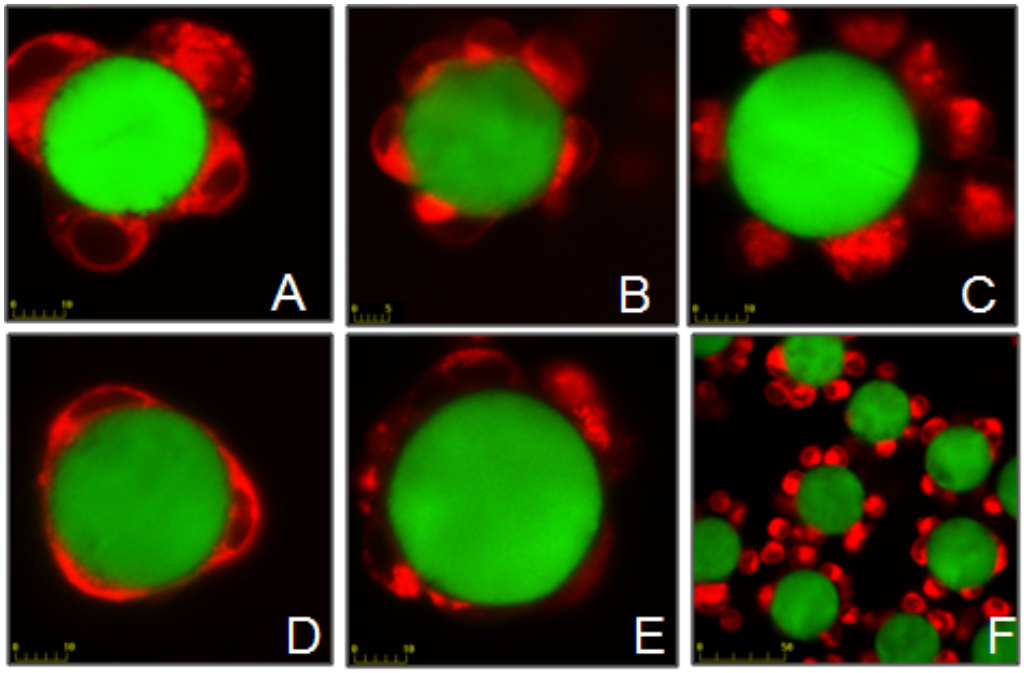

Figure 5. (A-E) Confocal merged green/red fluorescence images (λ_{ex} 488 nm and 567 nm) of (A) Vero-1, (B) CHO, (C) HELA, (D) HuH-7 and (E) COS-7 cells grown on the surface of LbL coated microcarriers. (F) Image of CHO cells loaded beads at lower magnification. The cytosol of the cells was colored red using LysoTracker Red. Note that non-encoded beads were used. The scale bar represents 10 µm in images A to E and 50 µm in image F.

Though we did not encounter real problems in growing CHO-320, Vero-1, Hela and HuH-7 cells on the encoded microcarriers, it may be less straightforward to grow other cell types. However, we would like to point out that LbL technology is highly flexible and easily allows adapting the surface properties for optimal cell growth. The attachment and proliferation of cells on polyelectrolyte flat membranes were recently nicely demonstrated [55,56]. Attractive and new in this study is that cells were grown on a magnetic polyelectrolyte surface which is highly functional in the sense that the LbL coating serves both as cell support layer and to orient of the microcarriers.

Decoding of cell loaded microcarriers

For decoding, the cell loaded microcarriers still have to be oriented. Also, one could wonder whether the cell layer surrounding the microcarrier does not optically interfere with the readout of the code Importantly, although the surface of the beads was covered with cells, we found that the orientation of the beads was not hampered and the code inside the beads remained perfectly detectable by confocal laser scanning microscopy, as shown in Figure 6.

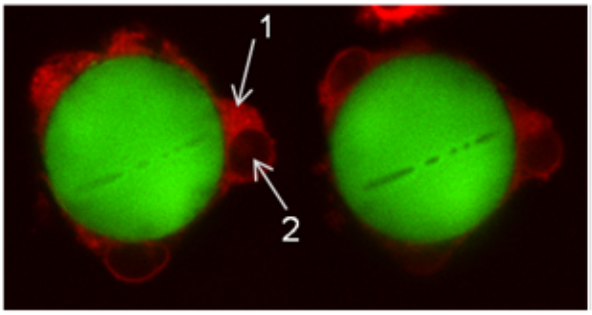

Figure 6. Confocal merged green/red fluorescence images (λ_{ex} 488 nm and 567nm) of Vero-1 cells grown on the surface of a (dot) encoded LbL coated microcarrier. In the magnetic field the microcarrier takes a correct position which makes the code readable. Arrow 1 indicates the cytosol of the Vero-1 cells which was colored red using LysoTracker Red, arrow 2 shows the nucleus of the cells. In the supplementary information one can find a movie which shows a z-scan of a Vero-1 cell loaded (encoded) microcarrier.

Figure 6 shows that a red colouring in the cytosol of the Vero-1 cells does not compromise the decoding of the beads. In a next step we wondered whether green fluorescence in the cells covering the microcarriers, which may be the case when the cell loaded microcarriers are used in assays, would not interfere with the decoding of the beads. Therefore, green fluorescent protein (GFP) expressing Vero-1 cells (Fig. 7) and red

fluorescent protein (RFP) expressing CHO-320 cells (data not shown) were grown on the encoded microcarriers. We observed that the code inside the beads remained detectable even when the cells covering the microcarriers exhibited green or red fluorescence.

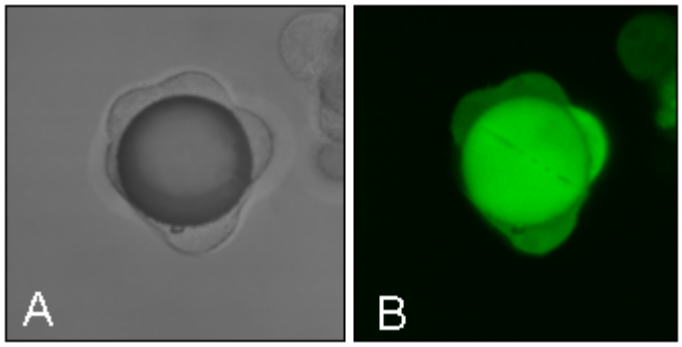

Figure 7. (A) Transmission and (B) confocal green fluorescence (λ_{ex} 488 nm) images of (dot) encoded LbL coated microcarriers loaded with GFP expressing Vero-1 cells.

Detecting ligand responsive cells on encoded microcarriers

In a next step we evaluated whether we could perform an ELISA assay on the cells that were grown on the surface of the encoded microcarriers. Therefore, Leukemia Inhibition Factor (LIF) responsive HEK-293 cells were grown on encoded microparticles upon LIF treatment. The expressed surface tag on these cells could be detected by use of a tag-specific antibody and a secondary (red fluorescently labeled) antibody (see Fig. 8). Note that beads covered with non-responsive HEK-293 cells did not show any red fluorescence (data not shown). The data in Figure 8 demonstrate that cell based ELISA assays can be performed on cells grown on the LbL coated encoded microcarriers.

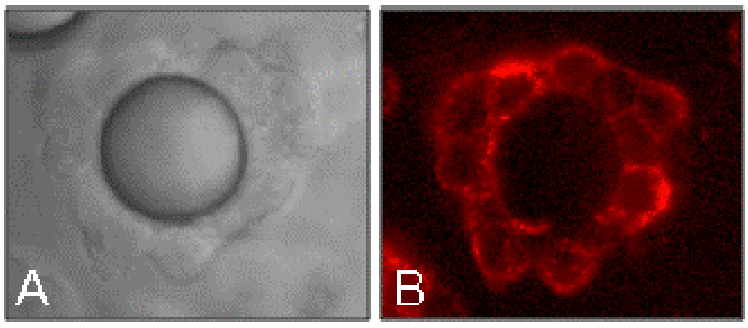

Figure 8. (A) Transmission and (B) red fluorescence (λ_{ex} 567 nm) images of LbL coated microcarriers loaded with LIF-responsive HEK-293 cells. The cells were treated with LIF resulting in the expression of a surface tag which was visualized by means of red fluorescently labeled antibodies.

CONCLUSION

In summary, this study shows that CHO-320, Vero-1, Hela, COS-7, HuH-7 and HEK-293 cells can be cultured at the surface of digitally encoded microcarriers coated with a number of layers of polyelectrolytes. Coating of the microcarrier's surface by LbL technology is highly flexible as it is straightforward to adapt the physicochemical composition of the coating with the aim to optimize the growth of a certain cell type. Incorporation of ferromagnetic chromium dioxide nanoparticles (CrO_2 NP) in the LbL coating allowed positioning of the microcarriers in a magnetic field, though they were heavily loaded with cells. The LbL coating serves as a cell supporting layer as well as to orient the microcarriers. We observed that the code inside the microcarriers remained readable even when the cells covering the microcarriers exhibited green or red fluorescence due to the expression of GFP and RFP respectively. It was also shown that tags expressed on the surface of the cells on the microcarriers could be detected by use of a tag-specific antibody and a secondary (red fluorescently labeled) antibody demonstrating that cell-based ELISA assays can be performed on cells grown on the LbL coated encoded microcarriers

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REFERENCES

1. Bailey S.N., Sabatini D.M., Stockwell B.R., Microarrays of small molecules embedded in biodegradable polymers for use in mammalian cell-based screens. Proc Natl Acad Sci U S A **2004**, 101(46), 16144-9.

2.Yan Y.X., Boldt-Houle D.M., Tillotson B.P., Gee M.A., D'Eon B.J., Chang X.J., Olesen C.E., Palmer M.A., Cell-based high-throughput screening assay system for monitoring G protein-coupled receptor activation using beta-galactosidase enzyme complementation technology. J Biomol Screen **2002**, 7(5), 451-9.

3. Bailey S.N., Wu R.Z., Sabatini D.M., Applications of transfected cell microarrays in high-throughput drug discovery. Drug Discov Today **2002**, 15(7), 113-8.

4. Ziayddian J., Sabatini D.M., Microarrays of cells expressing defined cDNAs. Nature **2001**, 411, 107-110.

5. Wu R.Z., Bailey S.N., Sabatini D.M., Cell-biological applications of transfected-cell microarrays. Trends Cell Biol **2002**, 12(10), 485-8.

6. Howbrook D.N., Van der Valk A.M., O'Shaughnessy M.C., Sarker D.K., Baker S.C., Lloyd A.W., Developments in microarray technologies. Drug Discov Today **2003**, 15, 8(14), 642-51.

7. Venkatasubbarao S., Microarrays--status and prospects. Trends Biotechnol **2004**, 12, 630-7.

8. Angres B., Cell microarrays. Expert Rev Mol Diagn **2005**, 5(5), 769-79.

9. Hoheisel J.D., Microarray technology: beyond transcript profiling and genotype analysis. Nat Rev Genet **2006**, 7(3), 200-10.

10. Beske O.E., Goldbard S., High-throughput cell analysis using multiplexed array technologies. Drug Discov Today **2002**, 15(7), 131-5.

11. Nolan J.P., Mandy F.F., Suspension array technology: new tools for gene and protein analysis. Cell Mol Biol **2001**, 47(7), 1241-56.

12. Nolan J.P., Sklar L.A., Suspension array technology: evolution of the flat-array paradigm. Trends Biotechnol **2002,** 20(1), 9-12.

13. Mattheakis L.C., Dias J.M., Choi Y.J., Gong J., Bruchez M.P., Liu J., Wang E., Optical coding of mammalian cells using semiconductor quantum dots. Anal Biochem **2004**, 327(2), 200-8.

14. Evans M., Sewter C., Hill E. An encoded particle array tool for multiplex bioassays Assay Drug Dev Technol **2003**, 1, 199-207.

15. Braeckmans K., De Smedt S.C., Leblans M., Pauwels R., Demeester J., Encoding microcarriers: present and future technologies. Nature Rev Drug Discov **2002**, 1, 447-56.

16. Wilson R., Cossins A.R., Spiller D.G., Encoded microcarriers for high-throughput multiplexed detection. Angew Chem Int Ed Engl **2006**, 45(37), 6104-17.

17. Han M., Gao X., Su J.Z., Nie S., Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. Nat Biotechnol **2001**, 19(7), 631-5.

18. Cunin F., Schmedake T.A., Link J.R., Li Y.Y., Koh J., Bhatia S.N., Sailor M.J., Biomolecular screening with encoded porous-silicon photonic crystals. Nat Mater **2002**, 1, 39-41.

19. Service, R.F., Radio Tags Speed Compound Synthesis. Science **1995,** 270, 577
20. Moran E.J., Sarshar S., Cargill J.F., Shahbaz M.M., Lio A., Mjalli A.M.M., Armstrong R.W., Radio frequency tag encoded combinatorial library method for the discovery of tripeptide substituted cinnamic acid inhibitors of the protein tyrosine-Phosphatase PTP1B. J Am Chem Soc **1995**, 117, 10787-88.

21. McHugh T. M., Miner R. C., Logan L. H., Stites, D. P., Simultaneous detection of antibodies to cytomegalovirus and herpes simplex virus by using flow cytometry and a microsphere-based fluorescence immunoassay. J Clin Microbiol **1988**, 26(10), 1957-61.

22. Vaino A.R., Janda K.D., Euclidean shape-encoded combinatorial chemical libraries. Proc Natl Acad Sci U S A **2000**, 97(14), 7692-6.

23. Braeckmans, K., De Smedt, S.C., Roelant, C., Leblans, M., Pauwels, R. & Demeester, J. Encoding microcarriers by spatial selective photobleaching. Nat Mater **2003**, 2, 169-73.

24. Nicewarner-Pena S.R., Freeman R.G., Reiss B.D., He L., Pena D.J., Walton I.D., Cromer R., Keating C.D., Natan M.J., Submicrometer metallic barcodes. Science **2001,** 294 (5540), 137-41.

25. Pregibon D.C., Toner M., Doyle P.S., Multifunctional encoded particles for high-throughput biomolecule analysis. Science **2007**, 315, 1393-96.

26. Schmid G., Zilg H., Johannsen R., Repeated batch cultivation of rBHK cells on Cytodex 3 microcarriers: antithrombin III, amino acid, and fatty acid metabolic quotients. Appl Microbiol Biotechnol **1992**, 38(3), 328-33.
27. Nikolai T.J., Hu W.S., Cultivation of mammalian cells on macroporous microcarriers. Enzyme Microb Technol **1992**, 14(3), 203-8.

28. Van Wezel A.L., Van Steenis G., Van der Marel P., Osterhaus A., Inactivated poliovirus vaccine : current production methods and new techniques. Rev Infect **1984**, 6(2), 335-340.

29. Beske O., Guo J., Li J., Bassoni D., Bland K., Marciniak H., Zarowitz M., Temov V., Ravkin I., Goldbard S., A novel encoded particle technology that enables simultaneous interrogation of multiple cell types. J Biomol Screen **2004**, 9(3), 173-85.

30. Chen T., Hansen G., Beske O., Yates K., Zhu Y., Anthony M., Agler M., Banks M., Analysis of cellular events using CellCard System in cell-based high-content multiplexed assays. Expert Rev Mol Diagn **2005**, 5, 817-29.

31. Beske O., Goldbard S., Turpin P., The CellCard System: a novel approach to assessing compound selectivity for lead prioritization of G protein-coupled receptors. Comb Chem High Throughput Screen **2005**, 8(4), 293-9.

32. Beske O., Bassoni D., Goldbard S., Use of the CellCard System for analyzing multiple cell types in parallel. Methods Mol Biol **2007**, 356, 129-38.

33. Derveaux S., De Geest B.G., Roelant C., Braeckmans K., Demeester J., De Smedt S.C., Multifunctional layer-by-layer coating of digitally encoded microparticles. Langmuir **2007**, 23(20), 10272-9.

34. Derveaux S., Stubbe B.G., Roelant C., Leblans M., De Geest B.G., Demeester J., De Smedt S.C., Layer-by-layer coated digitally encoded microcarriers for quantification of proteins in serum and plasma. Anal Chem **2008**, 80(1), 85-94.

35. Fayazpour F., Lucas B., Huyghebaert N. , Braeckmans K., Derveaux S., Stubbe B.G., Remon J.P., Demeester J., Vervaet C., De Smedt S.C. Digitally Encoded Drug Tablets to Combat Counterfeiting. Adv mat **2007**, 19 (22), 3854 –58.

36. Caruso F., Caruso R.A., Mohwald H., Nanoengineering of inorganic and hybrid hollow spheres by colloidal templating. Science **1998**, 282(5391), 1111-4.

37.Decher G., Fuzzy nanoassemblies: Toward Layered Polymeric Multicomposites. Science **1997**, 277, 1232-37.

38. Sukhorukov G.B., Donath E., Davis S., Lichtenfeld H., Caruso F., Popov V.I., Mohwald H., Stepwise Polyelectrolyte Assembly on Particle Surfaces: A Novel Approach to Colloid Design. Polym Adv Technol **1998**, 9, 759-67.

39. Sukhorukov G.B., Donath E., Lichtenfeld H., Kinppel E., Knippel M.,Budde A., Mohwald H., Layer-by-Layer self assembly of polyelectrolytes on colloidal particles. Colloid Surf A-Physicochem Eng Asp **1998**, 137, 253-66.

40. Hammond P.T., Recent explorations in electrostatic multilayer thin film assembly. Curr Opin Colloid Interface Sci **2000**, 4, 430-42.

41. Picart C., Polyelectrolyte multilayer films: from physico-chemical properties to the control of cellular processes. Curr Med Chem **2008**, 15(7), 685-97.

42. Lvov Y., Decher G., Sukhorukov G**.,** Assembly of thin films by means of successive deposition of alternate layers of DNA and poly(allylamine)**.** Macromolecules **1993**, 26, 5396-99.

43. Schüler C., Caruso F., Decomposable hollow biopolymer-based capsules. Biomacromolecules **2001**, 2, 921-6.

44. Pei R.J., Cui X.Q., Yang X.R., Wang E.K., Assembly of alternating polycation and DNA multilayer films by electrostatic layer-by-layer adsorption. Biomacromolecules **2001**, 2, 463-68.

45. Shi X.Y., Sanedrin R.J., Zhou F. M., Structural Characterization of Multilayered DNA and Polylysine Composite Films: Influence of Ionic Strength of DNA Solutions on the Extent of DNA Incorporation. J Phys Chem B **2002**, 106, 1173-80.

46. Yamauchi F., Kato K., Iwata H., Layer-by-layer assembly of poly(ethyleneimine) and plasmid DNA onto transparent indium-tin oxide electrodes for temporally and spatially specific gene transfer. Langmuir **2005**, 21(18), 8360-7.

47. Benkirane-Jessel N., Schwinte P., Falvey P., Darcy R., Haikel Y., Schaaf P., Voegel J.C., Ogier J., Build-up of polypeptide multilayer coatings with anti-inflammatory properties based on the embedding of piroxicam–cyclodextrin complexes. Adv Funct Mater **2004**, 14, 174–82.

48. Thierry B., Kujawa P., Bilodeau L., Tabrizian M., Delivery platform for hydrophobic drugs: prodrug approach combined with self assembled multilayers. J Am Chem Soc **2005**, 127, 1626-7.

49. Chluba J., Voegel J.C., Decher G., Erbacher P., Schaaf P., Ogier J., Peptide hormone covalently bound to polyelectrolytes and embedded into multilayer architectures conserving full biological activity. Biomacromolecules. **2001**, 2(3), 800-5.

50. Jessel N., Atalar F., Lavalle P., Mutterer J., Decher G. Schaaf P., Voegel JC, Ogier J., Bioactive coatings based on a polyelectrolyte multilayer architecture functionalized by embedded proteins. Adv Mater **2003**, 15, 692–95.

51. Haynie D.T., Zhang L., Rudra J.S., Zhao W., Zhong Y., Palath N., Polypeptide multilayer films. Biomacromolecules **2005**, 6(6), 2895-913.

52. Jelinek A., Klöcking H.P., *In vitro* toxicity of surfactants in U937 cells: cell membrane integrity and mitochondrial function. Exp Toxicol Pathol **1998**, 50(4-6), 472-6.

53. Waelput W., Verhee A., Broekaert D., Eyckerman S., Vandekerckhove J., Beattie J.H., Tavernier J., Identification and expression analysis of leptin-regulated immediate early response and late target genes. Biochem J **2000**, 348, 55-61.

54. Tavernier J., Van der Heyden J., Verhee A., Brusselle G., Van Ostade X., Vandekerckhove J., North J, Rankin SM, Kay AB, Robinson DS. Interleukin 5 regulates the isoform expression of its own receptor alpha-subunit. Blood **2000**, 95(5), 1600- 7.

55. Boura C., Muller S., Vautier D., Dumas D., Schaaf P., Claude Voegel J., Francois Stoltz J., Menu P., Endothelial cell--interactions with polyelectrolyte multilayer films. Biomaterials **2005**, 26(22), 4568-75.

56. Boura C., Menu P., Payan E., Picart C., Voegel J.C., Muller S., Stoltz J.F., Endothelial cells grown on thin polyelectrolyte mutlilayered films: an evaluation of a new versatile surface modification. Biomaterials **2003**, 24(20), 3521-30.

Chapter 4

Evaluation of Digitally Encoded Layer-by-Layer Coated Microparticles for Reverse Transfection

ABSTRACT

”Reverse transfection” cells with pDNA or siRNA has been proposed as a useful tool for the simultaneous high-throughput analysis of the function of many different genes on a solid surface. The aim of the present work is to evaluate whether encoded microcarriers, coated with polyelectrolyte multilayers, are suitable to immobilize naked nucleic acids, non-viral nucleic acid complexes and viruses and whether the cells which grow at the surface of the microcarriers become (“reversely”) transfected.

Chapter 4

Evaluation of Digitally Encoded Layer-by-Layer Coated Microparticles for Reverse Transfection

INTRODUCTION

Technologies which allow efficient delivery of nucleic acids, such as plasmid DNA (pDNA), oligonucleotides and small interfering RNA (siRNA), are of major importance both in basic biological sciences as well as in clinical medicine. Generally speaking, both viral and non-viral nucleic acid carriers are currently under development. Viral vectors, such as for example retroviruses and adenoviruses, are still the most efficient nucleic acid delivery vehicles [1-4], however, cytotoxicity, immunogenicity and mutagenesis of transfected cells are major concerns [5]. Non-viral vectors like polymers and liposomes [6-8], which are mostly cationic in nature and therefore spontaneously assemble with negatively charged nucleic acids, have attracted major interest for the safe delivery of therapeutic nucleic acids into target cells [9,10], however, their application *in vivo* remains currently limited due to their poor efficacy [11,12].

In most *in vitro* transfection studies typically nucleic acids, either in ”naked” form or complexed to carriers, are added to cells which are growing e.g. at the bottom surface of wells of microtiterplates. Recently, immobilizing DNA onto a solid surface prior to cell seeding, with the aim of transfecting the seeded cells has been proposed. This method is termed “solid-phase nucleic acid delivery” [13],”reverse transfection” [14] or”substrate mediated nucleic acid delivery” [15,16] as schematically represented in panel A of Figure 1. A possible advantage of this approach is that the cells are in closer contact with highly

concentrated spots of (immobilized) nucleic acids which may improve the transfection efficiency.

In general, a successful reverse transfection necessitates both an appropriate immobilization of the nucleic acids on the surface as well as an appropriate release of the nucleic acids from the surface into the cells. Different strategies were developed to immobilize naked nucleic acids or (non-) viral nucleic acid complexes on cell culture surfaces such as the entrapment of the nucleic acids in a thin gelatin film deposited on the surface [14], specific binding of the nucleic acids to the surface through the biotin-avidin interaction [15-19] and immobilization through non-specific adsorption [20-22].

Recently, immobilizing nucleic acids in polyelectrolyte coatings deposited on surfaces, to allow reverse transfection, has been proposed as well [23,24]. Such polyelectrolyte coatings are deposited on the surface by the so named ”Layer-by-Layer” (LbL) technology which is based on the sequential adsorption of oppositely charged polyelectrolytes on a charged planar substrate. Upon adsorption of a polyelectrolyte layer, charge overcompensation takes place, leading to a reversal of the surface charge, promoting the adsorption of a next, oppositely charged, polyelectrolyte. As an example, is has been shown that a polyelectrolyte film, also named LbL film, composed of naked (negatively charged) pDNA and (bio) degradable (positively charged) polyamines allow the (gradual) release of pDNA from the coating through (gradual) breakdown of the polyamines [24,25]. As another example, Jessel et al. have shown that LbL films composed of poly(L-glutamic acid), poly(L-lysine) and charged cyclodextrins can release entrapped plasmid DNA which transfects the cells growing on the LbL films [26]. Besides naked DNA also nucleic acid/cationic polymer complexes [27,28] and viral vectors [29] can be immobilized in LbL films.

“Reverse transfection”, may become a useful research tool in e.g. cell-based microarrays. An advantage of the reverse transfection approach is its multiplex capacity: whole libraries of nucleic acids can be easily and simultaneously screened on (a broad range of) cells. Ziauddin and Sabatini have recently created a transfected cell microarray (panel A in Figure 1) with the aim to identify the cellular functions of the immobilized genes [14]. Such cell-based microarrays show spots of (mammalian) cells expressing the DNA constructs immobilized at those spots. Similarly, recombinant viral vector libraries [30-33] can be arrayed by spotting the viruses on a solid surface onto which cells are subsequently plated. This should result in an array of cells, expressing the recombinant genes of interest. The reverse

transfection approach was also used to knock down genes in the plated cells; Indeed, Mousses et al. arrayed spots (hundreds of micrometers in size) of synthetic siRNAs on glass slides for parallel knockdown of different genes [34].

In this chapter we aimed to evaluate whether reverse transcription can be obtained from nucleic acids immobilized at the surface of (encoded) microcarriers with the overall goal to develop an "encoded microcarrier based reverse transfection assay", as schematically illustrated in panel B of Figure 1. The two specific questions which we considered were the following: (a) Are LbL coated encoded beads suitable to immobilize respectively viral vectors, naked nucleic acids and complexed nucleic acids? (b) Are the adenoviral particles and nucleic acids immobilized in the LbL coatings at the surface of the beads able to selectively transfect the cells that grow on top of the LbL coated microcarriers?

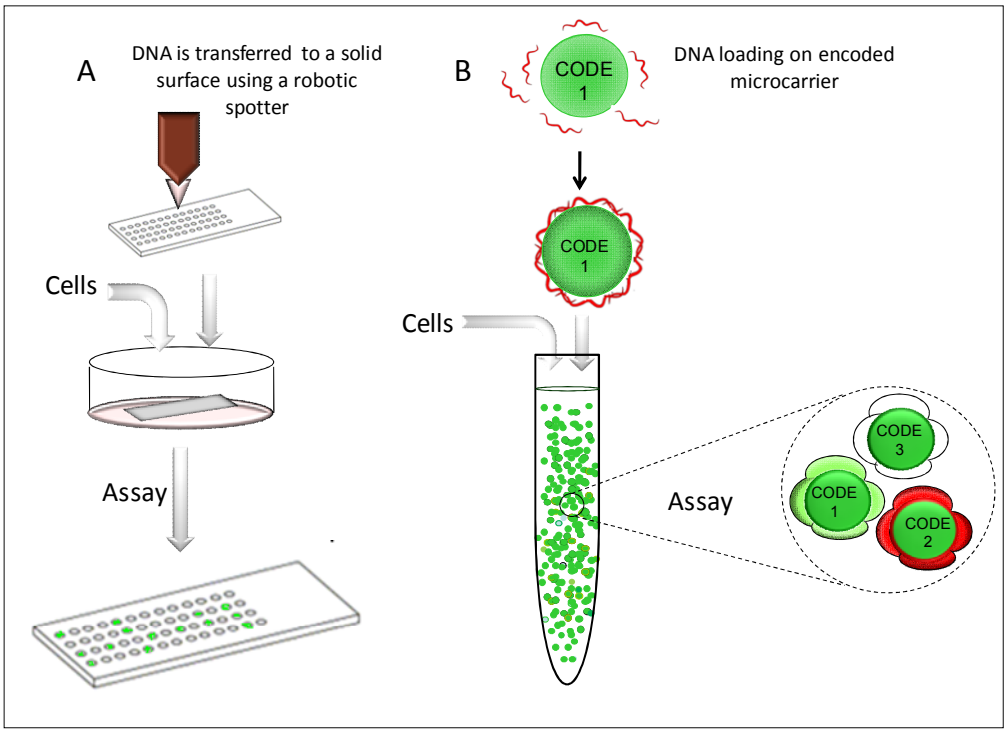


Figure 1. Reverse transfection of cells. (A) Schematical presentation of a DNA-microarray for the reverse transfection of cells. DNA is spotted onto a solid surface, each spot contains another type of DNA, the x,y-coördinate of the spot allows to identify which type of DNA is present at a specific location on the array. Subsequently cells are seeded and start to attach to the surface of the DNA microarray. DNA is released from the surface and only taken up by those cells that attach to regions where DNA is present. The transfected cells will express the protein corresponding to the DNA taken up by the cells. (B) Schematical presentation of an encoded microcarrier based reverse transfection assay. The DNA is applied on the surface of encoded microcarriers, the code in the carrier allows to identify which DNA is at the surface of a specific carrier. The DNA carrying beads are applied in a cell suspension; cells start to grow on the surface of the beads followed by reverse transfection of the cells.

MATERIALS & METHODS

Materials

Non-magnetic fluorescent carboxylated microspheres (CFP-40052-100, ø = 39 µm) were purchased from Spherotech (Libertyville, Illinois, USA). Poly (allylamine hydrochloride) (PAH; 70 kDa) and sodium poly (styrene sulfonate) (PSS; 70 kDa) were obtained from Sigma Aldrich (Steinheim, Germany). 4,4'-trimethylenedipiperidine and anhydrous dichloromethane (CH_2Cl_2) were purchased from Sigma-Aldrich (Bornem, Belgium). 1,4-Butanediol diacrylate was purchased from Alfa Aesar Organics (Karlsruhe, Germany). 1,2-Dioleolyl-3-trimethylammoniumpropane (chloride salt) (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids (AL, USA). Linear poly (ethylene imine) (LPEI, MW 22 KDa) was a kind gift from Prof. E. Wagner (Ludwig Maximilian University, Munich, Germany). The recombinant adenoviral vectors Ad-RFP and Ad-GFP, which express the red fluorescent protein (RFP) and green fluorescent protein (GFP) respectively, were obtained from Vector Biolabs (Philadelphia, USA). CHO-320 cells were a kind gift from Prof. Y.J. Schneider (Catholic University of Louvain). Vero-1 and CHO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Merelbeke, Belgium) containing 2 mM L-glutamine (L-Gln), 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). HuH-7_eGFPLuc cells stably expressing eGFP-luciferase were generated by transfecting HuH-7 cells with the vector pEGFPLuc (Clontech, Palo Alto, USA) as previously described [37]. DsRed2-C1 pDNA was obtained from Promega (Leiden, The Netherlands), amplified in *Escherichia coli* and purified by the Qiagen Plasmid Mega Kit (Venlo, The Netherlands). TOTO-3 was purchased from Invitrogen for labeling the pDNA. siRNA against EGFP and Dsred, "negative" siRNA (used in control experiments) and Cy5 labeled siRNA were purchased from Dharmacon (Chicago, USA). All siRNAs were purchased in their annealed form, dissolved in RNase free water at the concentration of 20 µM, aliquoted and stored at -80°C.

Layer-by-Layer (LbL) coating of the microcarriers

The polystyrene microspheres were coated with the polyelectrolytes PSS and PAH as reported previously [35]. Briefly, the microspheres were suspended in 1 ml PAH solution (2

mg/ml in 0.5 M NaCl) and continuously vortexed (1000 rpm, 25°C) for 15 min. The non-adsorbed PAH was removed by repeated centrifugation and washing. Subsequently, the microspheres were dispersed in deionised water containing CrO_2 nanoparticles (NPs, <500nm). The CrO_2 NPs are necessary to position the microspheres in a magnetic field to allow the reading of the code, as described in detail elsewhere [35]. The microsphere dispersion was continuously shaken for 15 min and the excess of CrO_2 NPs was removed by repeated centrifugation/washing steps. The next polyelectrolyte layers were applied similarly. As illustrated in Figure 2, the microspheres were coated with 5 or 6 layers in the following order: PAH / CrO_2 NP / PAH / PSS / PAH / PSS. Finally, the resulting LbL coated microspheres were resuspended in 1 ml of ultrapure demiwater at ± 400 000 microspheres/ml and subsequently encoded.

Encoding of the microcarriers

The LbL coated microspheres were encoded by spatial selective photobleaching as described previously [36]. Briefly, an in-house-developed encoding device was used, being a microscopy platform equipped with an Aerotech ALS3600 scanning stage, a SpectraPhysics 2060 Argon laser and an Acousto-Optic-Modulator (AA.MQ/A0.5-VIS, A.A-Opto-Electronique, Orsay Cedex, France). The encoding process consists of two steps, a writing step (i.e. the photobleaching process) and a magnetizing step, during which the CrO_2 loaded microspheres are exposed to an external magnetic field sufficient to provide them with a magnetic memory. The microspheres were fixed on a grid during the encoding process to prevent rotation between the two steps.

Synthesis of PbAE1

PbAE1 was synthesized as described previously [37]. Briefly, 37.8 mmol 1,4-butanediol diacrylate and 37.8 mmol 4,4'-trimethylenedipiperidine were separately dissolved in 50 ml CH_2Cl_2. The 4,4'-trimethylenedipiperidine solution was added dropwise to the 1,4-butanediol diacrylate solution under vigorous stirring. The reaction mixture was placed in an oil bath at 50°C and the polymerisation was allowed to proceed during 48 h under a nitrogen atmosphere. After cooling to room temperature, the reaction product was precipitated in diethyl ether saturated with HCl. The precipitate was filtered and thoroughly washed with diethyl ether. A white powder was obtained after overnight drying under

vacuum. Subsequently, the polymer was dissolved in acetate buffer (100 mM, pH 5.4) at a stock solution concentration of 0.5 mg/ml and filtered through a 0.22 µm membrane syringe filter prior to use.

Preparation of cationic liposomes

Cationic liposome composed of DOTAP:DOPE in a 1:1 molar ratio were prepared as described previously [38]. Briefly, lipids were dissolved in chloroform and mixed. The chloroform was subsequently removed by rotary evaporation at 37°C followed by flushing the obtained lipid film with nitrogen during 30 min at room temperature. The dried lipid film was then hydrated by adding Hepes buffer (20 mM, pH 7.4) till a final lipid concentration of 10 mM. After mixing in the presence of glass beads, liposome formation was allowed overnight at 4°C. Thereafter, the formed liposomes were extruded 11 times through two stacked 100 nm polycarbonate membrane filters (Whatman, Brentfort, UK) at room temperature using an Avanti Mini-Extruder (Avanti Polar Lipids).

Preparation of polyplexes

Polyplexes consisting of 22 kDa linear PEI were prepared as optimized previously [39]. Briefly, polyplexes were prepared in 20 mM Hepes pH 7.4 by adding different volumes of the polymer stock solution (0.5 mg/ml), dependent on the desired N/P ratio (10), at once to the pDNA solution. Subsequently, the mixture was vortexed for 10 sec and allowed to equilibrate for 30 min at room temperature prior to use. The final pDNA concentration in the polyplex dispersion was 0.126 µg/µl.

Preparation of lipoplexes

The extruded liposomes were mixed with pDNA, in a +/- charge of 4, the +/- charge ratio being defined as the ratio of the number of the positive charges (originating from DOTAP) to the number of the negative charges (originating from the pDNA). This mixture was then vortexed and incubated at room temperature for 30 min. The final concentration of pDNA in the lipoplex dispersion was 0.126 µg/µl.

Preparation of PbAE1:siRNA complexes

PbAE1:siRNA complexes at an N:P ratio of 30:1 were formed by mixing equal volumes of a PbAE1 solution and a 0.5 µM siRNA solution, followed by vigorously mixing. The resulting PbAE1:siRNA complexes were incubated at room temperature for at least 30 min before addition to the cells.

Size and zeta potential measurements

The average particle size and the zeta potential (ζ) of the liposome, lipo- and polyplexes were measured by photon correlation spectroscopy (PCS) (Autosizer 4700, Malvern, Worcestershire, UK) and particle electrophoresis (Zetasizer 2000, Malvern, Worcestershire, UK), respectively. The liposome, lipoplex and polyplex dispersion were diluted 40-fold in 20 mM Hepes buffer pH 7.4 while the PbAE:siRNA dispersion was diluted 2-fold in 0.1 M acetate buffer pH 5.4 prior to particle size and zeta potential measurements. The average (±standard error) size of the liposomes and lipoplexes was 134 ± 2 nm and 228 ± 2 nm, respectively. Their average ζ equalled 52 ± 2 mV and 46 ±3 mV, respectively. The size and ζ of the linear PEI polyplexes were 165 ± 4 nm and 33 ± 2 mV. The size and ζ of the PbAE:siRNA complex equalled 380 ± 9 nm and 31 ± 1 mV.

Immobilizing naked pDNA, polyplexes, lipoplexes, naked siRNA, PbAE:siRNA complexes and adenoviral particles at the surface of the encoded beads

For immobilizing adenoviral particles, naked or complexed nucleic acids, an appropriate amount of sample was added to a 10 µl dispersion of LbL coated (encoded) microcarriers in DMEM. The mixture was continuously vortexed (1000 rpm, 25°C) for 3 h. Subsequently, the microcarriers were separated from the (non adsorbed) free adenoviral particles or nucleic acids by repeated centrifugation and washing with DMEM (3 times). Cells were then grown on the loaded microcarriers as described below.

Growing cells on the microcarriers and reverse transfection

The LbL coated (encoded) beads, loaded with adenoviral particles or nucleic acids, were dispersed in cell culture medium (DMEM containing about 50 % FBS). Approximately 100 µl volume of the microsphere dispersion was applied in polycarbonate Erlenmeyer shake-flasks (Corning) that were treated with a silicone solution (Sigmacoat®; Sigma)

following the manufacturer's protocol. The silicone treatment should prevent the attachment of cells to the surface of the flasks. Subsequently, an appropriate amount of cell suspension (in DMEM supplemented with 2% P/S, 1% L-GLn and 10% FBS) was added to the flasks for 3 h at 37°C and 5% CO_2 to allow the attachment of cells to the surface of the beads. During this 3 h period the flasks were not shaken to allow the initial attachment of the cells. Subsequently we began to agitate the flasks on an orbital shaker under appropriate conditions to prevent the precipitation of the microcarriers. After 24 h the microcarriers carrying cells were studied under the microscope (CLSM; Bio-Rad MRC1024) to evaluate to which extent the cells became transfected or transduced.

Decoding of the cell loaded microcarriers

The cell loaded microspheres were decoded using a Bio-Rad MRC1024 microscope equipped with a 60x water immersion objective lens and a magnet. To visualize the beads and to read their code upon magnetic orientation an excitation light of 488 nm was used. To visualize the fluorescence in the cells at the surface of the microcarriers, excitation wavelengths of 567 nm and 647 nm were used.

RESULTS & DISCUSSION

Immobilizing naked nucleic acids on the surface of encoded microcarriers

First, we characterized to which extent the surface of the digitally encoded microcarriers could become loaded with naked (and complexed) nucleic acids. We especially evaluated how the charge and charge density of the surface influences the loading of the nucleic acids. Figure 2 schematically shows the composition of the surface of the microcarriers used in this study. Note that the surface of the "naked beads" (being microcarriers without LbL coating) is negatively charged due to the presence of dissociated carboxyl groups.

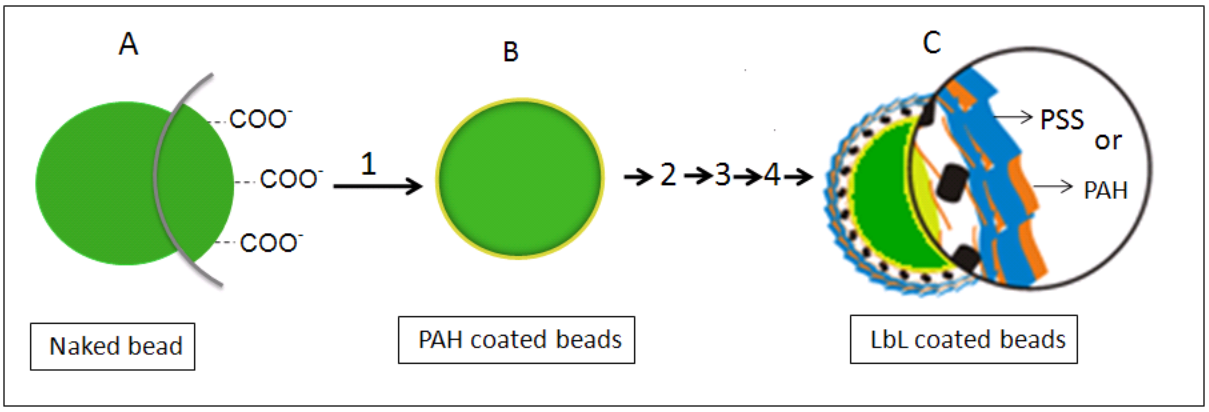


Figure 2. Composition of the surface of the encoded microcarriers used in this study. (A) The so named "naked beads" have negative charges at their surface due to the presence of carboxyl groups. (B) "PAH coated beads" are naked beads coated with the polycation PAH. (C) "LbL coated beads" are naked beads coated with different polyelectrolyte layers, alternatively with the polycation PAH and the polyanion PSS. As indicated in the figure, the outer layer of the LbL coating was respectively PAH (to obtain a positively charged surface) or PSS (to obtain a negatively charged surface).

Figure 3 and 4 visually show to which extent red (TOTO3) labeled naked pDNA becomes immobilized on microcarriers with a different surface. One can clearly see that only limited amounts of pDNA adsorb on the negatively charged beads while a positively charged (LbL coated) surface enhances the adsorption.

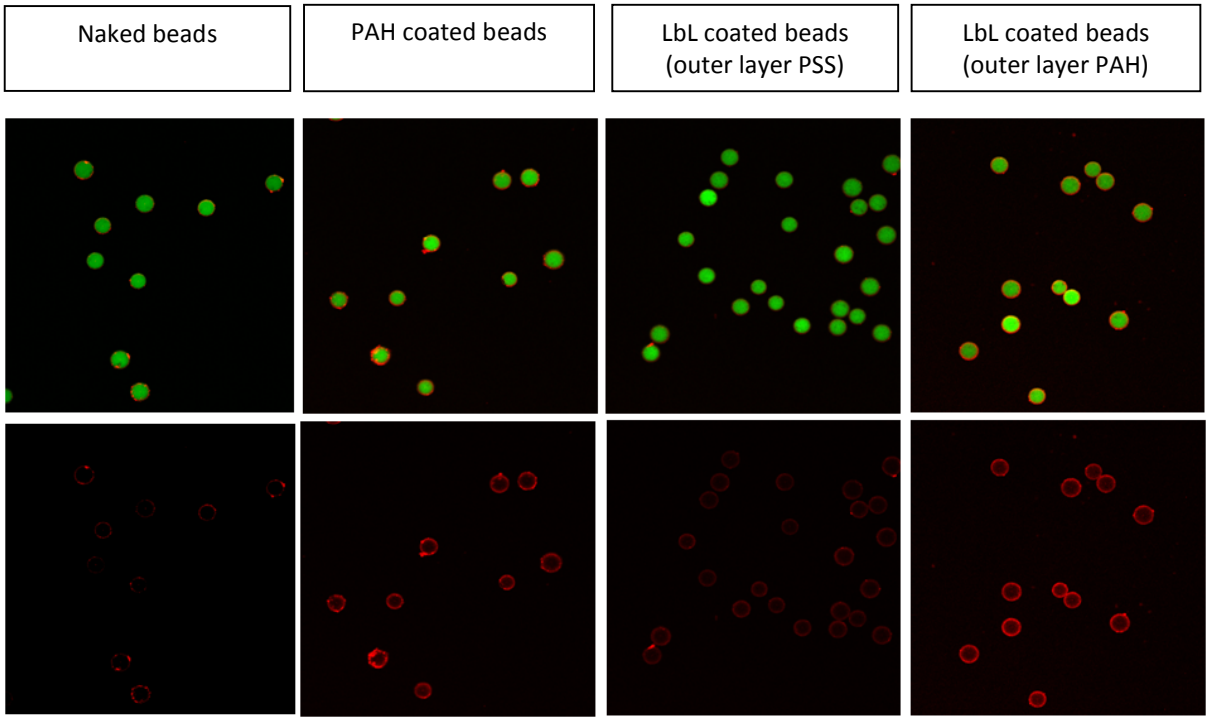


Figure 3. Red (TOTO3) labeled naked pDNA immobilized on microcarriers with a different surface. Merged green/red fluorescence images (top row) show the green fluorescence of the microcarriers (note that polystyrene microcarriers doped with a green fluorophore were used in this study); the red fluorescence image (bottom row) shows that naked pDNA becomes immobilized at the surface of the microcarriers. The beads in all the figures of this paper are approximately 40 µm in size.

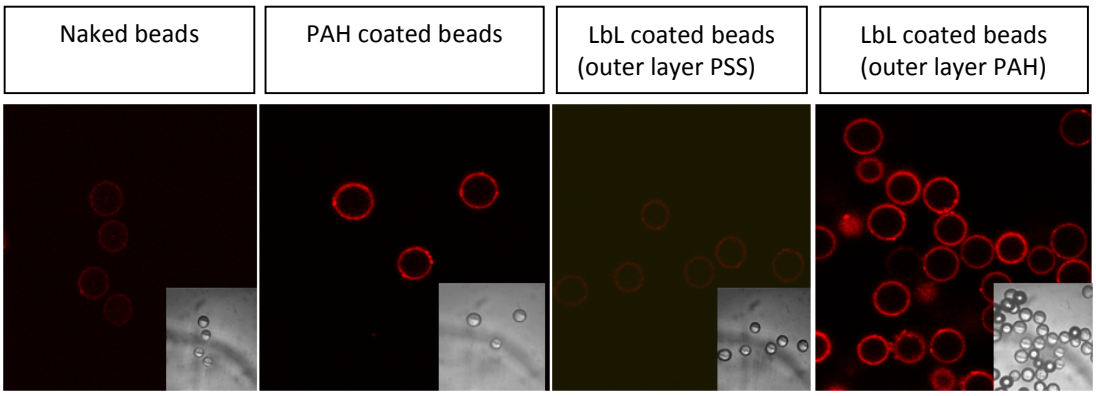
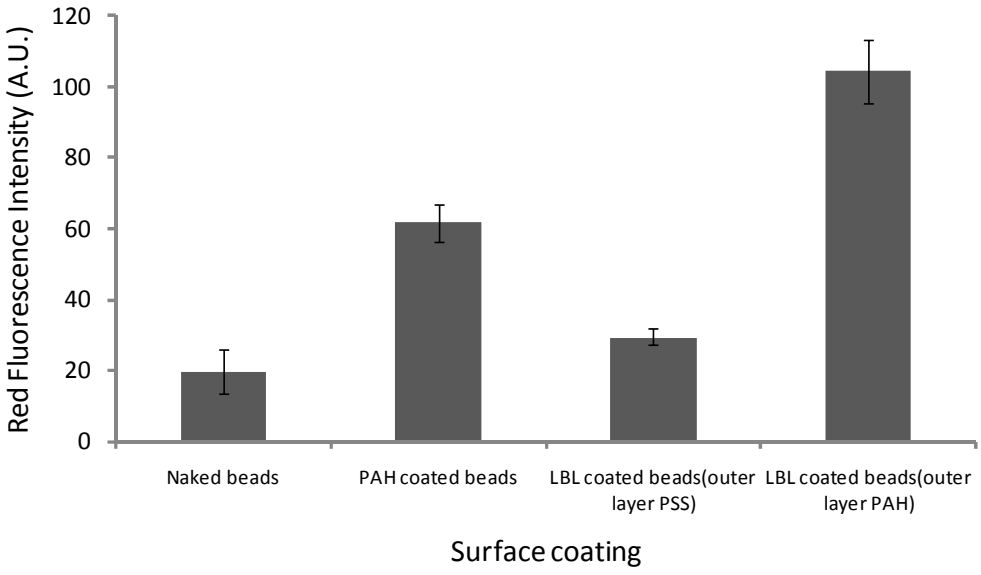


Figure 4. Red fluorescence images of the microcarriers at a higher magnification than in Figure 3. The insert shows the corresponding transmission image. It shows that all the beads in the transmission image become well covered with pDNA.

In Figure 5 the red fluorescence at the surface of the microcarriers was quantified. While only low levels of fluorescence were obtained in case of negatively charged microcarriers, as expected, a positive surface charge of the microcarriers significantly increases the measured fluorescence and thus improves the pDNA loading on the microcarrier surface. A positive outer layer clearly plays an important role for the efficiency of pDNA adsorption onto the surface of microcarriers. Additionally, Figure 6 also reveals that LbL coating of the beads seems to result in a more homogeneous loading of the beads with pDNA compared to microcarriers coated with a single PAH-layer (PAH coated beads).



Figur5. Quantification of the red fluorescence at the surface of the beads loaded with red (TOTO3) labeled naked pDNA

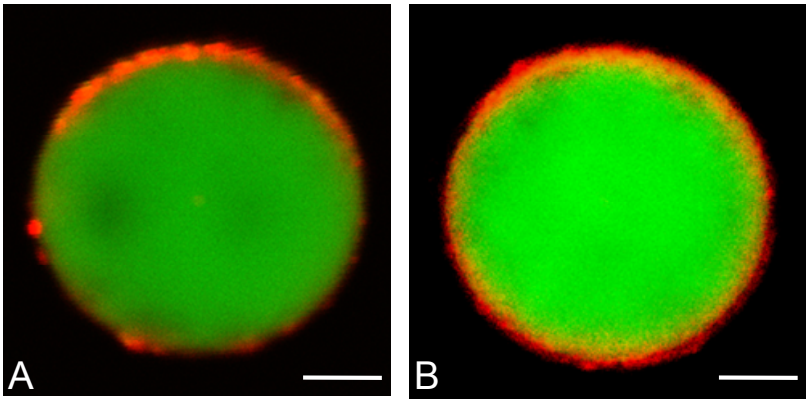


Figure 6. Red (TOTO3) labeled naked pDNA immobilized on respectively (A) PAH coated beads and (B) LbL coated beads (with PAH as outer layer). The scale bar represents 10 µm.

Similarly, Figure 7 shows the adsorption of naked red Cy5 labeled siRNA on microcarriers with a different surface. As observed for naked pDNA, only limited amounts of siRNA do adsorb to the negatively charged naked beads while a positively charged surface clearly enhances the adsorption.

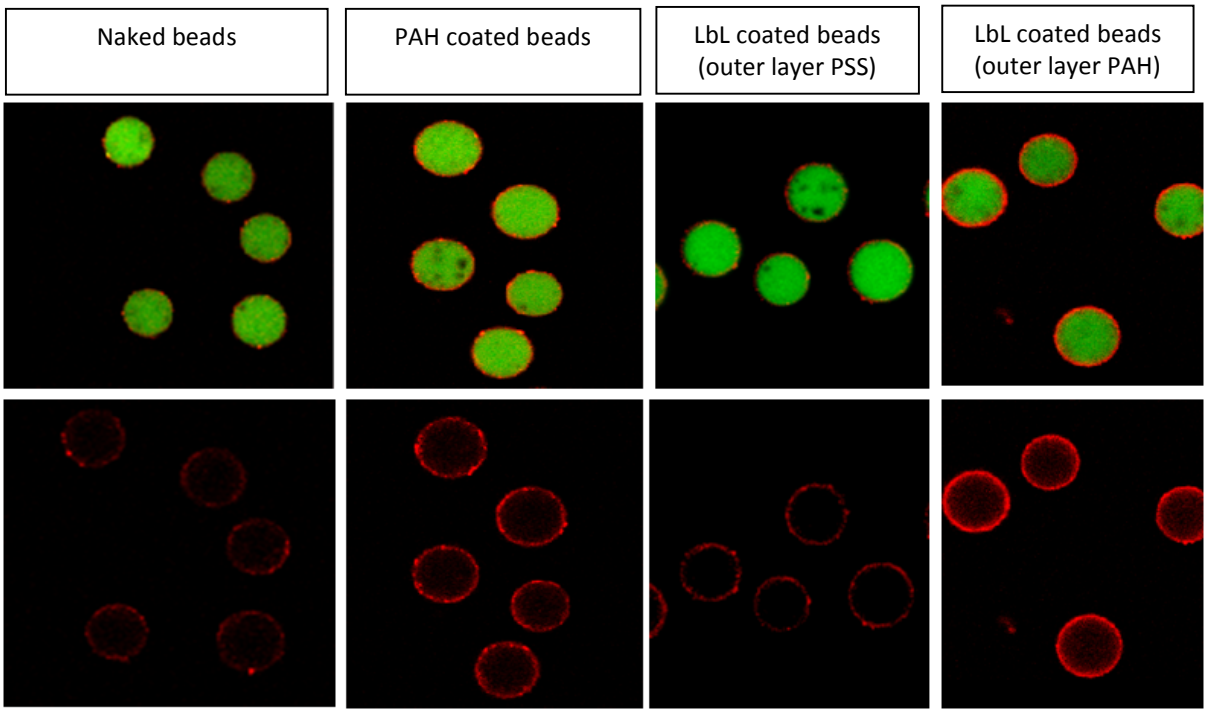


Figure 7. Red Cy5 labeled siRNA immobilized on microcarriers with a different surface. The merged red/green fluorescence image (top row) shows the green fluorescence of the polystyrene microcarriers. The red fluorescence image (bottom row) shows that siRNA becomes immobilized at the surface of the microcarriers.

Next, this was also confirmed by quantifying the red fluorescence (data not shown). However, similar as with pDNA, the adsorption of siRNA to the microcarrier surfaces is apparently not only dependent on the charge of the surface but also on the composition of the charged surface, as the LbL coated beads showed again a much more homogenous distribution pattern compared to PAH coated beads (Figure 7). Though, one should take care in relating the amount of red fluorescence signal at the surface to the amount of nucleic acids as the fluorescence signal may become also influenced by the chemical composition of the coating [40].

Immobilizing (non-viral) nucleic acid complexes on the surface of encoded microcarriers

Figure 9, 10 and 11 show that polyplexes and lipoplexes become immobilized at the surface of the beads. In contrast to the loading with naked pDNA and naked siRNA, the loading of the beads with poly- and lipoplex occurs more heterogeneously, as evidenced from the punctuated red fluorescence on the surface of the beads

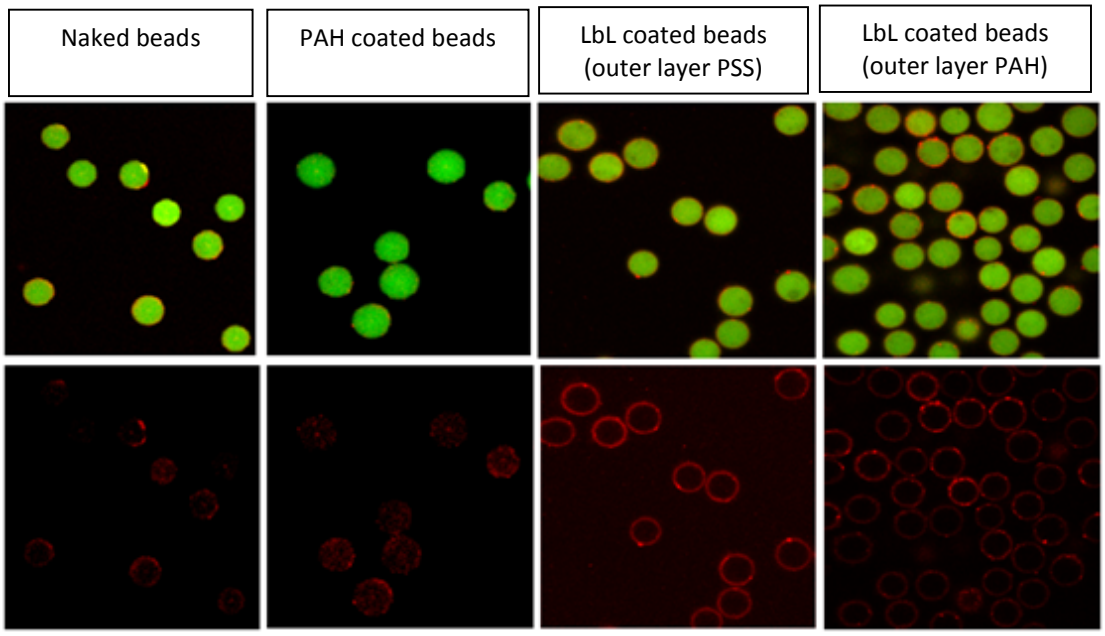


Figure 9. Red (TOTO3) labeled pDNA containing polyplexes (based on PEI) immobilized on microcarriers with a different surface. The merged red/green fluorescence image (top row) shows the green fluorescence of the polystyrene microcarriers. The red fluorescence image (bottom row) shows that polyplexes become immobilized at the surface of the microcarriers.

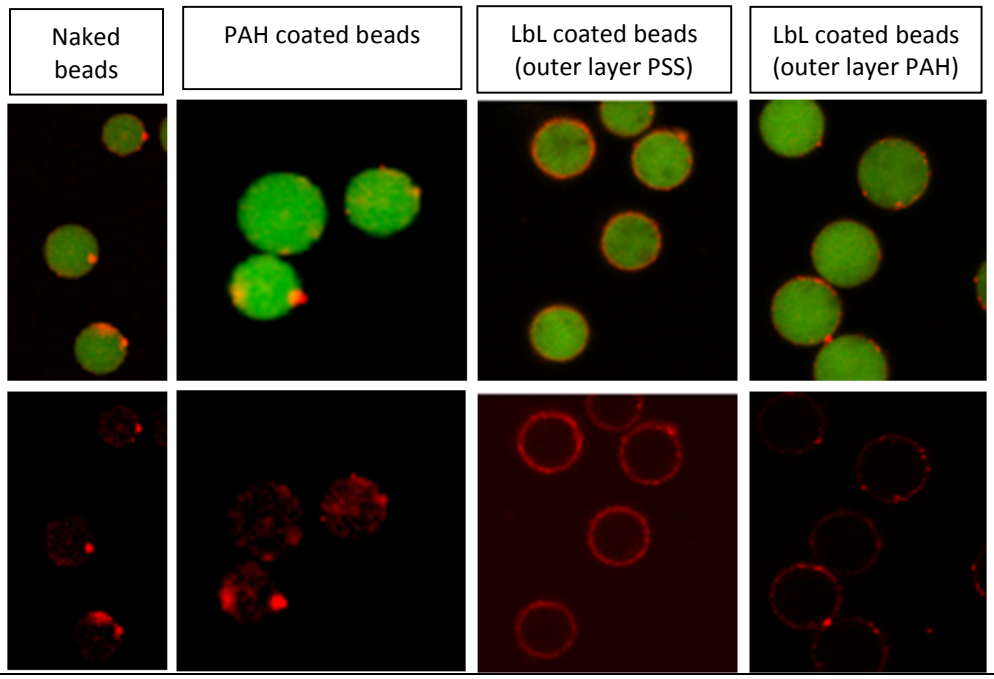


Figure 10. Red (TOTO3) labeled pDNA containing lipoplexes (based on DOTAP: DOPE) immobilized on microcarriers with a different surface. The merged green/red fluorescence image (top row) shows the green fluorescence of the polystyrene microcarriers. The red fluorescence image (bottom row) shows that the lipoplexes become immobilized at the surface of the microcarriers.

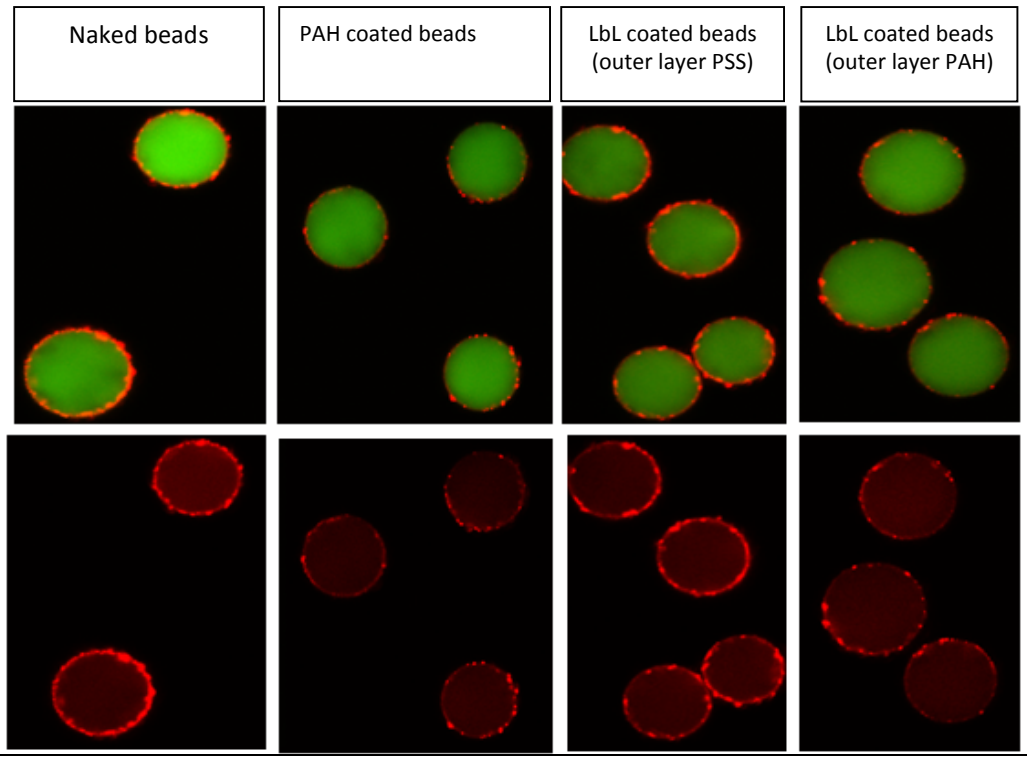


Figure 11. Red (Cy5) labeled siRNA containing polyplexes (based on PbAE1) immobilized on microcarriers with a different surface. The merged green/red fluorescence image (top row) shows the green fluorescence of the polystyrene microcarriers. The red fluorescence image (bottom row) shows that the siRNA polyplexes become immobilized at the surface of the microcarriers.

Figure 12 shows an image at higher amplification: it reveals the homogenous loading with naked siRNA in opposite to the more heterogeneous loading with siRNA polyplexes.

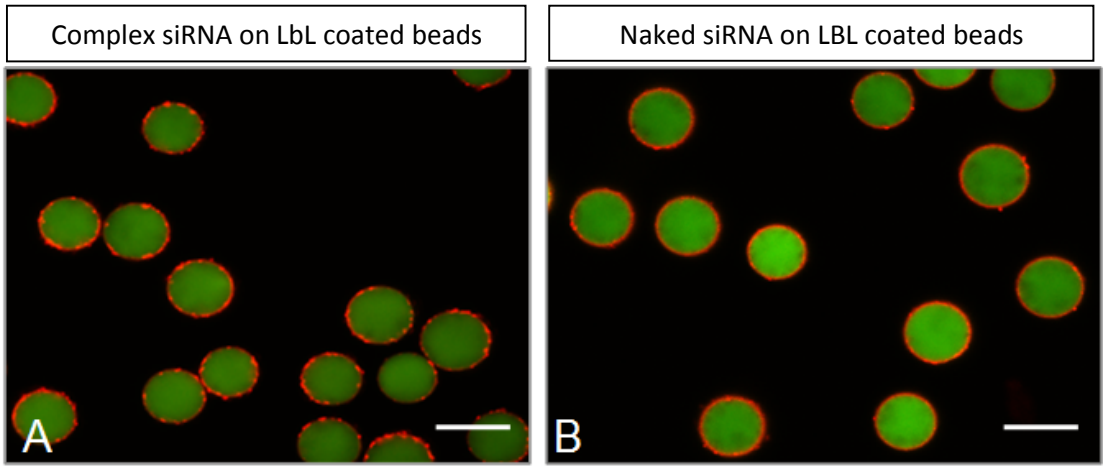


Figure 12. Fluorescent images of green microcarriers with LbL coating, loaded with red (cy5) labeled siRNA, either complexed (A) or free (B). The scale bar represents 50 µm.

Reverse transfection of cells by naked pDNA immobilized at the surface of encoded microcarriers

To evaluate the transfection efficiency of the immobilized nucleic acids, cells were grown on surface of the microcarriers. Figure 13A shows transmission and fluorescence images of microcarriers covered with cells; naked pDNA, encoding for a red fluorescent protein, was adsorbed to the surface of the LbL coated beads. The cells did not become transfected as the fluorescence image in Figure 13A did not show any red fluorescence in the cells. We hypothesize that a (too) strong binding of the naked pDNA to the surface of microcarriers may prevent the pDNA molecules to be released from the carriers surface and thus cellular internalization. The failure in the reverse transfection may be also attributed to the fact that negatively charged naked pDNA has difficulties in entering cells, due to negative charges at the cell membrane.

Reverse transfection of cells by complexed pDNA and siRNA immobilized at the surface of encoded microcarriers

Further we investigated whether cells grown on the beads could become reversely transfected with siRNA and pDNA complexes present at the surface of the beads. Segura et

al indeed showed that nucleic acid complexes immobilized on flat surface, could deliver the nucleic acids into cells [15,16,20]. Our group showed recently that (small, positively charged) PbAE1 based siRNA complexes were able to induce efficient siRNA-mediated gene silencing in cells grown in wells of microtiterplates, without causing significant cytotoxicity [37]. As cells immobilized at the surface of microcarriers start to overgrow each other after a number of days, siRNA release from the coating of the microcarriers into the cells should occur fast enough. Therefore we preferred to work with PbAE1:siRNA complexes as PbAE1 (bio)degrades relatively fast which may facilitate in time the release of the PbAE1:siRNA complexes from the coatings into the cells. The results showed that PbAE1:siRNA compelexes loaded on the surface of the beads were not able to induce efficient down regulation in cells, grown on microcarriers (Fig. 13B).

Similarly as for the PbAE1:siRNA complexes, significant transfection of the cells on the microcarriers did not occur when pDNA polyplexes, based on PEI, were loaded at the surface of the microcarriers (only a low level of gene expression could be observed; data not shown).

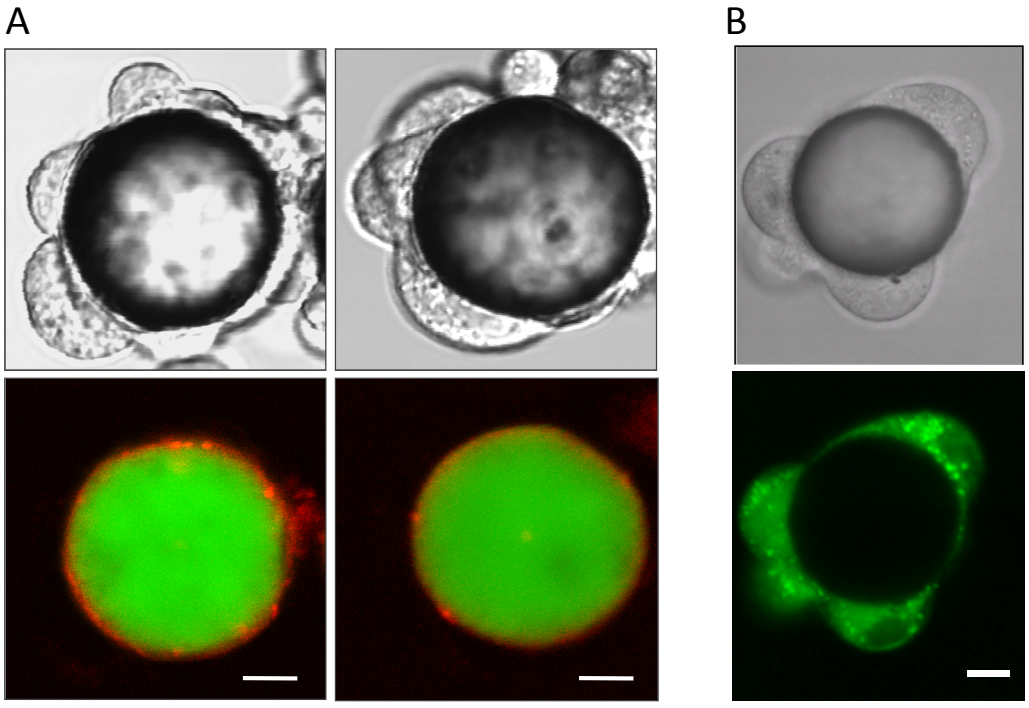


Figure 13. (A)Transmission (top row) and merged red/green fluorescence images (bottom row) of Vero cells grown at the surface of LbL coated beads (PAH as outer layer) loaded with TOTO3 labeled pDNA encoding for a red fluorescent protein. Clearly, the cells do not express the red protein.
(B) HuH-7_eGFPLuc cells grown at the surface of LbL coated beads loaded with PbAE1:siRNA complexes. siRNA were not able to induce efficient down regulation in cells as the cells are express the green protein. The scale bar represents 10 µm.

Reverse transduction of cells by adenoviral vectors immobilized at the surface of encoded microcarriers

Subsequently we investigated whether adenoviral vectors (bearing the genetic code for GFP or RFP) immobilized in the LbL coating surrounding the beads could transduce the cells grown on the surface of the beads. Recently, bioactive adenoviral vectors embedded in multilayered polyelectrolyte films on a flat surface were shown to efficiently transfect different cell lines [29]. Also Fischlechner *et al.* suggested virus coating of LbL coated colloids [41,42] and introduced these materials for use in multiplex suspension arrays to detect virus specific antibodies [43]. Hobson D.A *et al* proposed to use the extremely tight interaction between streptavidin and biotin to immobilize adenoviral vectors on the surface of wells and microparticles [18,19].

As Figure 14 shows, after 24 h a large percentage of cells that had settled onto the virus-coated microcarriers expressed RFP and thus became transduced. Importantly, only the cells attached to the surface of the beads became transduced (and not the cells being present in between the beads), as one can see when comparing the transmission and fluorescence images in Figure 15. It proves that the adenoviral particles did not detach from the microcarrier's surface during the time of the experiment, which would have resulted in (unwanted) transduction of the free cells.

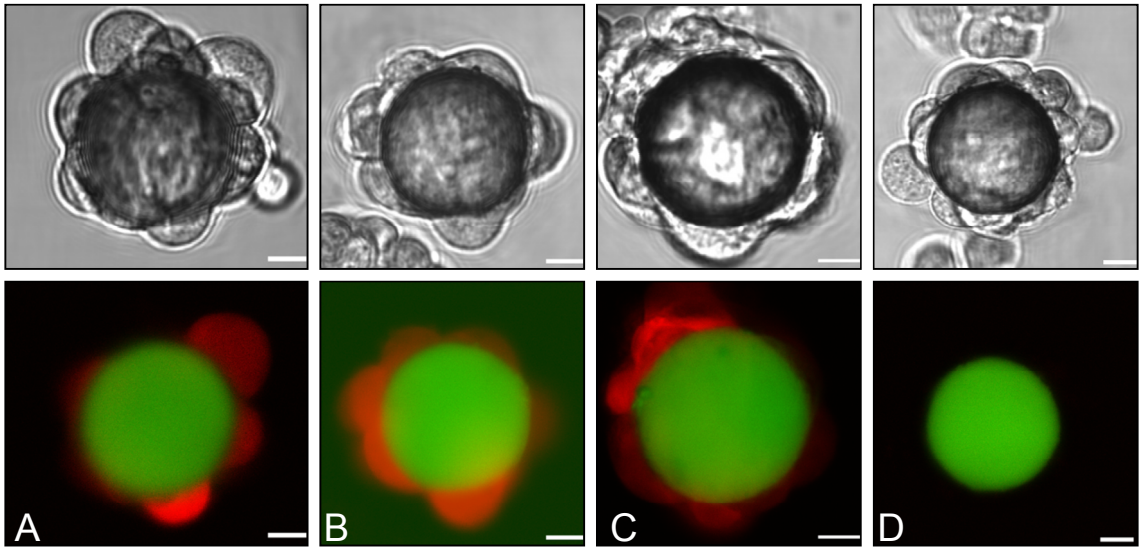


Figure 14. (A-C) Transmission (top) and merged red/green fluorescence (bottom) images of Ad-RFP coated microcarriers loaded with Vero-1 cells. In (D) microcarriers were used which did not contain viral particles in their coating (negative control). The scale bar represents 10 µm.

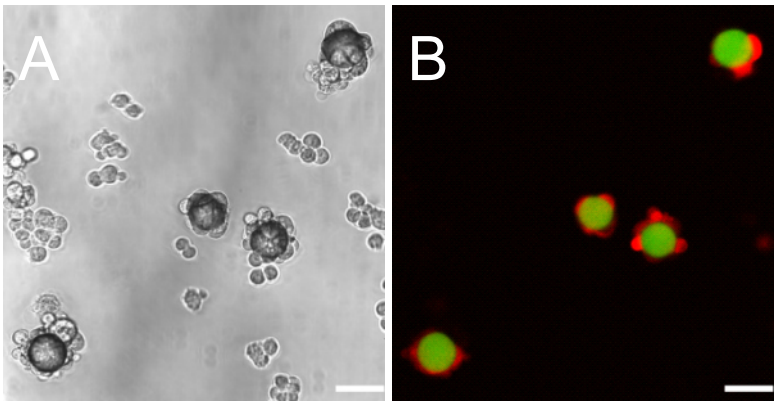


Figure 15. (A) Transmission and (B) merged green/red fluorescence images of a dispersion containing respectively Ad-RFP coated microcarriers loaded with Vero-1 cells and "free Vero-1 cells". The scale bar represents 50 µm.

One could argue, however, that the cells on the microcarriers became transducted by adenoviral vectors remaining free in the solution between the microcarriers in stead of by viral particles immobilized at the surface of the microcarriers. Therefore we performed transduction experiments on Vero-1 cells grown in 96 well plates using the "wash water" obtained in the preparation of the adenoviral coated microcarriers, thus containing the free adenoviral particles which did not become incorporated in the LbL surface. Clearly, washing the viral coated beads three times is sufficient to remove all free adenoviral particles as this solution did not transfect cells anymore (see fig 16). Note that the Ad-RFP loaded beads used in Figure 14 were washed three times and that the transduction of the cells on the beads could therefore only arrive from Ad-RFP particles immobilized in the coating of the beads (as there were no viral particles remaining in the surrounding solvent). The code in the microcarriers allows to identify with which viral-constructs the cells became transduced, and thus allows to know which protein target is expressed in the cells on a specific microcarrier. This approach may become interesting for making cell-based expression arrays with promises for proteomics and drug discovery.

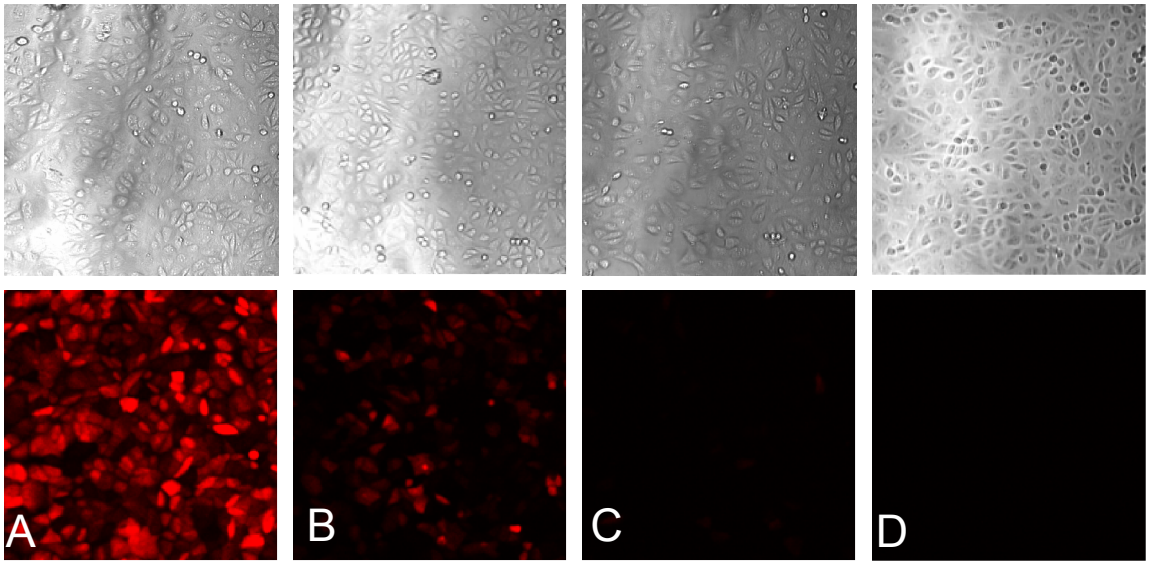


Figure 16. Transmission (top) and red fluorescence (bottom) images of Vero-1 cells seeded in 96 well plates transfected with respectively (A) the virus dispersion used to coat the microcarriers and (B) the first, (C) the second and (D) the third wash water as obtained during the preparation of the Ad-RFP coated microcarriers

CONCLUSION

In this chapter we showed that LbL coated encoded beads are suitable to immobilize respectively viral vectors, naked nucleic acids and complexed nucleic acids. However, naked nucleic acids and non-viral nucleic acid complexes immobilized in the LbL coatings at the surface of the beads did not (significantly) transfect the cells that grow on top of the LbL coated microcarriers. Highly likely this is due to the limited release of the naked and complexed nucleic acids from the microcarrier's surface. However, adenoviral particles immobilized in the LbL coating were able to selectively tranduce the cells that were grow on top of the "adenovirus coating". Indeed, we showed that adenoviral particles immobilized in the polyelectrolyte layer retained their ability to infect cells. Importantly, only the cells at the surface of the microcarriers, thereby in close contact with the adenoviral particles, became transduced, while free cells (i.e. cells present in the dispersion but not attached to a microcarrier) were not transduced. In conclusion, LbL coated encoded microcarriers may become a valuable tool to use in cell based microarrays in which the cells have to become transfected with nucleic acids. However, an optimal way should be found for appropriately immobilizing the nucleic acids at the surface of the microcarriers: on the one hand the binding should be strong enough in order to avoid the release into the surroundings of the

microcarriers, a too strong binding on the other hand may prevent that the nucleic acids become taken up by the cells.

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REFERENCES

1.Verma I.M., Somia N., Gene therapy -- promises, problems and prospects. Nature **1997**, 389(6648), 239-42.

2. Anderson J.L., Hope T.J., Intracellular trafficking of retroviral vectors: obstacles and advances. Gene Ther **2005** 12(23), 1667-78.

3. Ding W., Zhang L., Yan Z., Engelhardt J.F., Intracellular trafficking of adeno-associated viral vectors. Gene Ther **2005**, 12(11), 873-80.

4. Corvo M., Duque M., Viral gene therapy. Clin Transl Oncol **2006**, 8(12), 858-67.

5. Donahue R.E., Kessler S.W., Bodine D., McDonagh K., Dunbar C.,Goodman S., Agricola B., Byrne E., Raffeld M., Moen R., Bacher J., Zsebo K.M., Nienhuis A.W., Helper virus induced T cell lymphoma in nonhuman primates after retroviral mediated gene transfer. J Exp Med **1992,** 176, 1125–35.

6. Vasir J.K., Labhasetwar V., Polymeric nanoparticles for gene delivery. Expert Opin Drug Deliv **2006,** 3(3), 325-44.

7. Ma B., Zhang S., Jiang H., Zhao B., Lv H. Lipoplex morphologies and their influences on transfection efficiency in gene delivery. J Control Release **2007,** 123(3), 184-94.

8. Jo J., Tabata Y., Non-viral gene transfection technologies for genetic engineering of stem cells. Eur J Pharm Biopharm **2008**, 68(1), 90-104.

9. Li S.D., Huang L. Gene therapy progress and prospects: non-viral gene therapy by systemic delivery **2006**, 13(18), 1313-9.

10. Gao X., Kim K.S., Liu D., Nonviral gene delivery: what we know and what is next. AAPS J **2007,** 9(1), 92-104.

11. Wolff J.A., The "grand" problem of synthetic delivery. Nat Biotechnol **2002**, 20(8), 768-9.

12. Mastrobattista E., van der Aa M.A., Hennink W.E., Crommelin D.J., Artificial viruses: a nanotechnological approach to gene delivery. Nat Rev Drug Discov **2006** 5(2), 115-21.

13. Bielinska A.U., Yen A., Wu H.L., Zahos K.M., Sun R., Weiner N.D., Baker J.R. Jr, Roessler B.J., Application of membrane-based dendrimer/DNA complexes for solid phase transfection *in vitro* and *in vivo*. Biomaterials **2000**, 21(9), 877-87.

14. Ziauddin J., Sabatini D.M., Microarrays of cells expressing defined cDNAs, Nature **2001**, 411, 107-10.

15.Segura T., Shea L.D., Surface-tethered DNA complexes for enhanced gene delivery. Bioconjug Chem **2002**, 13(3), 621-9.

16. Segura T., Volk M.J., Shea L.D., Substrate-mediated DNA delivery: role of the cationic polymer structure and extent of modification, J Control Release **2003,** 18, 93(1), 69-84.

17. Segura T., Chung P.H, Shea L.D., DNA delivery from hyaluronic acid-collagen hydrogels via a substrate-mediated approach. Biomaterials **2005**, 26(13), 1575-84.

18. Hobson D.A., Pandori M.W., Sano T., In situ transduction of target cells on solid surfaces by immobilized viral vectors. BMC Biotechnol **2003**,3, 4.

19. Pandori M.W., Hobson D.A., Sano T., Adenovirus-microbeads conjugates possess enhanced infectively: a new strategy for localized gene delivery. Virology **2002**, 299, 204-12.

20. Bengali Z., Pannier A.K., Segura T., Anderson B.C., Jang J.H., Mustoe T.A., Shea L.D., Gene delivery through cell culture substrate adsorbed DNA complexes. Biotechnol Bioeng **2005**, 5, 90(3), 290-302.

21. Yoshikawa T., Uchimura E., Kishi M., Funeriu D.P., Miyake M., Miyake J., Transfection microarray of human mesenchymal stem cells and on-chip siRNA gene knockdown. J Control Release **2004**, 96(2), 227-32.

22. Cavanagh H.M., Dingwall D., Steel J., Benson J., Burton M., Cell contact dependent extended release of adenovirus by microparticles *in vitro*. J Virology methods **2001**, 95, 57-64.

23. Zhang J., Chua L.S., Lynn D.M., Multilayered thin films that sustain the release of functional DNA under physiological conditions. Langmuir **2004**, 20, 8015-21.

24. Johnston A.P., Read E.S., Caruso F., Multilayer films on planar and colloidal supports: sequential assembly of like-charged polyelectrolytes. Nano Lett. **2005**, 5(5), 953-6.

25. Jewell C.M., Zhang J., Fredin N.J., Lynn D.M., Multilayered films promote the direct and localized delivery of DNA to cells. J Control Release **2005**, 106(1-2), 214-23.

26. Jessel N., Oulad-Abdelghani M., Meyer F., Lavalle P., Haîkel Y., Schaaf P., Voegel J.C., Multiple and time-scheduled in situ DNA delivery mediated by beta-cyclodextrin embedded in a polyelectrolytemultilayer. Proc Natl Acad Sci U S A **2006**, 103(23), 8618-21.

27. Meyer F., Ball V., Polyplex embedding in polyelectrolyte multilayes for gene delivery. Biochim Biophys Acta **2006**, 1758 (3), 419-22.

28. Meyer F., Dimitrova M., Jedrzejenska J., Arntz Y., Schaaf P., Frisch B., Voegel J.C., Ogier J., Relevance of bi-functionalized polyelectrolyte multilayers for cell transfection. Biomaterials **2008**, 29(5), 618-24.

29. Dimitrova M., Arntz Y. , Lavalle P., Meyer F., Wolf M. , Schuster C., Haïkel Y., Voegel J.C., Ogier J., Adenoviral Gene Delivery from Multilayered Polyelectrolyte Architectures. Adv Funct Mater **2006**, 17, 233-45.

30. Wade-Martins R., Smith E.R., Tyminski E. Chiocca E.A., Saeki Y., An infectious transfer and expression system to genomic DNA loci in human and mouse cells. Nature Biotechnol **2001**, 19, 1067-70

31. Lotze M.T., Kost T.A., Viruses as gene delivery vectores: application to gene function, target validation, and assay development. Cancer Gene Ther **2002**, 9, 692-9

32. Elahi S.M., Oualikene W., Naghdi L., O'Connor-McCourt M., Massie B., Adenovirus-based libraries: efficient generation of recombinant adenoviruses by positive selection with the adenovirus protease. Gene Ther **2002**, 9(18), 1238-46.

33. Michiels F., van Es H., Van Rompaey L., Merchiers P., Francken B., Pittois K., Van der Schueren J., Brys R., Vandersmissen J., Beirinckx F., Herman S., Dokic K., Klaassen H., Narinx E., Hagers A., Laenen W., Piest I., Pavliska H., Rombout Y., Langemeijer E., Ma L., Schipper C., Raeymaeker M.D., Schweicher S., Jans M., Van Beeck K., Tsang I.R., van de Stolpe O., Tomme P., Arts G.J., Donker J., Arrayed adenoviral expression libraries for functional screening. Nat Biotechnol **2002**, 20(11), 1154-7.

34. Mousses S., Caplen N.J., Cornelison R., Weaver D., Basik M., Hautaniemi S., Elkahloun A.G., Lotufo R.A., Choudary A., Dougherty E.R., Suh E., Kallioniemi O., RNAi microarray analysis in cultured mammalian cells. Genome Res **2003**, 13(10), 2341-7.

35. Derveaux S., De Geest B.G., Roelant C., Braeckmans K., Demeester J., De Smedt S.C., Multifunctional layer-by-layer coating of digitally encoded microparticles. Langmuir **2007**, 25, 23(20), 10272-9.

36. Braeckmans K., De Smedt S.C., Roelant C., Leblans M., Pauwels R., Demeester J., Encoding microcarriers by spatial selective photobleaching. Nat Mater **2003**, 2(3), 169-73

37. Vandenbroucke R.E., De Geest B.G., Bonné S., Vinken M., Van Haecke T., Heimberg H., Wagner E., Rogiers V., De Smedt S.C., Demeester J., Sanders N.N., Prolonged gene silencing in hepatoma cells and primary hepatocytes after small interfering RNA delivery with biodegradable poly(beta-amino esters). J Gene Med **2008**, 10(7), 783-94.

38. Sanders N.N., Van Rompaey E., De Smedt S.C., Demeester J. Structural alterations of gene complexes by cystic fibrosis sputum. Am J Respir Crit Care Med **2001** 164(3) 486-93.

39. Fayazpour F., Lucas B., Alvarez-Lorenzo C., Sanders NN., Demeester J., De Smedt SC., Physicochemical and transfection properties of cationic hydroxyethylcellulose/DNA nanoparticles. Biomacromolecules **2006**, 7(10), 2856-62.

40. Stubbe, B.G.; Gevaert, K.; Derveaux, S.; Braeckmans, K.; De Geest, B.G.; Goethals, M.; Vandekerckhove, J.; Demeester, J.; De Smedt, S.C. Evaluation of encoded layer-by-layer coated microparticles as protease sensors. Adv Funct Mater **2008**, 18**(**11**),** 1624-1631.

41. Fischlechner M., Toellner L., Messner P., Grabherr R., Donath E., Virus-engineered colloidal particles--a surface display system. Angew Chem Int Ed Engl. **2006**, 45(5), 784-89.

42. Fischlechner M., Zschörnig O., Hofmann J., Donath E., Engineering Virus Functionalities on Colloidal Polyelectrolyte Lipid Composites. Angew Chem Int Ed Engl **2005**, 44(19), 2892-5.

43. Toellner L., Fischlechner M., Ferko B., Grabherr R.M., Donath E., Virus-coated layer-by-layer colloids as a multiplex suspension array for the detection and quantification of virus-specific antibodies. Clin Chem **2006**, 52(8), 1575-83.

44. Echeverri C.J., Perrimon N., High-throughput RNAi screening in cultured cells: a user's guide. Nat Rev Genet **2006**, 7(5), 373-84.

45. Hook A.L., Thissen H., Voelcker N.H., Surface manipulation of biomolecules for cell microarray applications. Trends Biotechnol **2006**, 24(10), 471-7.

Chapter 5

Turning to Digitally Encoded Drug Tablets to Combat Counterfeiting

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ABSTRACT

Counterfeiting of drugs is a real threat to public health in both developing and industrialized countries due to the high risk and inevitable occurrence of patients taking, or being treated with drugs of inferior quality. “In-drug labeling”, i.e. the safe labeling of the drug instead of the packaging, would be a major step forward to combat counterfeiters. The digitally encoded microparticles described in this paper, called Memobeads, may become a major tool in combating counterfeiters of pharmaceuticals who are becoming increasingly sophisticated. Here we show that Memobeads can be incorporated in drug tablets, by far the most widely used drug dosage form in the world. We found that entrapping the Memobeads in starch-based granules protects them from deformation during tabletting, even when high compression forces are applied.

Compared to other types of encoded microcarriers, Memobeads have a number of assets for “in-tablet” labeling. One asset being that ingested Memobeads are highly unlikely to be toxic to humans; another asset being that the number of unique Memobeads is virtually unlimited, an obvious advantage considering the huge number of pharmaceuticals at risk of being counterfeited.

Chapter 5

Turning to Digitally Encoded Drug Tablets to Combat Counterfeiting

INTRODUCTION

Pharmaceutical products are an important part of the healthcare system. Therefore, drug counterfeiting can cause a wide array of serious medical and economic damages. Although the problem is recognized by official health organizations like FDA and WHO, actions taken so far to combat drug counterfeiting have been insufficient [1-4]. There are different types of counterfeit drugs that can affect the patient. The counterfeit products look identical to the legitimate ones, and may either lack the active ingredient, contain active ingredients different from those listed on the label, be diluted and/or contain harmful impurities. Other counterfeit drugs are intentionally mislabeled to extend the expiration date [5].

Various researchers estimate different percentage of counterfeit medications in total pharmaceutical market. The World Health Organisation (WHO) has estimated that 10% of global medicines are counterfeit [3]. But because of the incomplete database on counterfeit drug, it is hard to estimate the true extent of the problem and the real level could be higher [6] .In parts of Africa and Asia this figure exceeds 50% [7]. The 192,000 patients killed by fake drugs in China in 2001 or the 3000 died people in Nigeria in 1995 because of fake vaccines gives an indication of the scale of human suffering [8,9].

With increasing free trade policies and the new phenomenon of purchasing drugs from online pharmacies, counterfeit drugs are becoming widespread. In some cases, internet pharmacies sell products of unknown quality [10]. Only 12% of e-pharmacies displayed quality accreditation seals [11,12]. Today drug counterfeiting is a global problem and it is not limited to developing countries [4]. Generally in developing countries, life-saving medications such as

antibiotics, painkillers and vaccines are being counterfeited and distributed via the local market. In industrialized countries the counterfeiters generally target “lifestyle drugs” such as hormones and anti-psychotics via the internet [13-16]. The problem is growing and there is no simple solution or a single "magic bullet" technology to combat counterfeiting. To solve this major world wide problem coordinated action between governments, law enforcement agencies, health professionals, the pharmaceutical manufacturers, wholesalers and patients is required [17-19]. The WHO has issued several guidelines against counterfeiting [20-24]. Currently there are a number of anti-counterfeiting technologies for pharmaceuticals available or under development. These technologies range from the very simple and low cost to highly sophisticated and expensive ones. To obtain higher security, a combination of technologies can be used. Strategies differ by products and pharmaceutical manufacturers should choose the most effective strategy based on different parameters for each product.

Anti-counterfeiting technologies

Anti-counterfeiting technologies basically can be applied on two different levels: the package and the product. In packaging-based measures, the security device is used on packaging, whereas the second approach is focused on labeling of the dosage form. Using anti-counterfeiting technology at the level of dosage form is useful even when the products are separated from their package. The product labeling could be visible, which is called “on product” marking or applied inside the product (invisible), named “in product” labeling. Anti-counterfeiting technologies can also be classified into “overt”, “covert” and “forensic” technologies [25]. Each of them is effective to some extent as a counterfeit-proof.

(a) ***Overt*** features: these security features on product or packaging, e.g. holograms, color shifting inks and watermarks, are apparent and visible; they require inexpensive instruments for reading out the information but are easily mimicked [26]. Evidence shows that visible security features or coding are not sufficient to protect products from counterfeiting. Using some kind of colour which makes the feature more difficult to reproduce or combine an overt design with a covert one can add extra security to this technique.

A new technique offers security microstructures that can be created by electron beam lithography (EBL) [27]. On-product marking is also an overt technology which allows placing special images or codes on oral dosage form. Colorcon has developed a new on-

tablet marking technique. With this technique the desired image or code is written upon the tablet surface by laser light [28].

Merck KGaA manufactures pearlescent pigments to use in hard and soft gelatin capsules and in film-coated tablets. These pigments can be distinguished by their characteristic shape, color and pearlescent effect and makes counterfeiting more difficult. The pigments consist of titanium oxide (E171), iron oxide (E172) and potassium silicate (E555) known as "mica" being widely approved food and pharmaceutical colorants (brand name Candurin® pigments) [29,30].

(b) ***Covert*** features or hidden markers on the product packaging (like certain inks and dyes that fluoresce or absorb ultraviolet light, invisible bar codes) are not visible to the eye and require simple equipment, such as an UV lamp for identification [31-33]. Recently, a technology has been developed which can print invisible micro-points in the packaging design. Detection of this micro-point in all print structure of packaging is based on a reader and special software [34,35]. Microtags are other covert devices which can be applied to packaging components. They can include multivariate levels of information. These microtags with the size about 75 microns also can be precisely engineered for forensic authentication[36].

(c) ***Forensic*** (or well hidden) markers can be based on a diversity of technologies, such as chemicals, biologicals or DNA taggants, that can be added on the packaging label or even into the drug itself. These markers are much more difficult to reproduce and expensive to read, because sophisticated analytical instrumentation is required [26]. Compared to the other methods, DNA (or other nucleic acid) taggants seems to be most effective as a counterfeit-proof. This technology involves the inclusion of a DNA marker into the product label or on the product surface. Applied DNA Sciences (APDN) provides patented DNA-based technologies to prevent and identify counterfeits. According to the company the patented technology, SigNature, can be used on almost any consumer product. A primary test can be performed using a detection pen, which will cause a color change if the SigNature ink is present [37]. Also another company, DNA Technologies (Australia) Pty Ltd, has a patent for DNA taggants, called DNA Matrix™ [38].

To combat counterfeiting and to allow tracking and tracing a drug throughout the supply chain, the packaging of an increasing number of drugs, such as Viagra®, is being "protected" by radio frequency tags (RFID), barcodes and/or serialization [32,39-41].

Unfortunately, such tracking and tracing technologies are only effective if the drugs are not repackaged. However, manufacturers often do not ship drugs directly to hospitals and dispensing pharmacies. For example, drugs are often sold to wholesalers or to repackagers who move drugs from bulk to unit-of-use containers. These multiple transactions can provide a means for counterfeit drugs to enter the legitimate drug supply chain. To overcome this, "in-drug labeling", i.e. the labeling of the dosage form (tablet, capsule, cream, solution…) itself instead of the packaging of the drug could help to combat counterfeiters.

Incorporation of taggants or chemical markers in the drugs themselves is much less developed and, to our knowledge, not yet used. A major reason is clearly that in-drug labeling requires both extensive toxicological screening of the taggant as well as formulation compatibility testing. So far only a very limited number of taggants have been proposed for in-product labeling of pharmaceuticals. For example, color-coded particles (5 – 45 µm) which are regarded as food safe (available under the brand name SECUTAG®). The basis of the SECUTAG® is melamine alkyd polymer particles. The code is produced by the use of sandwich method, in which 4-10 variably coloured layers are stratified. The identification of the colour code needs a microscope or an automatic reader [42]. ARmark™ covert markers are another authentication technology system developed by Adhesives Research Inc. for in-product labeling. The markers created by using nanoentonography are identified via digital micro-imaging hardware and customized software programs [43].

There is no guarantee of avoiding counterfeiting when the security device is just on the packaging. In this chapter we are launching a new concept for the in-product labeling of tablets, by far the most widely used drug dosage form in the world. We recommend digitally encoded micro-particles, known as Memobeads, for this purpose. Memobeads are micron-sized fluorescent polystyrene beads which are graphically encoded by "spatial selective photobleaching" of the fluorescence [44,45]. In this study we evaluate (a) how Memobeads can be incorporated into tablets and (b) whether the digital code withstands the compression forces applied during tabletting. We also critically discuss the toxicological aspects of orally administered Memobeads.

MATERIALS & METHODS

Memobeads

Surface carboxylated polystyrene beads of 39 µm (Sperotech, Libertyville, USA), dyed with a fluorophore were graphically encoded by "spatial selective photobleaching" of the fluorescence [44,45]. Photobleaching is a photo-induced process by which fluorescent molecules lose their fluorescent properties, which results in fading of the fluorescent colour. By using a specially adapted laser scanning confocal microscope, patterns can be photobleached at any depth inside the fluorescently dyed microsphere. Any geometry can be bleached, such as a symbol, an alphanumeric code, a barcode and even a logo. Details on the encoding procedure can be found elsewhere [46]. Certainly, as beads move and rotate, for the code to be clearly visible the beads need to be properly positioned at the time of decoding. We have shown previously that by providing the microspheres with orientation information, such as a (permanent) magnetic moment, it is possible to automatically position the spheres correctly for readout. For this purpose, as schematically shown in Figures 1C and 1D, ferromagnetic chromium dioxide particles were incorporated in the surface of the beads by the so called Layer-by-Layer (LbL) technology.

Layer-by-Layer (LbL) technology is based on the alternate adsorption of oppositely charged polyelectrolytes/nanoparticles onto a charged substrate. The LbL layer in this study was build with poly(allylamine hydrochloride) (PAH) as polycation and poly(styrene sulfonate) (PSS) as polyanion. The surface carboxylated microspheres (negatively charged) were therefore suspended in 1 ml PAH solution (2 mg/mL in 0.5M NaCl); the suspension was continuously vortexed (1000 rpm, 25°C) for 15 min. The non-adsorbed PAH was removed by repeated centrifugation (30 sec at 1500 rcf) and washing (with deionized water). Subsequently, the microspheres were dispersed in deionized water containing the negatively charged chromium dioxide nanoparticles (CrO_2 NP, 375 nm average diameters). The dispersion was continuously shaken for 15 min and the excess of CrO_2 NPwas removed by repeated centrifugation/washing steps. The microspheres were further coated with 4 layers in the following order: PAH / PSS / PAH / PSS. These LbL coated microspheres were resuspended in 1 ml of deionized water and subsequently encoded (as described above) and magnetized. These beads are magnetized in a strong magnet at the time of encoding. For

decoding, the beads are subjected to a weak magnetic field, thereby orienting the beads in a perfect position for reading out the code [47].

Production of a pharmaceutical formulation containing the encoded beads

Layering of encoded beads on non-pareil seeds. Microcrystalline cellulose spheres were used as non-pareil seeds (300 g of Cellets® 700 (700-1000 µm) (Pharmatrans, Basel, Switzerland)). Layering was performed in a fluid-bed apparatus (GPCG 1, Glatt, Binzen, Germany) used in the bottom spray mode with the Wurster setup (nozzle diameter 0.8 mm; spray rate of 4.6 g/min for 25 min; atomising pressure 1.5 bar; product temperature 45°C). The layering solutions were prepared by dissolving first a binder and then suspending the encoded beads in demineralised water. As binder, polyvinylpyrolidone (PVP, Kollidon® 25, BASF, Ludwigshafen, Germany) (1%, w/w) or hydroxypropylmethylcellulose (HPMC, Opadry®, Colorcon, Dartford, UK) (9%, w/w) were used. The content of the encoded beads in the layering solution was adjusted to obtain a yield of 8 beads/pellet after layering. The layering solution was stirred manually during the process. Before layering, the pellets were preheated until a product temperature of 45°C was reached.

Encoded tablets produced by granulation and compression*.* Hydroxypropylmethylcellulose (Methocel® E15 LV, Colorcon, Dartford, UK) and UNI-PURE® EX starch (National Starch and Chemical Company, New Jersey, US)) were mixed for 15 min in a Turbula mixer (model T2A, W.A. Bachofen, Basel, Switzerland). The HPMC and starch concentration in the powder mixture was 7 and 93% (w/w, dry mass), respectively. The powder mixture was granulated for 10 min at 60 rpm by means of a planetary mixer (Kenwood Chef, Hampshire, UK) with demineralised water (40 %, w/w, wet mass) in which encoded beads were suspended. Subsequently the wet mass was put through a sieve (0.94 mm) and then the granules were dried in the oven for 12 h at 40°C. The dry granules were sifted and the fraction between 250 and 710µm was further blended with 0.5% (w/w) magnesium stearate (< 90 µm) (BUFA, Brussels, Belgium) in a Turbula mixer for 1 min. With these obtained granules tablets (500 mg) were then prepared using an eccentric compression machine (Korsch EKO, Berlin, Germany) equipped with a flat faced double punch of 12 mm at compression forces ranging from 53 to 178 MPa.

Encoded tablets produced by direct compression*.* A suspension of Memobeads was dried in the oven for 12 h at 40 °C to obtain a solid sample. To destroy the aggregate of the beads,

dry sample was first shacked in a Turbula mixer with glass beads. This powder was gradually mixed with 250 g α-lactose monohydrate (Tablettose®, Meggle, Wasserburg, Germany) in a Turbula mixer for 30 min. The mixture was blended with 0.5% (w/w) magnesium stearate for another 1 min. Afterwards tablets (500 mg) were prepared using the compression machine described above at compression forces ranging from 53 to 223 MPa.

Recovering the Memobeads from the tablets and decoding. The Memobeads were recovered from a tablet (500 mg) by dissolving a part (about 50 mg) of a tablet in 500 µL distilled water. After centrifugation, the supernatant containing dissolved materials was removed. 100 µL of distilled water was added to the undissolved material containing beads. The sample was vortexed and applied on a microscope slide and imaged by a confocal laser scanning microscope (model MRC1024 UV, BioRad, Hemel Hempstead, UK) equipped with a water immersion objective lens (Plan Apo 60x, NA 1.2, Nikon). The 488 nm line of a krypton-argon laser was used for excitation. Note that a weak magnetic field was applied to the microscope table to allow perfect positioning of the Memobeads for readout (see above).

Cell toxicity of Memobeads

The cell toxicity of the Memobeads was evaluated by the EZ4U assay (Biomedica GmbH, Vienna, Austria) [48]. A large amount of LbL coated microcarriers (> 10^6) was dispersed for 48, 72 and 140 h in cell culture. In this way, compounds that are possibly present in the LbL coating and the polystyrene core of the microcarriers, and which may be toxic to cells, were “extracted” in the cell culture medium. Cells were subsequently grown in the cell culture medium obtained in this manner. An EZ4U test was performed after 48, 72 and 140 h (in the exponential phase of growth). Briefly, 50 µl of EZ4U substrate, together with 450 µl culture medium, was added to each well and incubated for 2-5 h. The absorbance was measured with a Wallac Victor^2 absorbance plate reader (Perkin Elmer; Waltham, MA) set at 450 nm with a reference wavelength of 620 nm. Negative and positive control experiments were included by using cell culture medium that had not been exposed to Memobeads and by using cell culture medium that was supplemented with 10 mg/ml of phenol, respectively.

Release of chrome (Cr) and fluorophore from Memobeads suspended in gastric fluid

An amount of Memobeads (approximately 10/ml and 50000/ml in the determination of the release of Cr and fluorophore, respectively) was suspended in 200 ml simulated

gastric fluid [49] for 2 h at 37°C. Subsequently, the Memobeads were transferred into 200 ml simulated intestinal fluid for 3 h at 37°C. The dispersions were continuously shaken. The Cr concentration in the supernatant was determined by Hidrocontrol (Antwerpen, Belgium). The fluorescence of the supernatant was measured by the highly sensitive detector of a Fluorescence Correlation Spectroscopy (FCS) instrument which can even measure single fluorophores and which is considered to be a highly sensitive fluorimeter [50,51].

RESULTS

Memobeads digitally encoded with drug information

As shown in Figure 1A, barcodes but also information like the name of the drug, the name of the producer, the batch number, the expiration date, can be clearly written inside homogeneously fluorescently dyed microspheres of 39 µm by "spatial selective photobleaching" (i.e. photobleaching of certain regions).

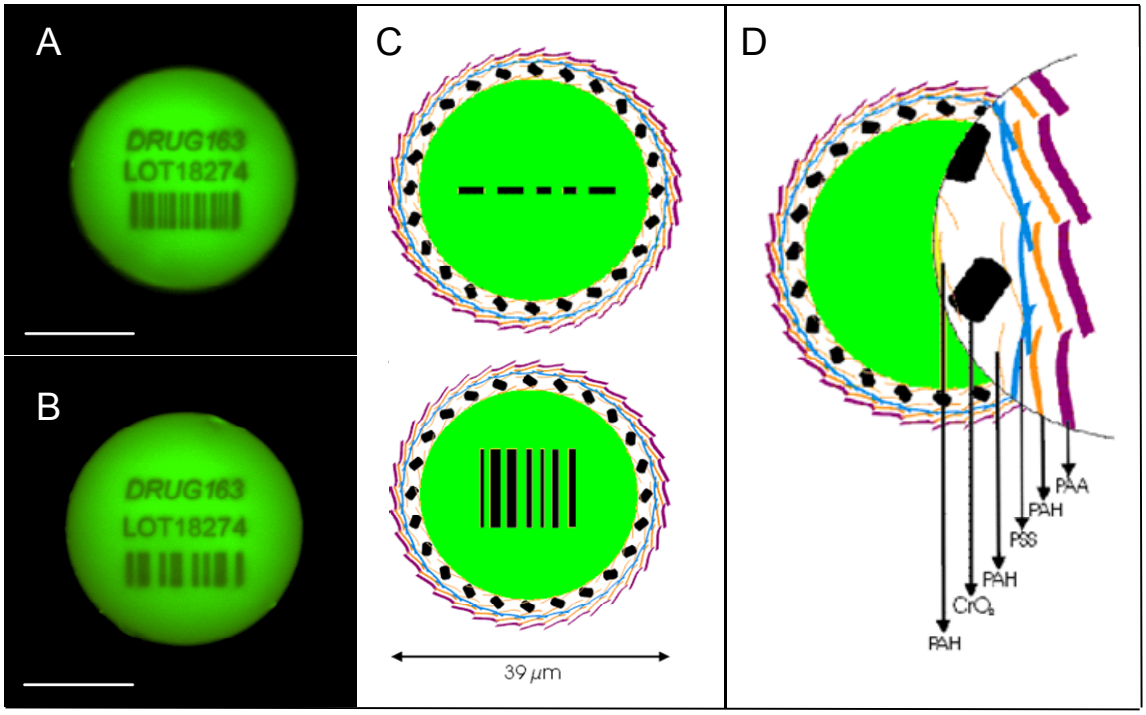

Figure 1: Composition of a Memobead. (A and B) Confocal images of the middle plane of encoded (39 µm diameter) Memobeads; the scale bar is 20 µm. (C) Schematic presentations of "dot-encoded" (top) and "bar-encoded" (bottom) Memobeads; the surface of the Memobeads contains ferromagnetic CrO_2 nanoparticles and polyelectrolytes. (D) The coating is obtained by sequential adsorption of poly(allylaminehydrochloride) (PAH), CrO_2, PAH, poly(styrenesulfonate) (PSS), PAH and PSS at the surface of the Memobeads by Layer-by-Layer (LbL) technology [52]. The ferromagnetic properties allow orienting the beads correctly to make the code clearly visible at the time of decoding [46].

Note that the information is written in (the middle plane of) the beads, as illustrated in Figure 2. Because the pattern in the carriers is bleached by a scanning laser beam "pixel by pixel" and "line by line", the time needed for writing the code depends on the complexity of the pattern. Therefore Memobeads with a simple one-dimensional "dot code" (see Fig. 1C), which requires only a single line-scan, were used in the experiments of this study.

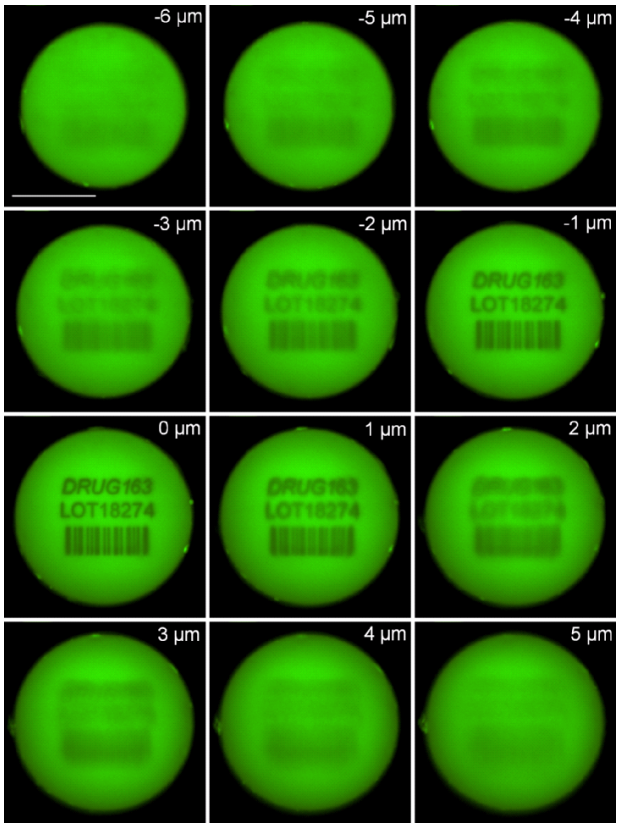

Figure 2: Confocal optical sections of a Memobead. The code is written in the central plane of the Memobead. At that position (0 µm) the code is clearly visible. At another depth in the Memobead (i.e. above (> 0 µm) or below (< 0 µm) the encoded central plane) the code becomes unreadable. The code is truly "hidden" inside the Memobead and can only be revealed by a confocal microscope focused at exactly the right depth inside the Memobead. The scale bar is 20µm.

Encoding pellets and tablets with Memobeads

Drugs can be formulated in pellets which subsequently can be filled in capsules. To produce encoded pellets we started layering of pellets with beads. Figure 3 is an image of an encoded microcarrier layered on a pellet, prior to isolation and decoding of the microcarrier.

We studied the influence of different parameters on layering of pellets with the encoded microcarriers. Based on our results layering with smaller beads have better efficiency (10µm better than 40µm (data not shown). Shear stress at different drying time

has no strong influence on layering of pellet (Fig. 4). Comparing different way of drying (in oven and fluid-bed) shows that by drying in oven we lose fewer beads, layered on the pellets (Fig 5A). The encoded beads layered on the pellets could easily be recovered and demonstrated their code to be intact (Fig 5B). However, a lot of encoded beads are lost during processing and the layering of pellets with encoded beads has low efficiency. These experiments proved that the product labelling by encoded microcarrier layered on the pellets is possible but not efficient. Therefore we focused further on using encoded microcarrier inside of the granules for in product labelling of tablets, the most widely used pharmaceutical formulation.

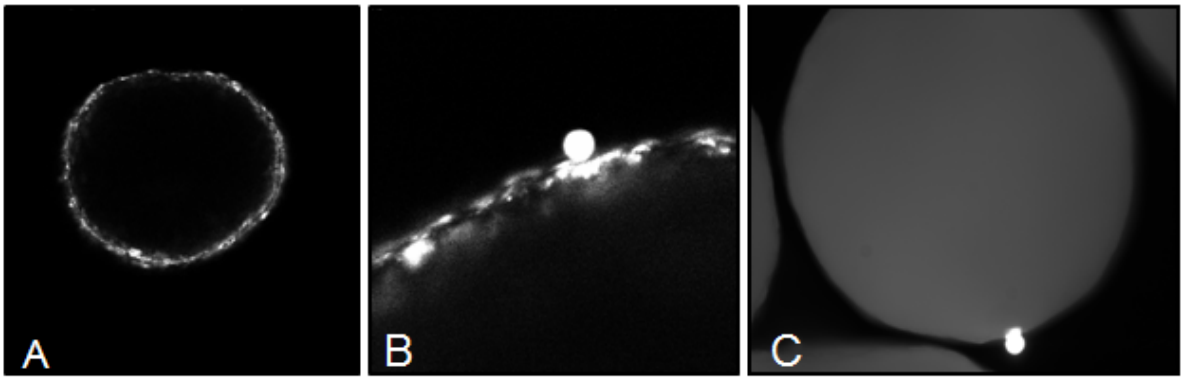

Figure 3. (A) A pellet with HPMC layer around it. (B, C) 10µm fluorescent bead on pellet

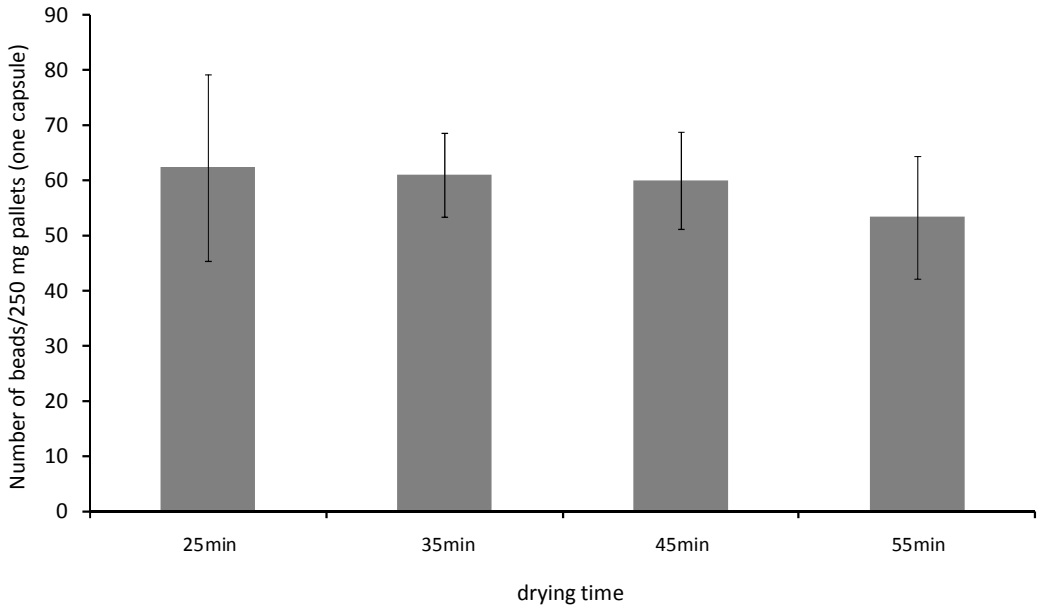

Figure4. The number of beads at different drying time

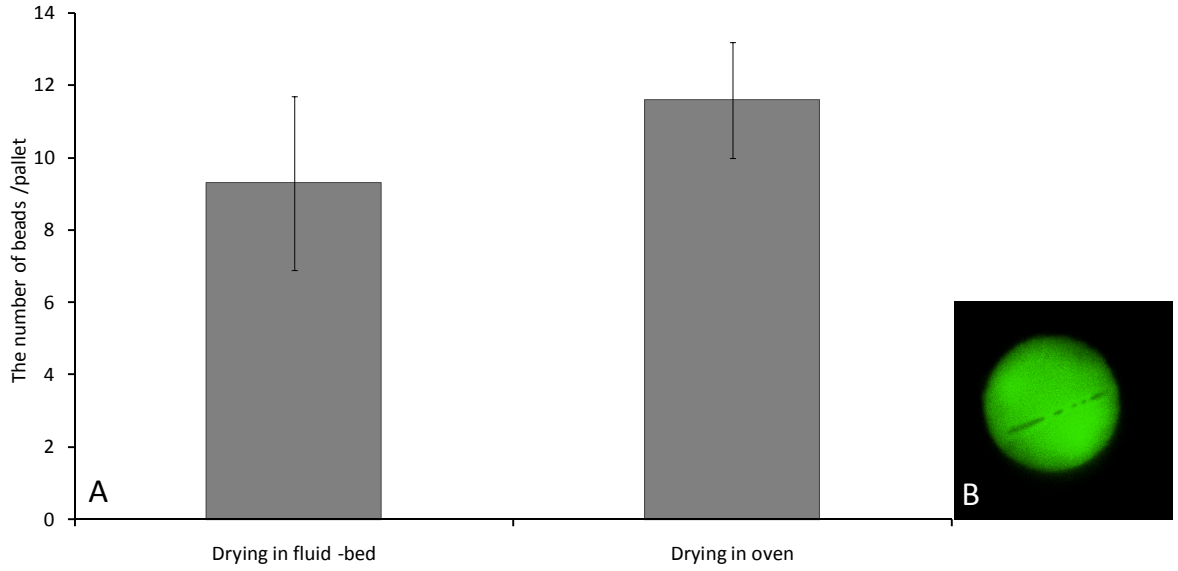

Figure 5. (A) The number of beads in different way of drying. (B)Dot-encoded beads recovered from pellet.

The production of tablets in pharmaceutical industry is mainly via direct compression of a drug/excipient powder mixture. This method is rather simple and has became popular in recent years as it saves time, labor, requires neither heat nor moisture, and all this may actually enhance the stability of the drug. Figure 6 shows Memobeads recovered from tablets produced by direct compression under different compression forces. Clearly, only at low compression forces the dot code does not deform; high compression forces destroy the code.

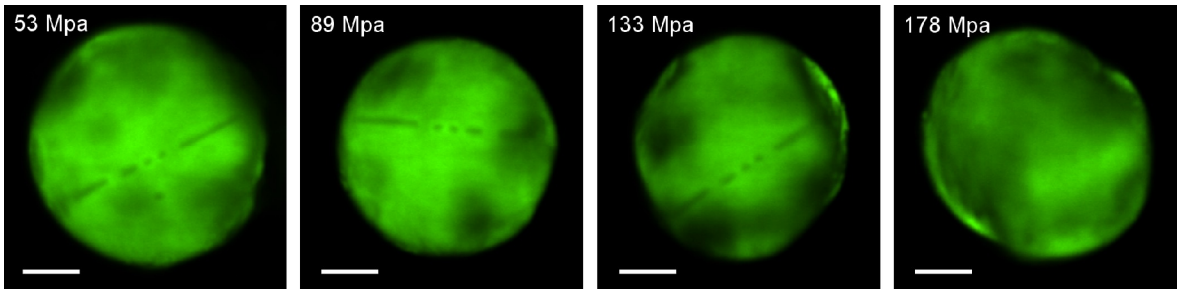

Figure 6: Dot-encoded beads as obtained from tablets made by direct compression of the powder mixture. The compression forces used during tabletting are indicated on the images. The Memobeads do not survive a compression force of 178 MPa as the code is no longer visible. The scale bar is 10 µm.

In order to enhance flow characteristics of the powder, avoid segregation of compounds, enhance compression behaviour and avoid dust generation, powder particles are often "granulated" before further compression. During this agglomeration process irregular-shaped granules (approximately 200-1000µm in size) are formed, containing drug

and other excipients (Fig. 7A). Figure 7B shows a (non confocal) fluorescence image of a part of a HPMC/starch-based granule that contains three (fluorescent) Memobeads; note that, because a non-confocal fluorescence microscope was used, the codes in the beads are not visible. Figure 7C shows confocal images of dot-encoded Memobeads which were isolated from tablets made by compression of the HPMC/starch granules. Clearly, the Memobeads remain spherical and, especially, the dot code does not deform and remains perfectly readable.

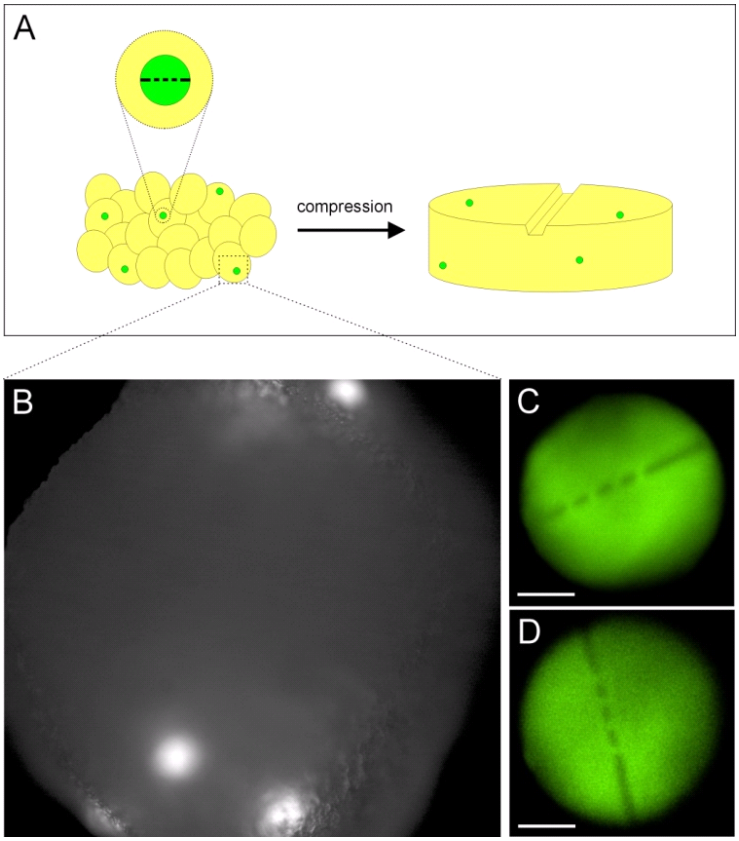

Figure 7: Dot-encoded beads as obtained from tablets made by compression of HPMC/starch granules. (A) Schematic presentation of the compression of granules (yellow, left image) into a tablet (right image); the granules and therefore the tablets contain encoded Memobeads (green). (B) Image obtained with an epifluorescence microscope of a part of an HPMC/starch granule (diameter about 0.5mm) in which three (fluorescent) Memobeads can be seen. (C, D) Confocal image of dot-encoded Memobeads obtained from the tablets (compression force used was 178 MPa). The scale bar is 10 µm.

More importantly, even when the granules were compressed at higher compression forces (up to 178 MPa), the dot codes in the Memobeads survived. One should note that the beads also remained perfectly capable of orientation by the magnetic field (a requirement for being able to see the code in the beads). This indicates that the chromium dioxide nanoparticles in the surface of the Memobeads (Fig. 1C) are not relocating by the high

compression forces. This is not surprising since they are strongly bound by electrostatic interactions with the polyanions and polycations of the LbL coating (Fig. 1D). Indeed, changing the positions of the nanoparticles would result in another orientation of the beads when brought into the magnetic field at the time of readout. Also note that, compared to two-dimensional codes, dot codes could be considered as the most "sensitive" towards deformation as deforming a small region in the microspheres would probably make the code unreadable.

As described above, the dot code withstands higher compression forces in case where the Memobeads are first embedded in a granule (Fig. 7C). It seems that the HPMC/starch matrix of the granules (Fig. 7B) physically protects the polystyrene matrix of the Memobeads from deformation by the high forces.

Uptake by the gut of orally ingested Memobeads

Memobeads in the tablets will be orally ingested by the patients. Obviously, the question arises whether Memobeads are harmful to humans. "Memobead related toxicity" could be due to (a) accumulation of the beads into the tissues after absorption by the digestive track and (b) compounds (like fluorescent dye, chromium, chromium dioxide particles, styrene mono- and oligomers, polyelectrolytes; see Fig. 1C) released from the Memobeads while they are present in the gastric and intestinal fluids. When debating the toxicity of Memobeads in the tablets it is important to keep the following in mind. As the Memobeads have no pharmacological or adjuvant function and are only intended for counterfeiting detection, the daily intake of Memobeads will be very limited. Assuming the presence of 10 Memobeads per tablet and further assuming one takes 10 tablets a day, it is reasonable to say that the total number of Memobeads administered per day will about one hundred. Note that the total mass of 100 Memobeads is estimated to be only about 3 μg.

Let us now consider the extent Memobeads are expected to be adsorbed by the gut. The lining of the gut is designed for the absorption of ions/molecules (nutrients) with a molecular mass of less than about one thousand [53]; it is essentially impermeable to particulate matter. However, a limited amount of particulate material can be transported across the intestinal barrier. Some interesting information is available on this topic particularly from pharmaceutical research, as gastrointestinal absorption of nano- and microparticles is very well understood, allowing the oral delivery of vaccines, peptides and

proteins. Indeed, membranous (M) cells (like in the domes of the Peyer's patches) are well known to be endocytotic allowing them to take up bacteria and *very small* particles. Although the uptake of bacteria and bacteria-sized particles by the M cells is well documented [54], little is known about the uptake of larger particles. Eldridge et al found that orally administered biodegradable poly(lactic-co-glycolic acid) (PLGA) microspheres with a diameter of 10 microns or larger did not become absorbed by the gut walls, whereas microspheres smaller than 10 microns were specifically taken up into the Peyer's patches. Microspheres between 5-10 microns in diameter remained fixed in the patches for an extended period (up to 35 days), while microspheres smaller than 5 microns were disseminated within macrophages to the mesenteric lymph nodes, the blood circulation and the spleen [55]. Studies on polystyrene and PLGA particles, both in cell culture and in animals *in vivo*, have shown that particle uptake decreases with particle size. M-cells may take up particles up to 10 µm in size [56,57], while particles up to 2 µm have been reported to be taken up by absorptive enterocytes [58]. LeFevre et al. administered 6 µm and 16 µm polystyrene microspheres orally to mice. They found that the larger particles did not accumulate in intestinal Peyer's patches, mesenteric lymph nodes, or other organs of the reticuloendothelial system or in the blood, even after the maximum dosage of millions of particles per day for 60 days. However, under similar administration the smaller particles were found in Peyer's patches, mesenteric lymph nodes, and lungs [59].

Not only size but also surface properties determine the susceptibility for uptake of particles by M cells: hydrophilic particles are known to adhere less to M-cells than hydrophobic ones [60] and since the intestinal surface is negatively charged [61], positively charged particles are expected to interact more with the cells than neutral or negatively charged particles.

Based on the observations above it is reasonable to assume that particles larger than about 10 µm, like Memobeads which are around 40 µm in size, are not taken up by the gut. We also note that, even if larger Memobeads were preferred they could be easily obtained by the same encoding/decoding approach.

Toxicity of the ingredients released from Memobeads

Let us further consider to what extent Memobeads are expected to release foreign compounds in the gastrointestinal fluids which could become absorbed by the gut.

In mass production, polystyrene is the major component of the Memobeads. Polystyrene is very often used for food packaging and is considered to be non- toxic as it is insoluble in body fluids. Polystyrene is, however, often contaminated with styrene monomers which are of potential concern [62]. The lethal oral dose (LD50) for styrene monomers is about 5 g/kg in rats [63]. The WHO guideline for styrene monomers in drinking water is 20 µg/l [62]. Assuming 100 beads are ingested daily (having a total mass of about 3 µg) it is reasonable to assume that the daily absorption of styrene monomers from Memobeads will be orders of a much lower magnitude than the daily absorption of styrene monomers tolerated when drinking water.

The surface of the Memobeads contains ferromagnetic chromium dioxide nano-particles (Fig. 1C) which could be released from the beads once they are in the gastrointestinal fluids. Clearly, this is highly unlikely as they are tightly bound in the coating of the Memobeads (see above). Chromium dioxide is insoluble in water and organic solvents and may thus be regarded as essentially non toxic. The oral LD50 in rats for chromium (II) chloride and chromium acetate hydrate are 1.870 and 11.260 mg/kg, respectively [64]. Chromium is normally found in water at concentrations less than 2 µg/L and no more than 50 µg/L is allowed in drinking water [65]. We determined the amount of chromium released from Memobeads after being dispersed for 2 h in simulated gastric fluid and subsequently 3 h in simulated intestinal fluid. Typically, the chromium concentration remained below 10 µg/L (Fig 8); note that in these experiments approximately 2000 Memobeads were suspended in 200 ml fluid which is much higher than the number of Memobeads we expect a patient will take per day.

As shown in Figure 1C, the chromium dioxide nanoparticles are adhered to the surface by a layer of polyelectrolytes. Clearly, both PAH and PSS are only present in trace amounts (far less than 1 % w/w). Toxicological data are not available for either PAH or for PSS. However, even in the unlikely event that PSS and PAH were released from the Memobeads in the gastrointestinal fluids the absorption of PAH and PSS by the gut is expected to be negligible as they are both high molecular weight compounds. One should also note that poly(styrenesulphonate) is even administered orally for the treatment of hyperkalaemia and PAH appears promising as phosphate binder to treat hyperphosphataemia among chronic renal failure and dialysis patients [66,67].

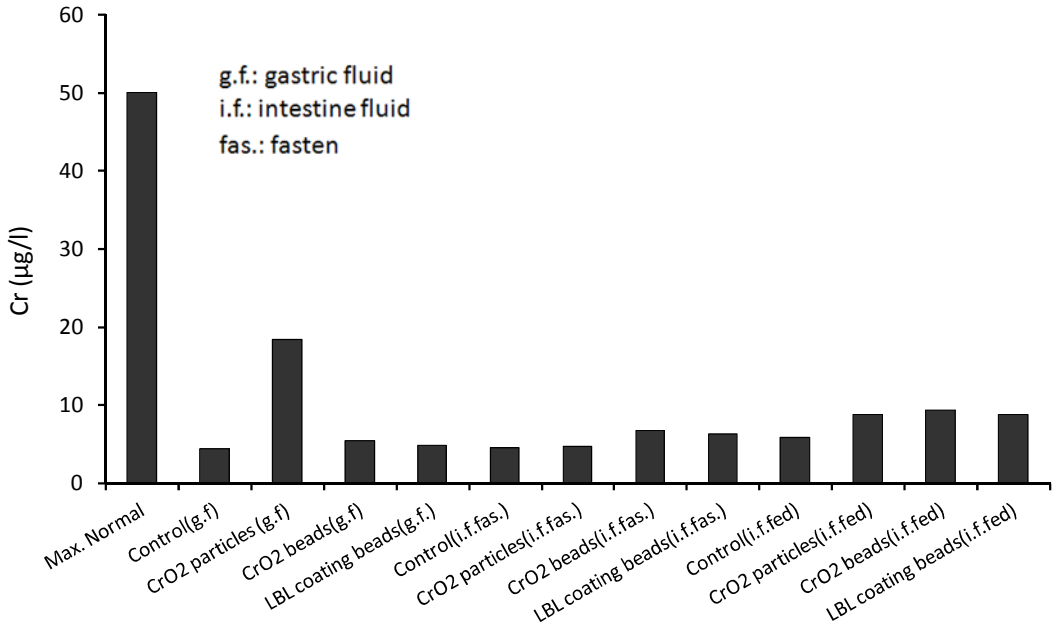

Figure 8. Release of Cr from encoded beads at different condition

Memobeads are loaded with a fluorophore. By fluorescence recovery after photobleaching [68] we found that >70% of the fluorophores is completely immobilized in the polystyrene matrix [69]. Such a large immobile fraction is indeed a prerequisite for being able to write a permanent code. Since only a small fraction of the fluorophores is (very slowly) mobile, we do not expect a significant leaking of fluorescent molecules out of the Memobeads into the environment. Indeed, we observed that the fluorescence of 200 ml gastric/intestinal fluid in which 50,000 Memobeads per ml were suspended for 5 h remained as low as the fluorescence of the blank sample (data not shown; the fluorescence of the supernatant was measured by the highly sensitive detector of a Fluorescence Correlation Spectroscopy instrument which can even measure single fluorophores) [70].The cytotoxicity of the Memobeads was also screened on A549 and Vero-1 cells. Therefore, as described in the materials and methods section, a monolayer of cells was cultured in a “Memobead extract” and the cytotoxicity of the medium was evaluated. As shown in chapter 3, the cells survived well indicating that Memobeads do not release compounds that are toxic to cells.

DISCUSSION

One can raise the question why Memobeads are better suited for the in-product labeling of tablets compared to other kinds of optically- or graphically-encoded microcarriers as reviewed by Braeckmans et al. [45]. As shown in Figure 8, in "colloidal encoding" of microcarriers two type of beads are used [71,72]: (a) large "support" beads (approximately 100 µm in size) and (b) smaller (fluorescent) silica "encoding" beads (0.2 – 5 µm in diameter). A fluorescent code is generated on each support bead through the physical attachment of 50 to 400 encoding beads, which contain specific combinations of fluorescent dyes. Although, numerous uniquely encoded beads can be generated in this way, colloidal-encoded microcarriers are very likely unsuitable for in-product tagging of tablets as there is a real risk that the small encoding beads will be absorbed by the gut.

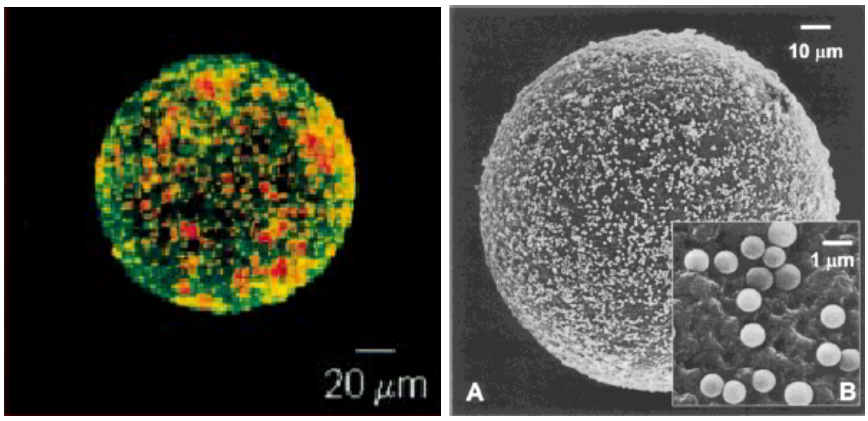

Figure 8. Colloidal encoding. *(Left)* Fluorescence microscope image of fluorescent encoding beads non-covalently attached to the surface of a large support bead. *(Right)* Transmission electron microscope image [72].

Microcarriers can be also optically encoded by incorporating distinct proportions of different fluorescent dyes. This "color encoding" [73,74] strategy is indeed successfully used in Xmap technology (for multiplexing purposes in diagnostics) in which 5 µm sized polystyrene beads are internally loaded with precise proportions of red and orange organic dyes or quantum dots [45]. However, compared to the digital information as written in Memobeads, the code in both colloidally encoded microcarriers and "multicoloured" microparticles is far more limited in capacity to store information on a specific drug. Encoding by spatial selective photobleaching, at the other hand provides for a virtually unlimited number of unique codes.

Decoding of Memobeads incorporated in tablets is in principal not very difficult. Clearly, dissolution of the tablets and subsequent centrifugation or filtration on a suitable membrane is sufficient to recover the Memobeads from the tablets. Subsequently, a simple fluorescence microscope (equipped with a pseudo-confocal module which allows imaging of the middle plane of the beads where the code is written; see Fig. 2) and a weak magnetic field at the microscope table would allow technicians to decode the beads. Memobeads are, however, also suited for the in-drug labeling of oral dosage forms other than tablets (like capsules, drug suspensions, drug solutions,….) and topical dosage forms (like creams, ointments,…) as long as they are compatible with the conditions during production. Clearly, Memobeads are not suited for the in-product labeling of injectables; 40 µm sized particles are too large and will block the microcapillaries in e.g. the lungs. Also, as they are large and not biodegradable, they would not be excreted by the kidneys and would thus accumulate in the body.

CONCLUSION

By spatial selective photobleaching (digitally) encoded micron-sized beads can be created. We have shown that Memobeads can be easily incorporated in tablets by compression of either Memobead-containing drug granules or a drug powder mixture containing Memobeads. Especially, the dot codes in the Memobeads did not deform during tabletting even when high compression forces were applied. Also, the permanent magnetic coating surrounding the Memobeads withstands the compression forces, a necessity for proper orientation of the beads at the time of readout.

Compared to other types of optically encoded microcarriers, Memobeads have a number of assets for in-tablet labeling. Compared to multicolored microparticles, encoding by spatial selective photobleaching has the advantage of being able to provide a virtually unlimited number of unique codes, an obvious advantage considering the huge number of pharmaceuticals which are at risk of being counterfeited. Compared to colloidal encoding, which makes use of nanometer sized beads for encoding; the relatively large Memobeads have the advantage that the risk of becoming absorbed after oral intake by the gut is minimal. Also, as argued above, ingestion of Memobeads is highly unlikely to be toxic for humans. The styrene (monomer) and styrene oligomers mass in 100 beads (assuming 100

beads are taken daily) is expected to be less than 0.05 µg which equals the quantity tolerated in 2.5 ml of drinking water. The total mass of chromium dioxide in 100 beads is less than 1 µg, corresponding to the amount tolerated in 20 ml of drinking water. Also, the fluorescent dye is not expected to be toxic as it is fully immobilized in the polystyrene matrix and will thus not be released from the Memobeads in the gastrointestinal fluids.

Counterfeiting of drugs is a real threat to public health in both developing as well as the industrialized countries. While probably all anti-counterfeiting technologies can be circumvented, the encoded particles described in this paper may help in combating counterfeit manufacturers who are becoming increasingly sophisticated in the use of high-tech systems. Decoding Memobeads is not too difficult. However, encoding the beads, at a sufficiently high speed (i.e. > 10 000 beads a second) requires an extremely high degree of skill, equipment and know-how which should discourage counterfeiters.

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REFERENCES

1. Wertheimer A.I., Santella T.M., Counterfeit drugs: defining the problem and finding solutions. Expert Opin Drug Saf **2005**, 4(4),619-22.

2. Burns W., WHO launches taskforce to fight counterfeit drugs. Bull. World Health Organ **2006**, 84, 689-90.

3. Department of Essential Drugs and Other Medicines. Guidelines for the Development of Measures to Combat Counterfeit Drugs. Geneva, Switzerland: World Health Organization, **1999**. WHO/EDM/QSM/99.1.

4. Sreedhar D., Subramanian G., Udupa N., Counterfeit drugs: problem of developing and developed countries. Current Science **2006**, 90, 1054-5.

5. Wertheimer A.I., Santella T.M., Chaney N.M., Counterfeit Pharmaceuticals – Update on Current Status and Future Projections. Business briefing pharmagenerics **2004**, 1-8

6. WHO. General information on counterfeit medications.
www.who.int/medicines/services/counterfeit/overview/en/index.html

7. Surendran A., World agencies try to stem flood of fake drugs. Nat Med **2004**, 10:111.

8. Cockburn R., Newton P.N., Agyarko E.K., Akunyili D., White N.J.,The global threat of counterfeit drugs: why industry and governments must communicate the dangers. PLoS Med **2005,** 2, 302-8.

9. Paradise P.R., Trademark counterfeiting, product piracy, and the billion dollars threat to the U.S. economy. **1999**, 175 (book)

10. WHO. Counterfeit medicines
http://www.who.int/mediacentre/factsheets/fs275/en/

11. Bessell T.L., Silagy C.A., Anderson J.N., Hiller J.E., Sansom L.N., Quality of global e pharmacies: can we safeguard consumers? Eur J Clin Pharmacol **2002**, 58(9), 567-72.

12. Montoya I.D, Jano E., Online pharmacies: safety and regulatory considerations. Int J Health Serv **2007**, 37(2), 279-89.

13. Newton P.N., Green M.D., Fernandez F.M., Day N.P., White N.J., Counterfeit anti-infective drugs. Lancet Infect Dis **2006,** 6, 602-13.

14. Aldhous P., Counterfeit pharmaceuticals: murder by medicine. Nature **2005,** 434,132-6.

15. Rudolf P.M., Bernstein I.B., Counterfeit drugs. N Engl J Med **2004**, 350, 1384-6.

16. Wondemagegnehu E., Pharmaceutical counterfeiting: A WHO perspective in pharmaceutical fraud and counterfeiting, SMI conference Documentation, London SMI Publishing **2003**

17. Combating counterfeit drugs: a concept paper for effective international collaboration, WHO, Health Technology and Pharmaceuticals, 27 January, 2006.

http://www.who.int/entity/medicines/services/counterfeit/CombatingCounterfeitDrugs_Conceptpaper.pdf

18. Ham M.T., Health risks of counterfeit pharmaceuticals. Drug Saf **2003**, 26(14), 991-7.

19. Harper J., Counterfeit medicines survey report, Council for Europe. Council of Europe **2006** (book).

20. WHO. Counterfeit Drugs: Guidelines for the Development of Measures to Combat Counterfeit Drugs, **1999**, Geneva: WHO. 1–60.

21. Anon. World Health Report 1999 Making a Difference, **1999**, Geneva: WHO. 49.

22. Quality Assurance Program HTP/EDM Revised Drug Strategy, **2000**.
http://www.who.int/medicines/library/qsm/who-edm-qsm-99-3/who-edm-qsm-99-3.pdf

23. WHO. The Declaration of Rome **2006**
http://www.who.int/entity/medicines/services/counterfeit/RomeDeclaration.pdf

24. WHO. World Health Organisation-Western Pacific Region Rapid Alert System.
http://218.111.249.28/ras/default.asp **2006**.

25. Jotcham R., Understanding and evaluating security technologies for pharmaceuticals in combating pharmaceutical fraud and counterfeiting, SMI Conference Documentation, London SMI Publishing **2003**.

26. Deisingh, A.K. Pharmaceutical counterfeiting. Analyst **2005**, 130**,** 271-9.

27. Lee R.E., Micro-technology for anti-counterfeiting. Microelectron Eng **2000**, 53, 513-6.

28. Colorcon announces new on-tablet laser inscription technology to protect patients and prevent counterfeiting **2007**, http://www.colorcon.com/best/index.htm

29. http://www.candurin.merck.de/servlet/PB/menu/1232980/index.html

30. Merck's Candurin pearlescents approved as food colorants, Focus on Pigments **2007**, (1), 7.

31. Anderson R.J., Security engineering. A Guide Building Dependable Distributable Systems, New York: Wiley, **2001** (book)

32. United States Food and Drug Administration (FDA). Combating counterfeit drugs **2004**. http://www.fda.gov/oc/initiatives/counterfeit/report02_04.html

33. Coyle W., Anti-Counterfeiting Drug Initiative: Fluorescent Materials **2003**, 1-4

34. Meylan R., Using manufacturing marks to identify counterfeits. Med Device Technol **2007**, 18(1), 48-50.

35. Meylan R., Protecting Pharmaceutical Products from Counterfeiting using Digital Imaging Technology and off-the-shelf Camera Phones. Industrial Pharmacy **2007**, 14, 10-3

36. Gabriele P.D., Emphasis on product ID: easing counterfeiting concerns with covert microtags: Adhesives Research, Inc., Medical design technology online feature articles, January-**2006**

37. Bird K., DNA markers in packaging as an anti counterfeit measure, Breaking news on packaging technologies & markets **2007**, http://www.adnas.com/signature.

38. DNA Technologies (Australia) Pty Ltd. http://www.dnatecaus.com/about.htm.

39. O'Connor M.C., Pfizer Using RFID to Fight Fake Viagra **2006**, http://www. Pfizer.com

40. Johnston R.G., An Anti-Counterfeiting Strategy Using Numeric Tokens, Int J Pharm Med **2005**, 19 (3), 163-71.

41. James J.S., FDA, companies test RFID tracking to prevent drug counterfeiting. AIDS Treat News **2005**, 417, 5-8.

42. SECUTAG Anti-Counterfeit, Anti-Pirary, Product Authentication and Identification System http://www.security-codes.com/anti-piracy.htm

43. ARmark™ Authentication Technologies - World's Smallest Piece of Flat Art Unveiled
Glen Rock Pa., **2007**, http://www.adhesivesresearch.com/content/view/404/102/

44. De Smedt S.C., Pauwels R.W.J., Demeester J.,Roelant C.H.S. Encoding of microcarriers WO Patent 00/63695, **2000**.

45. Braeckmans, K., De Smedt, S.C., Leblans, M., Pauwels, R., Demeester, J., Encoding microcarriers: present and future technologies. Nat Rev Drug Discov **2002**, 1, 447-56.

46. Braeckmans, K., De Smedt, S.C., Roelant, C., Leblans, M., Pauwels, R. & Demeester, J. Encoding microcarriers by spatial selective photobleaching. Nat Mater **2003,** 2, 169-73.

47. Derveaux S.,De Geest B.G., Roelant C., Braeckmans K., Demeester J., De Smedt S.C. Multifunctional layer-by-layer coating of digitally encoded microparticles. Langmuir **2007,** 23, 10272-79.

48. Jelinek A., Klocking H.P., In vitro toxicity of surfactants in U937 cells: Cell membrane integrity and mitochondrial function. Exp. Toxicol Pathol **1998**, 50**,** 472-6.

49. USP 24, Mateau P., Mikenus M. et al., J Dairy Sci **1997**, 80, 1031-7

50. Meseth U., Wohland T., Rigler R., Vogel H., Resolution of fluorescence correlation measurements, Biophys J. **1999**, 76, 1619-31.

51. Chen Y., Muller J.D., Berland K.M., Gratton E., Fluorescence fluctuation spectroscopy, Methods **1999**, 19, 234-52.

52. Sukhorukov G.B., Donath E., Lichtenfeld H., Knippel E., Knippel M., Budde A., Mohwald H., Layer-by-layer self assembly of polyelectrolytes on colloidal particles. Colloid Surface A. **1998**, 137,253-66.

53. Florence A.T., Attwood, D., Physicochemical principles of pharmacy (3rd edition ed) Macmillan press, London, **1998**

54. Gebert A., Rothkotter H.J., Pabst R., M cells in Peyer's patches of the intestine. Int Rev Cytol **1996**, 167,91-159.

55 Eldridge J.H., Meulbroek J.A., Staas J.K., Tice T.R., Gilley R.M., Adv Exp Med Biol **1989**, 251, 191-202.

56. Eldridge J.H., Hammond C.J., Meulbroek J.A., Staas J.K., Gilley R.M., Tice T.R., Controlled vaccine release in the gut-associated lymphoid tissues. I. Orally administered biodegradable microspheres target the peyer's patches. J. Control Release **1990**, 11, 205-14.

57. Tabata, Y., Inoue, Y., Ikada, Y., Size effect on systemic and mucosal immune responses induced by oral administration of biodegradable microspheres. Vaccine **1996**, 14, 1677-85.

58. McClean S., Prosser E., Meehan E., O'Malley D., Clarke N., Ramtoola Z., Brayden D., Binding and uptake of biodegradable poly –DL-lactide micrand nanoparticles in intestinal epithelia. European J. Pharm Sci-US **1998**, 6, 153-63

59. LeFevre M.E., Hancock D.C., Joel D.D., Intestinal barrier to large particulates in mice. J Toxicol Environ Health **1980**, 6, 691-04.

60. Jepson M.A., Simmons N.L, Savidge T.C, James P.S, Hirst B.H., Selective binding and transcytosis of latex microspheres by rabbit intestinal M cells. Cell and Tissue Research **1993**, 271, 399-405.

61. Andrianov A.K, Payne L.G., Polymeric carriers for all oral uptake of microparticulates. Adv Drug Deliv Rev **1998**, 34, 155-170.

62. Roe F.J., What does carcinogenicity mean and how should we test for it? Food Chem Toxicol **1993**, 31, 225-9.

63. Styrene fact sheet. US Environmental Protection Agency, **1994**.

64. Registry of toxic effects of chemical substances: chromium (II) chloride, chromium (II) dioxide. National Institute of Occupational Safety and Health, **1995**.

65. Guidelines for drinking-water quality. World Health Organization, **1993**.

66. Albaaj F., Hutchison A.J., Hyperphosphataemia in renal failure: Causes, consequences and current management. Drugs **2003**, 63, 577-96.

67. Reynolds J.E.F., Martindale: The extra pharmacopoeia. 30th edn, (Pharmaceutical Press, London, **1993**).

68. Braeckmans K., Peeters L., Sanders N.N., De Smedt S.C., Demeester J., Three-dimensional fluorescence recovery after photobleaching with the confocal scanning laser microscope. Biophys J **2003**, 85**,** 2240-52.

69. Braeckmans K., Photobleaching with the confocal laser scanning microscope for mobility measurements and the encoded of microbeads, PhD thesis **2004,** chapter 6

70. Haustein E., Schwille P., Single-molecule spectroscopic methods. Curr Opin Struct Biol **2004**, 14, 531-40.

71. Trau M., Battersby B. J., Novel colloidal materials for high throughput screening application in drug discovery and genomics. Adv Mater **2001**, 13, 975-9.

72. Battersby B.J., Bryant D., Meutermans W., Matthews D., Smythe M.L., Trau M., Toward larger chemical libraries: encoding with fluorescent colloids in combinatorial chemistry. J Am Chem Soc **2000**, 122, 2138-9.

73. Han M., Gao X.H., Su J.Z. & Nie S., Quantum –dot-tagged microbeads for multiplexed optical coding of biomolecules. Nature Biochnol **2001**, 19, 631-5.

74. Alivisatos A.P., Less is more in medicine- sophisticated forms of nanotechnology will find some of their first real- world application in biomedical research, disease diagnosis and possibility, therapy. Sci Am **2001**, 285, 66-73.

Summary and General Conclusions

Summary and General Conclusions

SUMMARY

The development of highly efficient and functional gene transfection techniques is important for both therapeutic objectives as well as a research tool in molecular biology. Nucleic acids can be delivered alone (naked nucleic acids) or can be packaged using viral or non viral vectors. Although viral vectors are currently the most efficient tool for gene transfer into cells, there are a number of problems associated with the use of these vectors. Therefore, there is increasing interest in non viral gene delivery which necessitates safe and efficient (non viral) vectors. In this framework, chapter 2 of this thesis evaluates two types of cationic hydroxyethylcellulose for gene delivery purposes.

Immobilizing DNA to a supporting surface prior to cell seeding, instead of adding the DNA to cells already cultured on a support (bolus addition) has been proposed as a mechanism to improve the transfection efficiency. This “reverse transfection” method is at the basics of the so named “reversely transfected cell microarray” which may become a powerful tool for high-throughput screening of gene function *in vitro*. In this framework, chapters 3 and 4 of this thesis evaluate the immobilization of nucleic acids and cells to photophysically encoded microcarriers.

In **Chapter 1** we overviewed different nucleic acid delivery systems. “Reverse transfection” and the use of this method for high throughput screening of gene function *in vitro* were reviewed. Additionally several strategies were discussed to immobilize nucleic acids on solid surfaces. We described the LbL technique and the use of this technique to immobilize nucleic acids on solid surfaces. Digitally encoded microparticles with LbL coating and their possible use as non-positional microarray in multiplexing assays were introduced as well.

In **Chapter 2** the cationic hydroxyethylcelluloses PQ-4 and PQ-10 were evaluated for gene delivery. PQ-4 and PQ-10 were previously characterized in detail and already have applications in cosmetics and drug delivery devices for topical applications. Size distribution and surface charge properties of the nucleic acid/ cationic hydroxyethylcellulose polyplexes were determined by DLS and zeta potential measurements.The complexation of pDNA to the cationic polymers and dissociation of the polyplexes by polyanions (dextran sulfate or poly-L-aspartic acid) were studied by gelelectrophoresis and the picogreen fluorescence assay. Transfection efficiencies using COS-7 cells were evaluated by using the SEAP assay. The cell viability towards the polyplexes was determined using EZ4U assay. Their DNA binding and transfection properties were compared with the branched and linear polyethylenimie (PEI) which are widely investigated for DNA delivery purposes. Gelelectrophoresis and fluorescence experiments indicated that PQ-4 and PQ-10 indeed can bind and condense pDNA. Compared to PEI, the interaction between pDNA and cationic HEC seems to be stronger. Although, the gelelectrophoresis data showed that PQ-10 binds all the pDNA, the fluorescent quenching assay indicates that PQ-10 does not condense pDNA as tightly as the other cationic polymers, still allowing picrogreen to intercalate to a certain extent in the pDNA.

We found that PQ-4 and PQ-10 based pDNA complexes show low toxicity as more than 90% of the cells remain alive. However, compared to PEI based polyplexes, transfection of cells by cationic hydroxyethylcellulose is significantly lower which was, highly likely, due to a too strong complexation with the nucleic acids (for PQ-10) and an non-efficient uptake of the complexes by the cells (for PQ-4).

In drug screening and molecular biology people are interested to evaluate the activity of compounds in different types of cells. On the other hand performing multi-parametric and multiplexed assays provides significant advantages to singlet assays such as reduction of technical operation, execution time and over all costs. For this purpose “cell microarrays” are under development. In a “positional cell array” cells are grown at specific locations on a chip: from the x,y-coördinates of the place on the chip a particular cell is growing one knows which cell type it is. In **Chapter 3** we proposed to develop a “non-positional cell array”, using photophysically encoded microcarriers (“Memobeads”); each of these encoded microcarriers can accommodate one cell type, the code in the carrier allows to identify the cell type growing at the surface of the carrier.

Codes were written inside fluorescent polystyrene beads by selective photobleaching. To make the polystyrene beads magnetic (necessary for encoding and decoding) and to provide them with a surface suitable for cell growth, the encoded polystyrene microspheres were coated by Layer-by-Layer technology with suitable polyelectrolytes. The cell loaded microspheres were decoded using a confocal laser scanning microscope.

The encoded beads did not show significant toxicity and several cell lines such as CHO, Vero, Hela and COS-7 could be grown on the beads. Importantly, the cells covering the beads did not hamper the decoding of the beads: they still permitted the magnetic orientation of the memobeads at the time of read-out. Fluorescence present in the cells (due to the expression of fluorescent proteins) did not hamper the decoding of the encoded beads. Furthermore, in **Chapter 4** we have shown that the polyelectrolytes at the surface of the encoded microcarriers are suitable for immobilizing DNA, siRNA and adenoviral particles; subsequently, on top of the immobilized nucleic acids/viral particles cells can be grown. We further showed that adenoviral vectors immobilized on encoded microcarriers maintain their ability to infect cells (reversed transfection) and the cells growing on the polyelectrolyte layer can become transduced with adenoviral particles hosted by the polyelectrolyte layer. In addition, the adenovirus-coated microparticles have demonstrated to be stable and, importantly, the adenoviral particles only transfect those cells which are at the surface of the carrier on which the viral particles are immobilized.

Another innovative application for the photophysically encoded microcarriers is described in **Chapter 5** where we recommend to use them for the “in-product labelling” of tablets to avoid counterfeiting. Counterfeiting of drugs is a real threat to public health in both developing and industrialized countries as patients may become treated with drugs of inferior quality. “In-drug labelling”, i.e. the labelling of the drug instead of the packaging, would be a major step forward to combat counterfeiters.

The Memobeads were incorporated in tablets either by compression of Memobead containing drug granules or by direct compression of a drug powder/Memobead mixture. We showed that the codes in the Memobeads in tablets produced by granulation did not deform during tabletting. Even when the granules were compressed at higher compression forces the Memobeads remain spherical and, especially, the code did not deform and remained perfectly readable. In tablets produced by direct compression the code in the

beads seemed to become destroyed when higher compression forces were applied. We also found evidence that, after oral intake, the encoded microparticles are highly unlikely toxic to humans.

GENERAL CONCLUSIONS

The first goal of this thesis was to investigate the potential of two cationic polysaccharides (PQ-4 and PQ-10) for DNA delivery. We have shown that, compared to PEI based polyplexes, they were less efficient in transfecting cells. However, as they had very low toxicity, further tailoring of the nature and extent of cationic side chains on cationic hydroxyethylcellulose may be a promising avenue to further enhance their DNA delivery properties.

As a second goal we investigated the applications of digitally encoded microcarriers for cell based assays. We succeeded to show that encoded microcarriers were suitable to grow cells on. Nor the coating at the surface of the beads (which facilitates the growth of the cells), neither the cells themselves hampered the decoding of the beads, even when the cells covering the microcarriers exhibited green or red fluorescence due to the expression of GFP and RFP respectively. We were able (a) to immobilize DNA, siRNA or adenoviral particles on the surfaces of the encoded microcarriers by the use of polyelectrolytes and, subsequently, (b) to grow cells on top of the nucleic acids/adenoviral particles. The DNA and siRNA immobilized on the surface of the microcarrier were not able to transfect cells. However, we showed that the cells growing on the polyelectrolyte layer could indeed become transduced with adenoviral particles hosted by the polyelectrolyte layer. In conclusion, a proof of principal to use photophysically encoded microcarriers as transfected cell microarray has been shown.

As a third goal we investigated the use of digitally endoced microcarriers as tool to combat counterfeiting of tablets. We showed that the codes in the Memobeads in tablets produced by granulation did not deform during tabletting and that the code in the beads remained readable. We also found evidence that, after oral intake, the encoded microparticles are highly unlikely toxic to humans.

Samenvatting en Algemene Besluiten

Samenvatting en Algemene Besluiten

SAMENVATTING

De ontwikkeling van efficiënte en functionele gentransfectie technieken is enerzijds belangrijk vanuit therapeutisch standpunt en anderzijds als werkmiddel in moleculair biologisch onderzoek. Het toedienen van nucleïnezuren kan ofwel via naakte nucleïnezuren gebeuren ofwel kunnen deze ingepakt worden door gebruik te maken van virale of niet-virale vectoren. Hoewel virale vectoren de meest efficiënte dragers zijn die momenteel beschikbaar zijn voor de afgifte van DNA in cellen, brengt het gebruik van deze vectoren verscheidene problemen met zich mee. Er is dan ook een stijgende interesse in de ontwikkeling van veilige en efficiënte (niet-virale) gen afgifte systemen. In deze context werden in het eerste deel (hoofdstuk 2) van deze scriptie twee types van kationische hydroxyethylcellulose polymeren geëvalueerd als gen afgifte systemen.

Het immobiliseren van DNA op een drageroppervlak vóór het uitzaaien van de cellen in plaats van het DNA toe te voegen aan cellen die vooraf in cultuur gebracht werden op een drager (bolus toediening) werd voorgesteld als een mechanisme om de transfectie efficiëntie te verhogen. Deze “omgekeerde transfectie” methode vormt de basis voor de zogenoemde “omgekeerd getransfecteerde cel microarray” dat een krachtige middel is voor het op grote schaal (high throughput) screenen van genfuncties *in vitro*. In deze context werd in het tweede deel (hoofdstuk 3 en 4) van deze scriptie het immobiliseren van nucleïnezuren en cellen op fotofysisch gecodeerde microcarriers geëvalueerd.

In **Hoofdstuk 1** werd een overzicht gegeven van de verschillende nucleïnezuur afgifte systemen. “Omgekeerde transfectie” en het gebruik van deze *in vitro* methode voor high throughput screening van genfuncties werden meer in detail besproken. Daarnaast werd

een overzicht gegeven van verscheidene strategieën om nucleïnezuren te immobiliseren op vaste oppervlakken, met nadruk op de in deze scriptie gebruikte laag-na-laag of LbL-techniek. Ook digitaal gecodeerde micropartikels met LbL mantel en hun gebruik als niet-positionele microarrays in mulitplex assays werden geïntroduceerd.

In **Hoofdstuk 2** werden de hydroxyethylcellulose (HEC) polymeren PQ-4 en PQ-10 geëvalueerd voor gen afgifte. Beide kationische polymeren werden vroeger reeds in detail gekarakteriseerd en kennen al verscheidene toepassingen in cosmetica en als geneesmiddel afgifte systeem voor topicale toediening. Van de gevormde DNA/HEC polyplexen werden de grootte distributie en oppervlaktelading bepaald met behulp van DLS en zetapotentiaal metingen. De complexatie van pDNA met de kationische polymeren en de dissociatie ervan in aanwezigheid van polyanionen, zoals dextraansulfaat en poly-L-aspartaat, werden bestudeerd met behulp van gelelektroforese en een picogreen fluorescentie test. Vervolgens werd de transfectie efficiëntie en de cel viabiliteit geëvalueerd op COS-7 cellen via respectievelijk de SEAP en EZ4U assay. Als referentie werd gebruik gemaakt van vertakt en lineair polyethyleneimine (PEI), beide reeds uitgebreid bestudeerd en beschreven als afgiftesysteem voor DNA. Gelelektroforese en fluorescentie experimenten gaven aan dat PQ-4 en PQ-10 zoals verwacht in staat waren om te binden met DNA en het DNA bijgevolg te condenseren. In vergelijking met PEI bleek de interactie tussen pDNA en de kationische HEC polymeren sterker. Hoewel de gelelektroforese data aantoonden dat PQ-10 alle pDNA complexeert, blijkt uit de fluorescentie quenching test dat PQ-10 het pDNA minder sterk condenseert dan de andere kationische polymeren, aangezien picogreen tot op zekere hoogte nog steeds in staat is om in het pDNA te intercaleren.

We stelden vast dat de PQ-4 en PQ-10 gebaseerde pDNA complexen een lage toxiciteit vertoonden aangezien meer 90% van de cellen overleven. Ondanks deze bemoedigende resultaten bleken beide polymeren een significant lagere transfectie te vertonen in vergelijking met de PEI-gebaseerde polyplexen. Dit is hoogstwaarschijnlijk te wijten aan ofwel een te sterke complexatie met de nucleïnezuren, in geval van PQ-10, ofwel een inefficiënte opname van de complexen, in geval van PQ-4 polyplexen.

In geneesmiddelen screening en moleculaire biologie is men geïnteresseerd om de activiteit van bepaalde componenten in verschillende types van cellen te evalueren. Daarnaast leveren het uitvoeren van multi-parametrische en multiplexe assays significante voordelen t.o.v. singlet assays, waaronder een vermindering van het aantal technische

handelingen, van uitvoeringstijd en bovenal van kostprijs. Voor dit doeleinde zijn de zogenaamde “cel microarrays” in ontwikkeling. In een “positionele cel array” worden cellen gegroeid op specifieke plaatsten op een chip: aan de hand van de x,y coördinaten van de plaats op de chip waar de cellen groeien kan men afleiden over welk celtype het gaat. In **Hoofdstuk 3** werd voorgesteld om een “niet-positionele cel array” te ontwikkelen door gebruik te maken van fotofysisch gecodeerde microcarriers of “Memobeads”. Hierbij kan elke van deze gecodeerde microcarriers één celtype huisvesten, waardoor men vervolgens op basis van de aanwezige code, het celtype kan identificeren dat op het oppervlak van de Memobead groeit. Codes werden in de fluorescente polystyreen beads geschreven d.m.v. selectieve fotobleking. Om de polystyreen beads magnetisch te maken, wat noodzakelijk is voor het positioneren van de beads bij het coderen en aflezen, en om hen te voorzien van een geschikt oppervlak voor celgroei werden de gecodeerde polystyreen microsferen gecoat d.m.v. de LbL-technologie met de geschikte polyelektrolieten. Vervolgens werden de met cellen beladen microsferen gedecodeerd m.b.v. een confocale laser scanning miscoscoop. De gecodeerde beads vertoonden geen significante toxiciteit en verscheidene cellijnen, waaronder de CHO, Vero, HeLa en COS-7 cellijnen, konden gegroeid worden op het beadoppervlak. Een belangrijke vaststelling was dat deze cellen de decodering van de beads niet verstoorden aangezien de magnetische oriëntatie tijdens het aflezen nog steeds mogelijk was. Ook de aanwezigheid van fluorescentie in de cellen, veroorzaakt door de expressie van fluorescente eiwitten, verhinderde de decodering van de beads niet.

In **Hoofdstuk 4** werd bovendien aangetoond dat de polyelektrolietlaag op het oppervlak van de beads geschikt is om zowel DNA, siRNA als adenovirus partikels te immobiliseren. Vervolgens kunnen bovenop deze geïmmobiliseerde nucleïnezuren of virale partikels cellen gegroeid worden. Verder toonden we aan dat de geïmmobiliseerde adenovirale vectoren op de gecodeerde microcarriers hun infectie eigenschappen behouden (“omgekeerde transfectie”) aangezien de cellen die groeien op de polyelektrolietlaag getransduceerd worden door de adenovirussen aanwezig in de elektrolietlaag. Daarnaast werd aangetoond dat de adenovirus gecoate micropartikels stabiel zijn en dat enkel de cellen die geïmmobiliseerd zijn op het oppervlak van de met virus gecoate bead getransduceerd werden.

Een andere innovatieve toepassing van bovenstaande fotofysisch gecodeerde microcarriers werd beschreven in **Hoofdstuk 5** waar we voorstellen om deze beads te

gebruiken voor “in-produkt labelling” van geneesmiddeltabletten om namaak te voorkomen. Namaak van geneesmiddelen vormt een reële bedreiging van de publieke gezondheid in zowel ontwikkelingslanden als industriële landen aangezien patiënten door deze namaak risico lopen om behandeld te worden met geneesmiddelen van lagere kwaliteit. “In-drug labelling”, dit is het labellen van een geneesmiddel zelf in plaats van de verpakking, zou een grote stap voorwaarts zijn in de bestrijding van geneesmiddelen namaak. Hiervoor werden de Memobeads ingebouwd in de tabletten ofwel via compressie van de Memobead bevattende geneesmiddelgranules ofwel via directe compressie van een geneesmiddelpoeder/Memobead mengsel. We konden aantonen dat de codes in de Memobeads niet vervormd werden in de tabletten die bekomen waren via granulatie. Zelfs wanneer de granules met hogere druk behandeld werden behielden de Memobeads hun sferische vorm en werd de code niet vervormd of onleesbaar. Bij de tabletten gemaakt via directe compressie bleek de code vernietigd te worden wanneer hogere drukkrachten gebruikt werden. Daarnaast konden we ook aantonen dat de gecodeerde micropartikels na orale inname hoogstwaarschijnlijk niet toxisch zijn voor mensen.

ALGEMENE BESLUITEN

De eerste doelstelling van deze scriptie was nagaan of twee kationische polysaccharides (PQ-4 en PQ-10) potentieel hadden als DNA afgifte systeem. We konden aantonen dat beide polymeren in vergelijking met PEI-gebaseerde polyplexen minder efficiënt waren in het transfecteren van cellen. Ze vertoonden echter een lage toxiciteit, waardoor ze mogelijks wel geschikt zouden zijn voor gentherapie toepassingen na het verfijnen van de eigenschappen en lengte van hun kationische zijketens.

Een tweede doelstelling was het gebruik van digitaal gecodeerde microcarriers voor cel gebaseerde assays. We slaagden erin om aan te tonen dat de gecodeerde microcarriers geschikt waren om cellen op te groeien. Noch de coating op het oppervlak van de beads (wat de groei van de cellen vergemakkelijkt), noch de cellen zelf verhinderden het aflezen van de code, zelfs niet wanneer de cellen groen of rood fluorescent waren door de expressie van respectievelijk GFP of RFP. We waren bovendien in staat (a) om DNA, siRNA en adenovirale partikels op het oppervlak van de gecodeerde microcarriers te immobiliseren via het gebruik

van polyelektrolieten en (b) om cellen te groeien bovenop de geïmmobiliseerde nucleïnezuren of adenovirale partikels. Het DNA of siRNA dat geïmmobiliseerd werd op het oppervlak van de microcarriers bleek niet in staat om cellen te transfecteren. We konden daarentegen wel aantonen dat cellen die gegroeid werden op een polyelektrolietlaag die adenovirale partikels bevatte getransduceerd konden worden. Hiermee werd bewezen dat de fotofysisch gecodeerde microcarriers als “omgekeerd getransfecteerde cel microarray” gebruikt kunnen worden.

Als derde doelstelling onderzochten we tenslotte het gebruik van digitaal gecodeerde microcarriers als middel om de namaak van geneesmiddelen te verhinderen. We konden aantonen dat de codes van de Memobeads in tabletten geproduceerd via granulatie niet vervormd werden en dat de code bovendien leesbaar bleef. Daarnaast vonden we ook bewijzen dat de gecodeerde micropartikels hoogstwaarschijnlijk niet toxisch zijn voor mensen na orale inname.

چکیده و نتیجه گیری کلی

چکیده
و نتیجه گیری کلی

چکیده

توسعه و تکامل انتقال ژنی با کارآیی بالا از اهداف مهم در درمان و همچنین ابزار تحقیقاتی با ارزشی در بیولوژی مولکولی می باشد.

اسید های نوکلئیک می توانند بصورت تنها(اسید های نوکلئیک برهنه) و یا به صورت بسته بندی شده در یک حامل (ویروسی یا غیر ویروسی) انتقال داده شوند.

گرچه در حال حاضر ناقل های ویروسی مؤثرترین وسیله در انتقال ژن به داخل سلول هستند، مشکلات و محدودیتهایی به دنبال استفاده از این حامل ها وجود دارد.

به همین علت توجه روز افزونی بسمت حاملان ژنی غیر ویروسی برای انتقال بی خطر و مؤثر جلب شده است.

در همین راستا فصل دوم این پایان نامه به ارزشیابی دو نوع حامل (کایتونیک هیدروکسی اتیل سلولز) برای انتقال ژنی پرداخته است.

به تازگی ثابت کردن DNA بر روی سطوح قبل از کشت سلولی، به جای اضافه کردن آن به سلولهایی که قبلا" بر روی سطح کشت شده اند به عنوان مکانیسمی برای بهبود نتیجه انتقال ژنی پیشنهاد شده، این روش پایه تکنیکی به نام Reverse transfected microarray می باشد که می تواند ابزاری پر قدرت برای غربالگریهای چند گانه در عملکرد ژنها در مطالعات invitro باشد . به دنبال این بحث در فصل سوم و چهارم این پایان نامه به بررسی نحوه ی ثابت کردن اسید های نوکلئیک و سلولها بر روی میکرو حامل های کددار پرداخته ایم.

نمایی از مطالب توضیح داده شده در فصول مختلف به شرح ذیل است:

فصل یک نگاهی دارد به روشهای مختلف انتقال اسید های نوکلئیک، همچنین انتقال معکوس (Reverse transfection)و استفاده از این روش به عنوان روشی برای سنجش عملکرد ژنها در مطالعات invitro بعلاوه در مورد روشهای مختلفی که به واسطه آنها می توان اسید های نوکلئیک را بر روی سطوح ثابت کرد بحث شده.

روش LbL و استفاده از این روش برای ثابت کردن اسید های نوکلئیک بر روی سطوح توضیح داده شده. در این فصل همچنین مایکرو ذرات کد دار که باروش LbL پوشش دار شده اند و امکان استفاده از آنها به عنوان Non-positional microarray برای سنجش های چند تایی معرفی شده اند.

در فصل ۲ کایتونیک هیدروکسی اتیل سلولز ها (PQ4 و PQ10) برای انتقال ژن مورد بررسی قرار گرفته اند. خصوصیات این دو پلیمر که در صنایع دارویی و آرایشی کاربرد دارند، قبلا" مورد بررسی قرار گرفته . ما در اینجا اندازه ذرات و شارژ پلی پکلسهای حاصل از ترکیب آنها با اسید های نوکلئیک را با کمک دستگاه DLS و Zeta potential و تشکیل کمپلکس بین pDNA و کایتونیک پلیمرها و تفکیک پلی پلکسها با استفاده از پلی آینونها (دکستران سولفات و پلی اسپارتیک اسید) بوسیله ژل الکتروفورز و سنجش میزان فلور پیکوگرین مورد ارزیابی قرار گرفت.

کار آیی انتقال ژنی این حامل ها در سلولهای COS-7 با استفاده از سنجش SEAP انجام شد. سمیت پلی پلکسها با استفاده از تست EZ4U اندازه گیری شد.

انتقال ژنی بوسیله این حامل ها و همچنین اتصال آنها با DNA،با حامل شناخته شده پلی اتیلن (Linear-Branched) که بطور گسترده ای به این منظور مورد مطالعه قرار گرفته ، مقایسه گردید. ژل الکتروفورز و آزمایش فلورومتری نشان می دهد که PQ4 و PQ10 توانایی اتصال و فشرده کردن pDNA را بعنوان یک حامل دارا می باشند. این اتصال در مقایسه با PEI به نظر محکم تر می رسد. گرچه نتیجه ژل الکتروفورز نشان داد کهPQ10 می تواند کاملا" با تمامی DNAباند شود ولی نتایج فلوریمتری نشان می دهد که PQ10 توانایی فشرده کردن pDNA را به اندازه کایتونیک پلیمر های دیگر ندارد. و اجازه می دهد تا پیکوگرین(Picogreen) تا حدی بین بازهای pDNA نفوذ کند.

ما دریافتیم که کمپلکسهای حاصل از PQ4 و PQ10 با DNA سمیت بسیار کمی دارند و بیش از ۹۰٪ از سلولها زنده می مانند اما در مقایسه با پلی پلکسهای PEI ، انتقال ژنی کایتونیک سلولز ها به طور قابل ملاحظه ای کمتر می باشدکه به احتمال زیاد ناشی از تشکیل کمپلکس بسیار قوی با نوکلئیک اسید در مورد PQ10 و عدم نفوذ کمپلکس به سلول در مورد PQ4 می باشد.

در پژو هش های دارویی و مولکولار، پژوهشگران علاقمند به ارزیابی اثر ترکیبات مختلف بر انواع گوناگون سلولی می باشند. از طرفی انجام سنجش های چند گانه (Multiplexed assays) در مقایسه با سنجش های تکی (Singlet assays) باعث کاهش عملیات تکنیکی زمان و هزینه ها می شودو به همین منظور Cell microarrays توسعه یافتند.

در Positional cell array که سلولها بر روی Chip رشد می کنند ، هر نوع سلول از محل قرار گرفتن آن بر روی سطح Chip مشخص می شود. در فصل ۳ ما یک Non-positional cell array را با استفاده از مایکرو حامل های کد دار پیشنهاد کردیم.

هر یک از این ذرات کد دار می توانند جایگاه یک نوع سلول باشند و هر کد اجازه شناسایی سلولهای رشد کرده برروی سطح حامل را می دهد.

کد ها در داخل مایکرو حاملهای پلاستیکی فلورسنت با استفاده از تکنیک Selective photobleaching نوشته شده .

برای مغناطیسی کردن حامل ها (برای خواندن کد ها لازم است) و فراهم کردن سطحی مناسب برای رشد سلولی این میکرو ذرات به روش LbL و با پلی الکترولیتهای مناسب پوشش داده شده اند.

سلولهای روی هر مایکرو ذره از طریق خواندن کد آن ذره بوسیله میکروسکوپ confocal شناسایی می شود.

مایکرو حاملهای کد دار سمیت قابل ملاحظه ای نشان نداده و انواع سلولها مانند Vero، CHO، Hela و COS-7 می توانند روی آنها رشد کنند.

لازم به ذکر است که سلولهای پوشاننده مایکرو حامل ها مزاحمتی برای خواندن کدها نمی باشند و چرخش آنها در میدان مغناطیسی در زمان خواندن کد هنوز میسر می باشد.

همچنین فلورسنت موجود در سلولها (ناشی از تظاهر پروتیینهای فلورسنت) مزاحمتی برای خواندن کد نمی باشد .

در فصل ۴ ما نشان داده ایم که وجود پلی الکترولیت ها در سطح حامل های کد دار برای ثابت کردن DNA، و siRNA و ذرات آدنو ویروس مناسب است و بدنبال آن سلولها می توانند بر روی اسیدهای نوکلئیک رشد کنند.

ما نشان دادیم که آدنو ویروس های ثابت شده بر روی مایکرو حامل های کد دار قدرت خود را در آلوده کردن سلولها (انتقال معکوس) حفظ کرده و می توانند در سلول های رشد کرده بر روی پلی الکترولیتهاانتقال ژنی صورت دهند.

بعلاوه لایه ذرات آدنو ویروس نشان دادند که لایه ایی با ثبات بوده و تنها در سلول هایی که در سطح حامل ها هستند انتقال ژنی صورت می گیرد.

یک کاربرد جدید دیگر برای مایکرو حامل های کد دار در فصل ۵ توضیح داده شده که از آنها بعنوان بر چسب های داخل محصول استفاده شود تا بتوانیم از تقلبها پیشگیری کنیم.

تقلبات دارویی یک تهدید جدی برای سلامت عمومی بوده و بیماران چه در کشور های پیشرفته و چه در کشور های در حال توسعه می توانند مورد درمان با داروهای نامرغوب قرار گیرند.

در این روش برچسب دارویی به جای قرار گرفتن روی بسته بندی دارو داخل دارو قرار می گیردکه می تواند قدم بزرگی در راه مبارزه با تقلبها باشد.

Memobead ها در داخل قرص هایی که از طریق گرانولاسیون یا فشار مستقیم ساخته شده بودند قرار گرفتند.

مانشان دادیم کد های Memobead ها، در قرص های تولید شده بوسیله گرانولاسیون شکل خود را در طول پروسه ساخت قرص از دست ندادند.

حتی زمانی که گرانولها در فشار بالا فشرده شدند Memobead ها بصورت کروی باقی می مانند و کدها تغییر شکل ندادند. در قرص های تولید شده بوسیله فشار مستقیم ذرات کد دار در فشار بالا تخریب می شوند. ما همچنین به این نتیجه رسیدیم که مصرف خوراکی این مایکرو ذرات کد دار برای انسان سمی نمی باشد.

نتیجه گیری کلی:

هدف اولیه این پایان نامه تحقیق در مورد توانایی انتقال DNA بوسیله دو کایتونیک پلی ساکارید به نام های PQ4 و PQ10 بود.

ما نشان دادیم که در مقایسه با پلی پلکسهای با مبنای PEI این پلی پلکسهای جدید اثر کمی در انتقال ژنی دارند.

ولی از آنجا که آنها سمیت خیلی کمی دارند تغییراتی در ساختمان آن می تواند نویدی برای بهبود توانایی انتقال DNA در آنها باشد. بعنوان دومین هدف ما در مورد استفاده از مایکرو حامل های کد دار در سنجش های سلولی تحقیق کردیم .

ما موفق شدیم نشان دهیم که مایکرو حامل های کددار برای رشد سلولها بر روی آنها مناسب هستند.

نه پوشش سطح مایکرو حامل ها (که تسهیل کننده رشد سلولها هستند) و نه خود سلولها نمی توانند مانعی برسر راه خواندن کد های روی حامل ها باشند.

حتی زمانیکه سلولهای پوشاننده مایکرو حامل ها، فلورسنت سبز و یا قرمز ناشی از تظاهرات GFP و RFP را از خود نشان می دادند ما توانستیم کد ها را بخوانیم.

ما توانستیم الف) DNA و siRNA و ذرات آدنو ویروس ها را بر روی سطوح مایکرو حامل های کد دار ثابت کنیم و بدنبال آن ب) سلولها را روی نوکلئیک اسید ها و ذرات آدنو ویروس رشد دهیم .

siRNAوDNA ثابت شده بر روی سطح مایکرو حامل ها قادر به انتقال ژنی نبودند ولی ما نشان دادیم که در سلولهای رشد کرده بر روی ذرات آدنو ویروس ها انتقال ژنی صورت گرفته.

به طور کلی ما اصول اولیه استفاده از میکرو حاملهای کد دار را برای Transfected cell microarray نشان دادیم.

بعنوان سومین هدف در این پایان نامه ما در مورد استفاده از ماکرو حاملهای کد دار برای مبارزه با تقلبات دارویی تحقیق کردیم.

ما نشان دادیم که کدهای موجود در Memobead، در قرص های تولید شده بوسیله گرانولاسیون در طول مسیر ساخت قرص تغییر شکل نداده و قابل خواندن می باشد. ما همچنین شواهد یافتیم که مصرف خوراکی این مایکرو حاملها برای انسان به احتمال زیاد بی خطر می باشد.

Curriculum Vitae

Curriculum Vitae

Name	Fayazpour
Surname	Farzaneh
Nationality	Iranian
Place and date of birth	Tehran 13.08.1967
Marital status	married, daughter (Niloufar, 21.08.1997)

April, 1994	Islamic Azad University of Tehran - Master in pharmaceutical science
September, 2003	Ghent University - Master in pharmaceutical science with great distinction

Master thesis:

"Evaluation of an amylolytic enzyme produced by a microorganism"

Promoter: Prof. Dr. R. Yazdanparast, Institute of Biochemistry and Biophysics, University of Tehran, Iran

"Stability control of fungal enzymes in solution& Degradation and transfection properties of plasmid DNA"

Promotors: Prof. Dr. J. Demeester and Prof. Dr. S. C. De Smedt, Faculty of Pharmaceutical Sciences, University of Gent, Belgium

PEER REVIEWED ABSTRACT

1. **Fayazpour F**., Lucas B., Huyghebaert N., Braeckmans K., Derveaux S., Vandenbroucke R., Remon J.P Demeester J., Vervaet C., De Smedt SC., Opportunities for digitally encoded microcarriers in pharmacy and cell-based assays. **Eur J Pharm Sci, 2008**, 34 (1),29-29 (IF_{2007}: 3.127)

PEER REVIEWED PUBLICATIONS

1. **Fayazpour F.**, Lucas B., Alvarez-Lorenzo C., Sanders NN., Demeester J., De Smedt SC., Physicochemical and transfection properties of cationic hydroxyethylcellulose/DNA nanoparticles **Biomacromolecules, 2006**, 10, 2856-62. (IF_{2007}: 4.169)

2. Remaut K., Sanders N.N., **Fayazpour F.,** Demeester J., De Smedt SC., Influence of plasmid DNA topology on the transfection properties of DOTAP/DOPE lipoplexes. **J Control Release 2006**, 115(3):335-43. (IF_{2007}: 4.756)

3. **Fayazpour F**., Lucas B., Huyghebaert N., Braeckmans N., Derveaux S., Stubbe B.G., Remon J.P., Demeester J., Vervaet C. and De Smedt S.C., Turning to digitally encoded drug tablets to combat counterfeiting, **Advance Materials**, **2007**, 19, 3854. (IF_{2007}: 8.191)

4. **Fayazpour F**., Lucas B., Vandenbroucke R.E., Derveaux S., Tavernier J., Lievens S., Demeester J., De Smedt S.C., Evaluation of digitally encoded layer-by-layer coated microparticles as cell carriers **Advanced Functional materials, 2008,** 18(18), 2716-2723. (IF_{2007}: 7.496)

5. Derveaux S., **Fayazpour F.**, Adibkia K., Demeester J., De Smedt S.C., Faster and more sensitive bead based multiplexing by tyramide signal amplification, **Submitted**.

CONFERENCES WITHOUT PRESENTATION

1. The 5th International Conference and Workshop on Cell Culture and In-Vitro models for drug Absorption and Delivery, February 25 – March 5 (2004), Saarbrucken - Germany.

2. 8th European Symposium on Controlled Drug Delivery, 7-9 April (2004), Noordwijk aan Zee – The Netherlands.

3. Autumn Meeting of the Belgian-Dutch Biopharmaceutical Society, November 28th, 2003, Groningen University, The Netherlands

4. Autumn Meeting of the Belgian-Dutch Biopharmaceutical Society, December 2nd, 2005, Utrecht University, The Netherlands

CONFERENCES WITH PRESENTATION (presenting author)

1. Spring Meeting of the Belgian-Dutch Biopharmaceutical Society, 20 May 2005 Gent Belgium (poster)
Cells grown on encoded microcarriers.
Fayazpour, F., Derveaux S., Lucas, B., De Smedt, S., Demeester, J.

2. Nanotech Montreux 2005 November 15-17, 2005 – Montreux – Switzerland (oral presentation)
Digitally readable, barcoded microspheres: their use in medical diagnostics, genomics, drug discovery, cellular assays and sorting of biomolecules.
De Smedt S.C., Derveaux S., Fayazpour F., Lucas B., De Geest B.G., Braeckmans K., Demeester J., Leblans M. and Roelant C.

3. Spring Meeting of the Belgian-Dutch Biopharmaceutical Society, 22 May 2006, Beerse, Belgium (poster), Physicochemical and transfection properties of cationic hydroxyethylcellulose/DNA nanoparticles.
Fayazpour F., Lucas B., Alvarez-Lorenzo C., Sanders NN., Demeester J., De Smedt SC

4. 9th Annual Meeting of the American Society of Gene Therapy, May 31-June 4, 2006 - Baltimore, MD (poster)
Physicochemical and transfection properties of cationic hydroxyethylcellulose/DNA nanoparticles.
Fayazpour F., Lucas B., Alvarez-Lorenzo C., Sanders NN., Demeester J., De Smedt SC.

5. Autumn Meeting of the Belgian-Dutch Biopharmaceutical Society November 27th, 2006, Leiden University, The Nederland (poster)
Turning to digitally encoded drug tablets to combat counterfeiting.
Fayazpour F., Lucas B., Huyghebaert N., Braeckmans N., Derveaux, Remon J.P., Demeester J., Vervaet C. and De Smedt S.C.

6. 3rd Pharmaceutical science world congress, Pre-Satellite meeting, April 20-21, 2007 Amsterdam, The Netherlands (oral presentation)

Turning to digitally encoded drug tablets to combat counterfeiting.

Fayazpour F., Lucas B., Huyghebaert N., Braeckmans N., Derveaux S., Stubbe B.G., Remon J.P., Demeester J., Vervaet C. and De Smedt S.C.

7. 11th annual meeting of the American Society of Gene Therapy, May 28-June 1, 2008, Boston (poster)

Evaluation of digitally encoded layer-by-layer coated microparticles as cell carriers.

Fayazpour F., Bart Lucas, Roosmarijn E. Vandenbroucke, Stefaan Derveaux, Joseph Demeester, Stefaan C. De Smedt

8. 19th Helsinki Drug Research 2008, 9-11 June (oral presentation)

Opportunities for digitally encoded microcarriers in pharmacy and cell based assays.

Fayazpour F., Bart Lucas, Roosmarijn E. Vandenbroucke, Stefaan Derveaux, Joseph Demeester, Stefaan C. De Smedt

RESEARCH VISITS

1. January 2005, Department of Bioengineering (GEBI), Catholic University of Louvain (UCL), Pro. Dr. Schneider “Growing cells on microcarriers”

2. July 2007, INSERM/ULP, medicine faculty, Strasbourg, France Dr. Nadia Jessel, “Reverse transfection from polyelectrolyte multilayers”.