

Research Article

Immobilized hematopoietic growth factors onto magnetic particles offer a scalable strategy for cell therapy manufacturing in suspension cultures

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Hematopoietic therapies require high cell dosages and precise phenotype control for clinical success; scalable manufacturing processes therefore need to be economic and controllable, in particular with respect to culture medium and growth factor (GF) strategy. The aim of this work was to demonstrate the biological function, and integration within scalable systems, of a highly controllable immobilized growth factor (iGF) approach. GFs were biotinylated and attached to streptavidin coated magnetic particles. GF concentration during biotinylation, GF-biotin ratio, and GF lysine content were shown to control iGF surface concentration and enable predictable co-presentation of multiple GF on a single bead. Function was demonstrated for immobilized GMCSF, SCF, TPO and IL-3 in GF dependent cell lines TF-1 and M-07e. Immobilized GMCSF (iGMCSF) was analyzed to show sustained activity over eight days of culture, a two to three order of magnitude potency increase relative to soluble factor, and retained functionality under agitation in a micro-scale stirred tank bioreactor. Further, short exposure to iGMCSF demonstrated prolonged growth response relative to soluble factor. This immobilization approach has the potential to reduce the manufacturing costs of scaled cell therapy products by reducing GF quantities and offers important process control opportunities through separation of GF treatments from the bulk media.

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1 Introduction

The processing, or manufacture, of hematopoietic stem cells (HSCs) for therapeutic application has made significant progress in recent years. In the field of HSC transplant successful strategies have been developed for the use of expanded cell populations in support of an unexpanded unit to reduce time for myeloid recovery [1].

Emerging research suggests a single expanded cell population may soon be a viable treatment option [2]. Success is also being achieved with genetically modified hematopoietic cells for treating rare genetic abnormalities such as Wiskott-Aldrich syndrome or Adenosine deaminase deficiency [3–5]. Early stage research promises manufacture of mature lineage cells such as erythrocytes or platelets from progenitors. Wider adoption of such therapies will require scalable and controllable processes within the constraints of economic delivery.

A common factor in the manufacture of therapeutic cells is the reliance on soluble signaling factors, such as cytokines, to control growth and differentiation outcomes; current cell culture protocols primarily consist of defined cocktails of soluble factors. However, the use of soluble factors has limitations including rapid decay and homogenous dispersion in culture [6]. This prevents presentation of high concentrations of factor at the receptor relative to bulk distribution (reducing effective potency),

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Abbreviations: ECM, extracellular matrix; GF, growth factor; GMCSF, granulocyte macrophage colony-stimulating factor; HSC, hematopoietic stem cell; iGF, immobilized growth factor; IL-3, interleukin 3; PEG, polyethylene glycol; PDs, population doublings; SCF, stem cell factor; SD, standard deviation; TPO, thrombopoietin

and presentation of several discrete combinations of factors simultaneously to generate multiple microenvironments within a bulk culture. It also prevents recycling of active GFs independently from other factors in the milieu [7, 8]. Collectively these drawbacks reduce opportunities to decrease costs, of which media volume and supplemented factors form a substantial part, and hinder the development of more refined GF strategies to exert control over proliferation, commitment and heterogeneity of cell populations.

Surface adhered GFs are an alternative culture strategy to soluble. In vivo, a number of GFs (e.g. SCF and Flt-3L) can exist in both soluble and matrix (or membrane) bound forms [9, 10]. Matrix bound GFs associate with glycosaminoglycans (heparin or heparan sulfate) or proteins of the extracellular matrix (ECM) including fibronectin and collagen [7, 11]. Immobilization of GFs in vitro allows high localized concentrations of factor combinations which can induce synergetic signaling through mechanisms such as ligand multivalency and receptor clustering; this has been suggested to lead to distinct signaling pathways in comparison to their soluble counterparts [12]. Further, proteins such as SCF and Notch-1 ligand have distinct functional effects depending on their soluble or bound state [13–17]. Other functional attributes reported for iGFs include increased potency, prolonged response, and protection from proteolytic degradation and receptor-mediated endocytosis [18–21]. Immobilization of proteins within a culture system therefore has the potential to offer more control opportunities than soluble factors alone.

Achieving controlled cell fate outcomes from iGF is dependent on a suitably well mixed system, an appropriate mechanism of immobilization, and that the cell/iGF interaction will predictably scale. Soluble growth factors (sGF) will diffuse down a concentration gradient in a non-mixed or poorly mixed system; conversely iGF have a fixed locus leading to potentially heterogeneous exposure of individual cells. To address these criteria we have developed an immobilization strategy that is amenable to incorporation in a stirred tank bioreactor format. Immobilization of GFs in a culture process could be achieved via a wide range of methods and these are described comprehensively elsewhere [22]. The immobilization method selected was based on a Streptavidin coated surface treated with a biotinylated version of the GF of interest with a PEG linker between the biotin and the GF. This system has a number of attractive attributes including: click chemistry under benign reaction conditions, reagents compliant with clinical use, and the potential to tune steric presentation of GFs. To achieve scalability we immobilized onto magnetic particles that could be distributed at a controlled surface to volume ratio in a standard stirred tank bioreactor format. Such a culture format has extensive literature supporting control of culture conditions and scalability [23–24]. Further the magnetic

properties enabled temporal control of GF exposure independently of the bulk medium. This approach enabled us to demonstrate functionality and selective temporal control of key clinically relevant hematopoietic factors GM-CSF, SCF and TPO in a scalable production system.

2 Materials and methods

2.1 Growth factor immobilization

The immobilization method involves a two-step reaction:

2.1.1 Step 1 – Growth factor biotinylation

- 1.1. Lyophilized carrier free GFs (R&D Systems, UK) were re-suspended in PBS at a concentration of 50 µg/mL.
- 1.2. NHS-PEG₂₀₀₀-biotin (Nanocs Inc., USA) was suspended in anhydrous DMSO at the required molar concentration for the individual experiment being carried out and used immediately.
- 1.3. Equal volumes of GF and NHS-PEG₂₀₀₀-biotin were mixed in LoBind Protein Eppendorf tubes and vortexed for 2 h at room temperature.

2.1.2 Step 2 – Immobilization of biotinylated growth factors

- 2.1. 2.8 µm streptavidin coated magnetic particles (Solulink, USA) were placed on a magnetic stand (MagnaRack™ Magnetic Separation Rack, ThermoFisher Scientific) and the supernatant removed.
- 2.2. Biotinylated GF solution from step 1.3 was added to the prepared particles and vortexed overnight at 4–8 °C.
- 2.3. iGFs were washed three times in 500 µL of PBS using a magnetic stand and used immediately.

2.2 Quantification assays

2.2.1 Immobilized growth factor concentration

2×10^5 iGF particles were incubated in 50 µL of flow cytometry stain buffer containing the required concentration of conjugated monoclonal antibodies (R&D Systems, UK) for 1 h at room temperature. Where conjugated antibodies were unavailable, antibodies were custom conjugated using an APC-Antibody conjugation kit (Solulink, USA). In parallel, iGF particles were also stained with an antibody isotype control. iGF particles were then washed once with PBS and re-suspended in 100–500 µL of PBS.

Fluorescence intensity was measured using FACS Canto II flow cytometer (BD Biosciences, UK) by taking a minimum of 10 000 events at medium flow rate. Fluorescence reference standards (Quantum MESF; Bangs Labs Inc., USA) were run on the same day to determine the mean mass of GF per particle using the net geometric

mean of fluorescence intensity (sample geometric mean minus isotype geometric mean).

2.2.2 Soluble growth factor concentration

Soluble GF concentrations were determined with a BioPlex Magpix Multiplex Reader (Bio-Rad Laboratories, UK) using pre-mixed Human Magnetic Luminescence assay kits with SCF and GM-CSF analytes (R&D Systems, UK). Cell culture media were stored at -20°C until use.

2.2.3 Determination of particle number

Particle number was determined using the particle count setting of an automated cell counter, Countess (ThermoFisher, UK). Final particle concentration was determined using flow cytometry where a fixed volume of sample was analyzed using medium flow rate. Flow cytometry data was analyzed using FlowJo software (Treestar, USA) and cells/mL was generated from the particle population gate.

2.3 Functional assays

2.3.1 TF1 cell proliferation assay

TF1 cells (ATCC, UK) were maintained in culture at a density of 2×10^5 to 8×10^5 cells/mL with media and GM-CSF replenishment every two to three days [25]. Media consisted of RPMI 1640 (phenol red and glutamine free; ThermoFisher Scientific), 10% v/v FBS (Life Technologies), 5 mM Glutamax (ThermoFisher Scientific) and 5 ng/mL carrier free GM-CSF (R&D Systems).

Unless otherwise stated, TF1 cells were washed three times in GM-CSF free media and starved of GM-CSF for 24 h. Either soluble GM-CSF (sGM-CSF) or immobilized GM-CSF (iGM-CSF) was supplemented to starved TF1 cells and a growth rate was determined thereafter using flow cytometry.

2.3.2 M07e cell proliferation assay

M-07e cells (Creative Biosystems, USA) were maintained in culture at a density of 4×10^5 to 1×10^6 cells/mL with media, GM-CSF and SCF replenishment every two or three days. Media consisted of RPMI 1640 (phenol red and glutamine free), 10% FBS v/v, 5 mM Glutamax, 5 ng/mL GM-CSF and 10 ng/mL SCF.

M07e cells were washed three times in GM-CSF free media and starved of GM-CSF for 24 h. Soluble or immobilized GM-CSF, SCF and TPO were supplemented individually to starved M-07e cells and a growth rate was determined thereafter using absolute cell counts determined by flow cytometry.

2.3.3 Micro-bioreactor TF1 culture

A micro-scale stirred tank bioreactor platform (ambr[®]; Sartorius Stedim, UK.) was used to determine the scalability of the immobilization method. Physicochemical parameters were set to: 100% DO₂ (oxygen delivery), pH 7.4,

temperature 37 °C, stir speed 350 rpm. Non-sparged vessels were used with 1% antifoam additions (Antifoam C, Sigma Aldrich, UK) performed every 24 h beginning at 0 h. The ambr[®] was programmed to sample from vessels every 3 h, dispensing into a microtiter plate containing 4% paraformaldehyde (Sigma Aldrich, UK) which was then analyzed on a flow cytometer to determine cell and particle number. A complete media change was performed every 48–72 h and cells were reset to a density of 2×10^5 cells/mL.

For iGF conditions, particles were retained within the vessel by holding a magnet against the vessel, whilst performing media changes.

2.4 Determination of viable cell number

2.4.1 Flow cytometry

Cell number was determined by flow cytometry. A fixed volume of sample was analyzed using medium flow rate. Flow cytometry data was analyzed using FlowJo software (Treestar, USA) and cells/mL was generated from the cell population gate.

2.4.2 ViCell

The AMBR was programmed to sample cell suspensions every ≈ 4 h with a two-fold dilution with media to determine viable cell number using a ViCell (Beckman Coulter), an automated trypan blue exclusion method cell counter. An average of 50 images per sample was used to determine viable cell number.

2.4.3 Determination of population doublings

Population doublings per day were determined using the following formula:

$$\text{population doublings} = (\log_{10} [FD/SD]) \times 3.32$$

where FD = final density (live cells/mL);
 SD = seeding density (live cells/mL).

2.5 Statistical analysis

To determine the statistical significance of one variable between two groups, a student's two-tailed t -Test was performed. To determine the statistical significance of one variable between three or more groups a one-way ANOVA was performed followed by Dunnett's test for multiple comparisons. To determine the statistical significance of two variables between three or more groups a two-way ANOVA was performed followed by Sidak's test for multiple comparisons.

For all tests a $P \leq 0.05$ was deemed significant. All statistical tests were performed using GraphPad Prism 6 (GraphPad Software Inc., USA).

3 Results

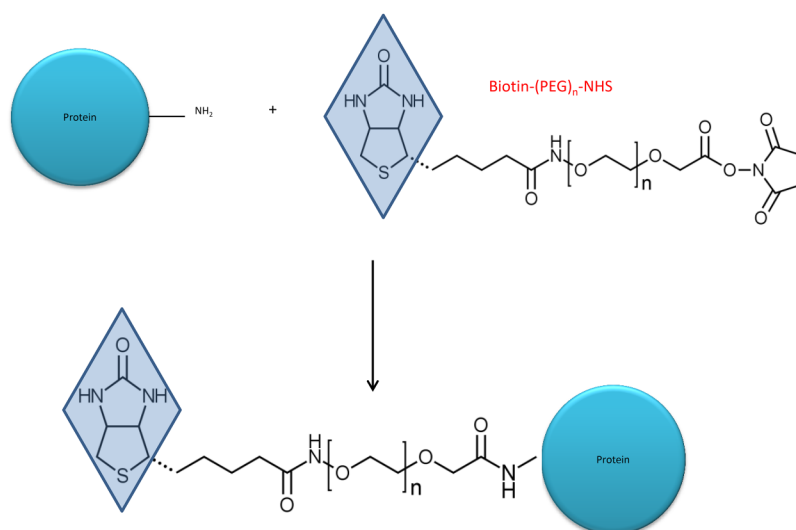
3.1 Immobilization and control of surface concentration of growth factors

A series of experiments were conducted to determine the effect of reagent concentrations on GF binding to the streptavidin coated beads. Target GFs were biotinylated via reaction of an NHS group on the biotin-PEG₂₀₀₀-NHS with primary amines of the lysine residues in the GF (Fig. 1, step 1). Initially we determined the effect of altering the molar ratio of biotin-PEG₂₀₀₀-NHS to lysine residues in the target GFs. It was anticipated that biotin-PEG₂₀₀₀-NHS in excess of a 1:1 ratio with target lysine

would result in free-biotin being carried forward to competitively blank streptavidin on the particles and therefore reduce GF-biotin bound to the particles, whilst below a 1:1 biotin-PEG₂₀₀₀-NHS to lysine ratio the number of biotinylated GFs available in the particle binding step would become a limiting factor (Fig. 1, step 2). In accordance with this, maximum surface yields of three GFs (GMCSF, SCF and TPO) were achieved by using the equivalent biotin-PEG₂₀₀₀-NHS molarity to the number of lysine residues within the GF (Fig. 2A). This is a potentially simple mechanism to control surface concentration of iGF.

The absolute concentration of biotin-PEG₂₀₀₀-NHS and GF during GF biotinylation (Fig. 1, step 1), at a fixed

Step 1: Protein Biotinylation



Step 2: Immobilisation

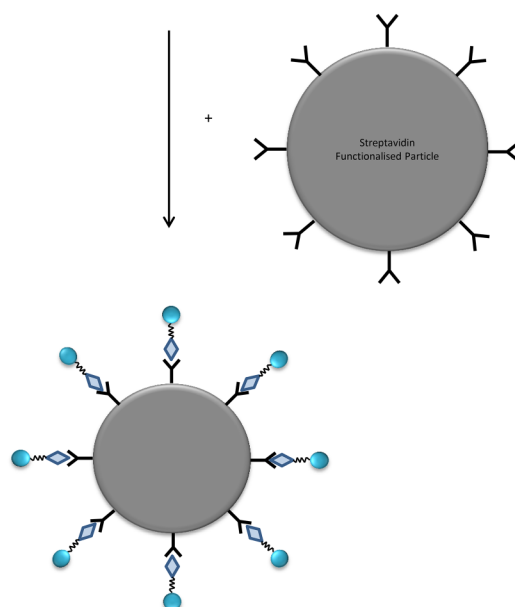
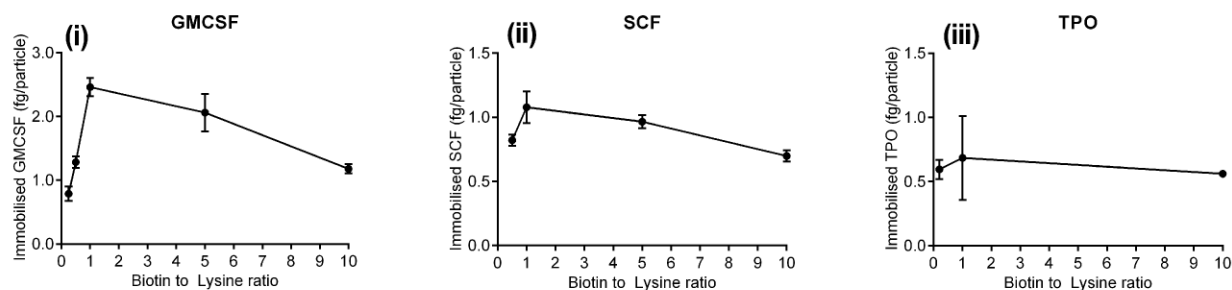
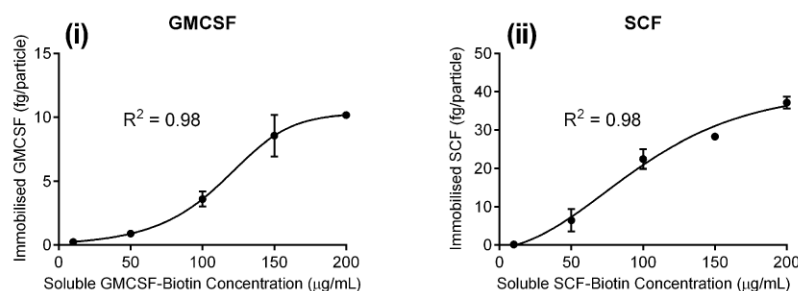


Figure 1. Immobilization of growth factors onto streptavidin functionalized microparticles. The immobilization method consists of a two-step reaction. In step 1, the GFs primary amines react with the NHS terminus of a hetero-bifunctional PEG molecule, NHS-(PEG)_n-biotin, to produce a biotinylated GF. Once the reaction is complete, streptavidin functionalized microparticles are added in step 2, allowing the biotin terminus of the biotinylated GF to bind to the streptavidin ligands of the microparticles resulting in immobilization.

A



B



C

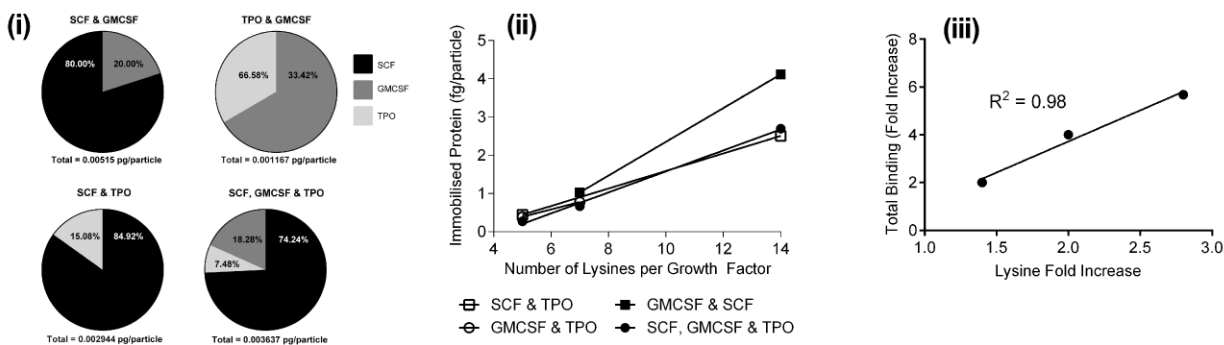


Figure 2. Methods for controlling surface concentration of immobilized growth factors. (A) The surface concentrations of three growth factors were controlled by regulating the NHS-(PEG)_n-biotin (biotin) molar excess during step 1 of the immobilization reaction. Maximum surface concentrations were achieved with a 1:1 biotin to lysine molar ratio for each of the growth factors investigated ($n = 3$ mean \pm SD). (B) The effect of soluble GF-biotin concentration at optimal GF:biotin ratios were investigated to regulate the surface concentration of immobilized growth factors. A sigmoidal relationship between soluble GF-biotin and iGF concentration for (i) GMCSF and (ii) SCF ($n = 3$ mean \pm SD). (C) The immobilization preferences for a mixture of two or more growth factors at equal molar concentrations were investigated. (i) The preference for immobilization was SCF>GMCSF>TPO from high to low (ii) Binding preference was linked to the residual lysine number of the growth factor (iii) A linear relationship between lysine number and concentration of immobilized GF was shown ($n = 3$).

molar ratio of 1:1 biotin-PEG₂₀₀₀-NHS to lysine residues, was evaluated to determine its relationship with the subsequent immobilized surface concentration. Surface concentration of iGF approached saturation at 200 μg/mL of soluble GF (sGF) (Fig. 2B). The relationship between the concentration of sGF and iGF per particle should theoretically be sigmoidal (R^2 of sigmoidal line = 0.98 for GMCSF and SCF) presuming the mechanism underpinning this is NHS degradation via hydrolysis at a constant rate and a rate of NHS binding with GF that is proportional to reagent concentration [26]. This presents a complementary method to manipulate immobilized surface concentration and indicates the importance of controlling

factors that influence reaction kinetics to achieve reproducible results. GF concentrations of 50 μg/mL were taken forward for particle preparation for functional studies; this utilized approximately 25% of the total GF presentation capacity of the beads.

The benefits of ligand co-signalling may require production systems to co-present two or more GFs on a single particle in controlled relative concentrations. It was hypothesized that if multiple biotinylated GFs at equal concentrations were incubated with the particles (Fig. 1, step 2) the relative bound surface concentrations would be determined by lysine residue number (and therefore biotinylated sites available for streptavidin binding). The

three GFs (GMCSF, SCF and TPO) mixed at equal concentrations, either in pairs or all three, resulted in surface binding of each GF in proportion to its lysine content (SCF>GMCSF>TPO) with a high degree of linear correlation ($R^2 > 0.98$; Fig. 2C). These indicated relative surface concentrations could be controlled via compensating for different lysine residues with changes in GF concentration.

3.2 Functionality, stability, and manipulation of immobilized GMCSF in a non-mixed culture system

Following demonstration that GFs could be effectively immobilized at controlled surface concentrations we sought to demonstrate iGFs retained functionality. Fur-

ther, we aimed to show iGFs could be manipulated independently of the bulk media with the use of magnets and retain prolonged functionality.

The functional activity of iGMCSF was investigated using a GMCSF dependent cell line, TF-1. A dose-response of sGMCSF and iGMCSF was compared to determine the relative response of TF-1. sGMCSF reached a maximal response at approximately 2 ng/mL in accordance with prior studies (Fig. 3A) [25]. iGMCSF created at very low surface concentrations (0.008–0.09 fg/bead) stimulated a linear increase in growth rate with a logarithmic increase in particle concentration, matching the soluble response at approximately 1×10^5 particles/mL at 0.09 fg/particle or 0.009 ng/mL (Fig. 3Bi). Conversion of the different particle concentrations and surface concentrations into total iGF presented per mL allowed calculation of a single dose

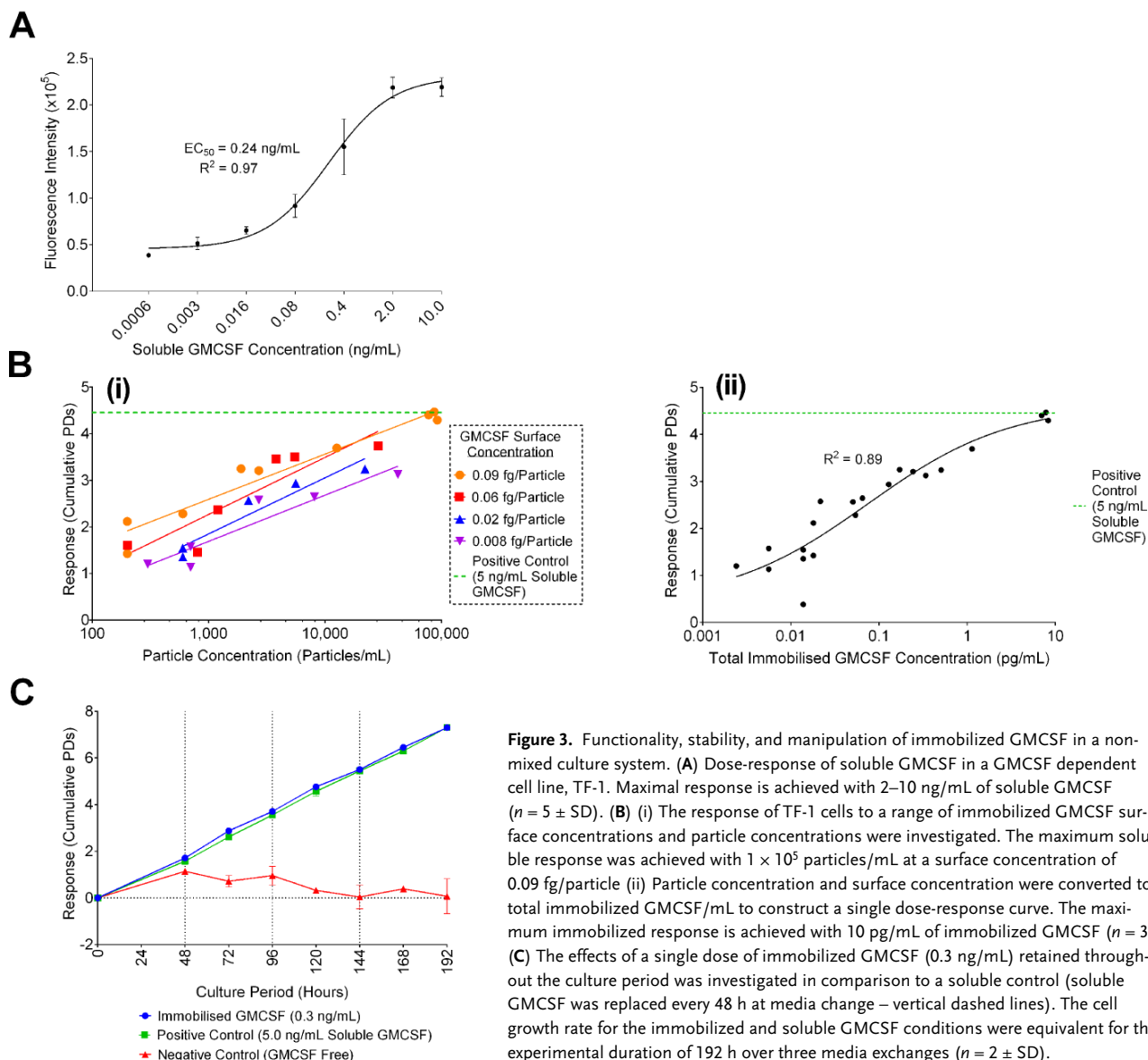


Figure 3. Functionality, stability, and manipulation of immobilized GMCSF in a non-mixed culture system. (A) Dose-response of soluble GMCSF in a GMCSF dependent cell line, TF-1. Maximal response is achieved with 2–10 ng/mL of soluble GMCSF ($n = 5 \pm \text{SD}$). (B) (i) The response of TF-1 cells to a range of immobilized GMCSF surface concentrations and particle concentrations were investigated. The maximum soluble response was achieved with 1×10^5 particles/mL at a surface concentration of 0.09 fg/particle (ii) Particle concentration and surface concentration were converted to total immobilized GMCSF/mL to construct a single dose-response curve. The maximum immobilized response is achieved with 10 pg/mL of immobilized GMCSF ($n = 3$). (C) The effects of a single dose of immobilized GMCSF (0.3 ng/mL) retained throughout the culture period was investigated in comparison to a soluble control (soluble GMCSF was replaced every 48 h at media change – vertical dashed lines). The cell growth rate for the immobilized and soluble GMCSF conditions were equivalent for the experimental duration of 192 h over three media exchanges ($n = 2 \pm \text{SD}$).

response (Fig. 3Bii). This indicated a maximal immobilized dose response of approximately 1–10 pg/mL, some three orders of magnitude more potent than the soluble equivalent. The formation of a relatively unified dose response curve from a range of different surface and particle concentrations suggest modulation of either surface concentration or particle number can be used to alter concentration of presented GF to a similar degree.

The functional stability of iGMCSF was tested by culture of TF-1 cells with a single set of GMCSF coated particles magnetically retained through serial bulk medium changes in a static system. A positive control was treated with a media exchange containing sGMCSF every 48 h; a negative control had no GMCSF. The cell growth rate for the iGMCSF and sGMCSF conditions were equivalent for the experimental duration of 192 h over three media exchanges (Fig. 3C). The surface concentration of iGMCSF was 1.56 fg/particle (or 0.3 ng/mL at 2×10^5 particles/mL) equivalent to a 94% decrease in total GF required to stimulate the maximal biological response. Including the three media changes the immobilized culture used 1.5% of the GF over the 192 h relative to the soluble control. sGMCSF concentration was monitored in all experimental systems via Magpix™ analysis to confirm no sGF leached from the immobilized system.

3.3 Functionality, stability, and manipulation of immobilized GMCSF in stirred tank bioreactors and effects of transient exposure to growth factor

The method presented can theoretically be scaled using surface area (particle number) to volume ratio in a suspension culture; we therefore aimed to validate the functional activity of iGMCSF under mechanical agitation in a micro-scale stirred tank bioreactor system (ambr®). Further, we proposed that continuous exposure of GF is unnecessary, or may have negative effects for continuous cell growth [27–28].

TF-1 cells were cultured in the ambr® system over 120 h with either sGMCSF or iGMCSF. Under stirred conditions the cells responded equivalently to continual exposure of both sGMCSF and iGMCSF over the 120 h, indicating agitation of the cells/particles was not a barrier to contact and signaling, and iGMCSF retained functionality over an extended period (Fig. 4A). At sequential time intervals after initiation of culture, GF was withdrawn to determine if stimulation and decay of cell growth was equivalent for GMCSF in both soluble and immobilized forms. Continued cell growth was observed for a significant time period after removal of sGMCSF or iGMCSF, however the duration and magnitude of growth was dependent on time of exposure and form of presentation (Fig. 4A). Cell growth measured at 50 h showed a significantly ($P = 0.01–0.05$) higher response than had been achieved with short exposure (2 and 4 h) to iGMCSF compared to equivalent short exposure to sGF

(Fig. 4B). Similarly, cell numbers continued to significantly increase ($P \leq 0.05$) for a longer period after a short exposure (2 and 4 h) to iGMCSF compared to the equivalent exposure to sGMCSF (Fig. 4C). iGMCSF concentrations used throughout the experiment were calculated to be equivalent to 0.4 ng/mL.

3.4 Functionality and dose response of immobilized GMCSF, SCF and TPO

The nature of the GF immobilization through lysine groups could potentially lead to reduced activity if the binding interfered with active site or induced conformational change. We therefore determined the functionality and dose-response of iGMCSF, iTPO and iSCF using an alternative cell line, M-07e, with relevant growth sensitivity to these GFs.

Both SCF and TPO GFs also remained biologically active once immobilized (Fig. 5). GMCSF and TPO both significantly exceeded the maximum sGF response ($P \leq 0.0001$) whereas SCF did not achieve the soluble response despite reaching the top of a response curve. Maximum responses were achieved with 2.32×10^4 particles/mL (1.4 fg/particle) for GMCSF, 2.65×10^4 particles/mL for TPO, and 8.42×10^6 particles/mL (1.9 fg/particle) for SCF (Fig. 5). Negative effects on cell viability were observed for highest concentrations of GMCSF and TPO ($>5.35 \times 10^4$ and $>8.82 \times 10^4$ particles/mL respectively). In this cell line 0.003 ng/mL of iGMCSF was required to accomplish the maximum soluble response achieved using 10 ng/mL of sGMCSF, providing a similar orders of magnitude potency gain to that observed in the TF-1 line. Additionally, 16 ng/mL of iSCF accomplished 65% of the maximum response achieved with 200 ng/mL of sSCF. This is consistent with literature indicating distinct roles for soluble and bound SCF [14, 15]. A single point concentration experiment with immobilized IL-3 showed that 0.0059 ng/mL (± 0.00069 ng/mL) of immobilized IL-3 produced approximately the same growth response (0.32 PD/day ± 0.0091 StDev) as 0.1 ng/mL soluble IL-3 (0.34 PD/day ± 0.011 StDev).

4 Discussion

An approach for incorporating iGF or other proteins into scalable suspension cell culture systems has been developed. The method enables significant reduction in the total quantity of some GFs required to produce a functional response relative to a soluble system. Further it allows control of local surface GF concentration as well as bulk GF concentration. The magnetic component of the particles allows complex temporal profiles of GF presentation independent from changes in the bulk media. The iGF appear to display alternate kinetics with respect to stimulation and decay of function. In concert these prop-

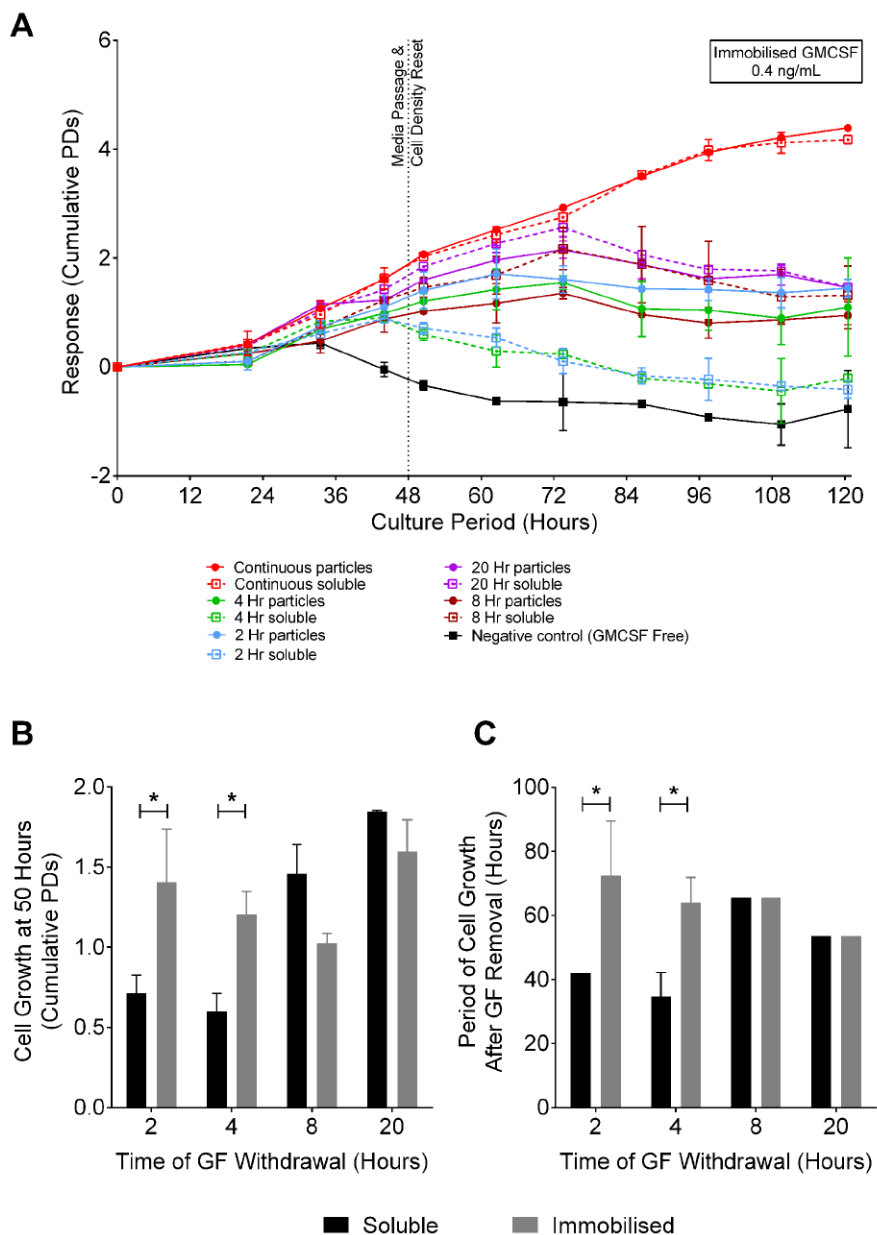


Figure 4. Functionality, stability, and manipulation of immobilized GMCSF in stirred tank bioreactors and effects of transient exposure to growth factor. **(A)** The effects of immobilized and soluble GMCSF exposure time on subsequent TF1 cell growth rates were investigated in an agitated scalable cell culture bioreactor. Identical cell growth rates were achieved with continuous exposure to immobilized and soluble GMCSF ($n = 2 \pm SD$). Vertical dashed line represents the time of a full media passage and cell reset to initial seeding density. **(B)** A comparison of cell growth rates at a single time-point from **(A)** (50 h) for immobilized and soluble GMCSF were investigated. Cell growth was significantly increased at 2 and 4 h of exposure to immobilized GMCSF when compared to soluble GMCSF ($n = 2 \pm SD$). Single asterisks represents a P value of 0.01–0.05. **(C)** The period of sustained cell growth following growth factor removal was calculated from **(A)**. A significant increase in the sustained cell growth was achieved with 2 and 4 h of exposure to immobilized GMCSF ($n = 2 \pm SD$). Single asterisk represents a P value of 0.01–0.05.

erties provide a platform for cost reduction and increased control in manufacture of complex GF dependent cell systems such as those being developed for haematopoietic products or broader cell therapies.

Multiple GF immobilization methodologies are available. The important criteria for the proposed application are compatibility with clinical grade production, functionality, controllability and economics. The GF immobilization technique retains biological function in three different GFs. Although it is possible that some of the iGF is inactive or unavailable depending on the orientation of binding, quantification suggests that despite such theoretical concerns a large potency and economic gain is

realizable through this approach to immobilization and recycling. The immobilization method has the potential for other protein targets important in hematopoietic cell expansion including Notch ligands, Wnt ligands and integrins [29]; this provides an opportunity to recapitulate some surface presented aspects of the stem cell niche in simple and well-mixed scalable systems and control a wider range of biological outcomes. The compatibility of the approach with agitated and well mixed systems is a key criterion; it ensures that particle-cell interactions should controllably scale and incorporates the advantages of spatially homogenous control of factors such as pH and dissolved gasses.

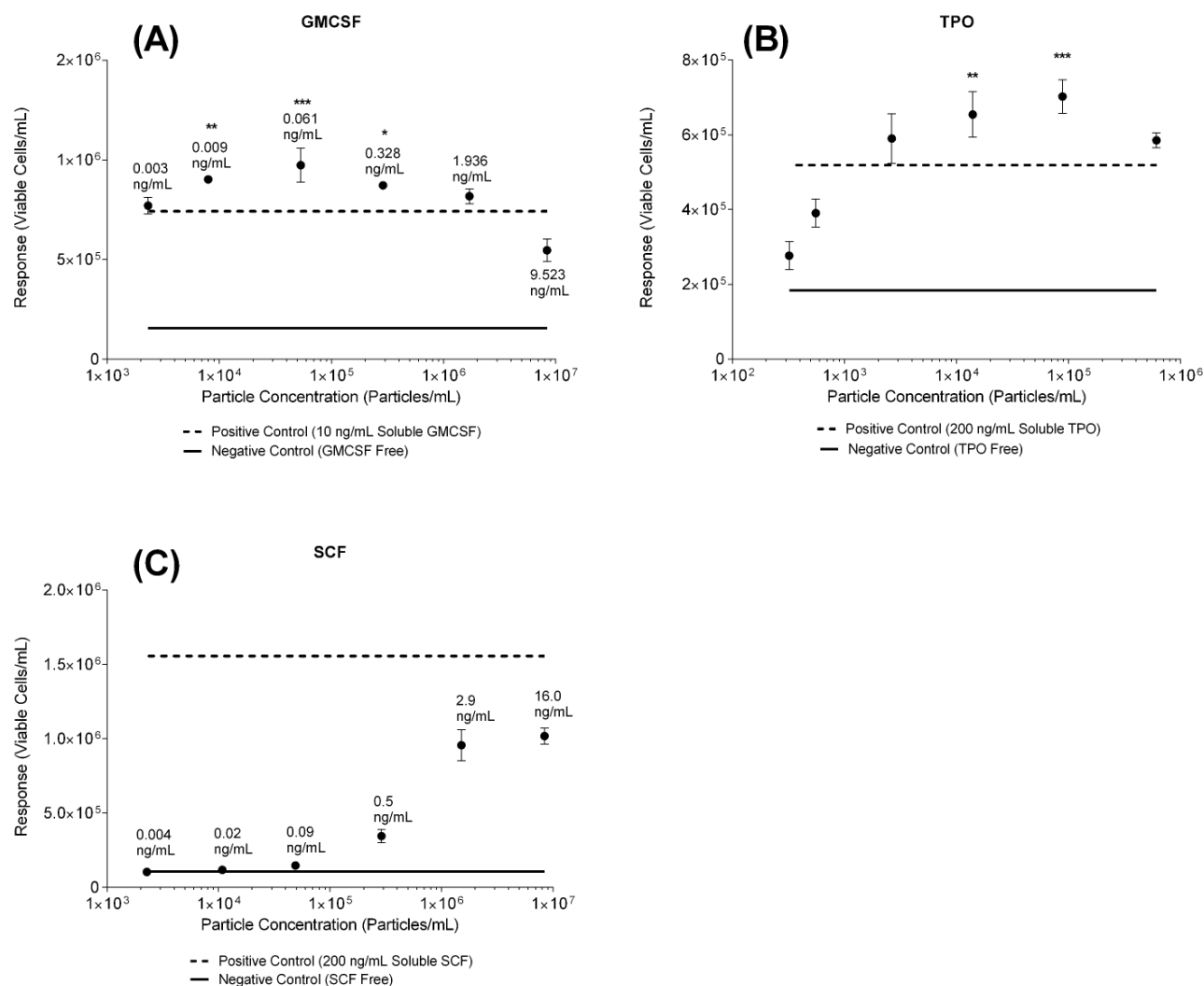


Figure 5. Functionality and dose response of immobilized GMCSF, SCF and TPO. The function and dose-response of three immobilized growth factors were investigated in a multi-factor dependent cell line (M-07e). All three factors remain biologically active once immobilized. Immobilized GMCSF and TPO both significantly exceed the maximal soluble response whereas SCF did not achieve the maximal soluble response. Where quantified, data points are labelled with total quantity of immobilized growth factor ($n = 3 \pm S.D.$). * $P = 0.01-0.05$, ** $P = 0.001-0.01$, *** $P = 0.0001-0.001$.

The demonstrated capability to control the surface presentation of iGFs allows the concentration and ratio of GFs to be tailored depending on the required functional response. The importance of spatial presentation of GFs for distinct functional responses has been reported, particularly in T-cell signaling [20]. This property of the system therefore increases the ability to tailor particles to different cell types and specific outcomes. iGF removal is also an important consideration for an economic intensified production system; the increased frequency of bulk media exchange with higher cell density (due to exhaustion of other medium components or build-up of toxic metabolites) will increase the potential cost impact of separating expensive GFs with alternate dynamics. For example, if a GF is subject to decay with time in the reactor, potentially cell mediated, presenting the GF intermit-

tently for 2 h would allow more specific productivity per GF than constant exposure. GMCSF data suggest that some GFs could be effective with particle concentrations in the order of 10^3 /mL given a suitably high surface concentration, providing further opportunity to reduce cost through minimizing particle use. The biological basis for the difference between iGF and sGF was not determined. However, there is a strong literature base indicating the potency and signaling longevity enhancements could be due to high local concentration of GF on the particles through mechanisms such as receptor multivalency and clustering [12, 30–32]. Short interaction times (50 ms) between enzyme and substrate are reported providing a basis for transient exposure sustaining cell growth [33]. It is also possible, given the extremely high potency, that some particle carry over could lead to sustained growth

after particle removal; monitoring particle carry over via flow cytometer indicated greater than 98% removal efficiency. Such a phenomenon would also not explain the observation that time of exposure to iGF had a 'U-shaped' relationship with growth response.

In conclusion, we have demonstrated a versatile and scalable technology for controlling iGF presentation in suspension cell culture platforms. The immobilization technique has economic and environmental benefits over soluble equivalents due to significant reductions of some GF quantities required to induce the maximal biological response and ability to recycle GFs independently from other bulk media components. The distinct functional effects of immobilizing GF and control potential of this system will allow more regulated engineering of complex GF dependent cell culture systems.

The authors declare the following potential conflict of interest. Improved cell culture using beads. Patent pending. Filed 19/05/2016. GB1608847.8

5 References

- [1] Dahlberg, A., Delaney, C., Bernstein, I. D., Ex vivo expansion of human hematopoietic stem and progenitor cells. *Blood* 2011, *117*, 6083–6090.
- [2] Milano, F., Heimfeld, S., Rifkin, I. B., Nicoud, I. et al., Infusion of a Non HLA-Matched off-the-shelf ex vivo expanded cord blood progenitor cell product following myeloablative cord blood transplantation is safe, decreases the time to hematopoietic recovery, and results in excellent overall survival. *Blood* 2014, *124*, 46.
- [3] Cicalese, M. P., Ferrua, F., Castagnaro, L., Pajno, R. et al., Update on the safety and efficacy of retroviral gene therapy for immunodeficiency due to adenosine deaminase deficiency. *Blood* 2016, *128*, 45–54.
- [4] Hacein-Bey Abina, S., Gaspar, H. B., Blondeau, J., Caccavelli, L. et al., Outcomes following gene therapy in patients with severe Wiskott-Aldrich Syndrome. *JAMA* 2015, *313*, 1550–1563.
- [5] Braun, C. J., Boztug, K., Paruzynski, A., Witzel, M. et al., Gene therapy for Wiskott-Aldrich syndrome – long-term efficacy and genotoxicity. *Sci. Transl. Med.* 2014, *6*, 227ra33.
- [6] Zandstra, P. W., Petzer, A. L., Eaves, C. J., Piret, J. M., Cellular determinants affecting the rate of cytokine depletion in cultures of human hematopoietic cells. *Biotechnol. Bioeng.* 1997, *54*, 58–66.
- [7] Tayalia P., Mooney, D. J., Controlled growth factor delivery for tissue engineering. *Adv. Mater.* 2009, *21*, 3269–3285.
- [8] Edelman, E., Nugent, M., Karnovsky, M., Perivascular and intravenous administration of basic fibroblast growth factor: Vascular and solid organ deposition. *Proc. Natl. Acad. Sci. USA* 1993, *90*, 1513–1517.
- [9] Broudy, V. C., Stem cell factor and hematopoiesis. *J. Am. Soc. Hematol.* 1997, *90*, 1345–1365.
- [10] McKenna, H. J., Stocking, K. L., Miller, R. E., Brasel, K. et al., Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood* 2000, *95*, 3489–3497.
- [11] Taipale, J., Keski-Oja, J., Growth factors in the extracellular matrix. *FASEB J.* 1997, *11*, 51–59.
- [12] Ito, Y., Covalently immobilized biosignal molecule materials for tissue engineering. *Soft Matter* 2008, *4*, 46–56.
- [13] Varnum-Finney, B., Brashem-Stein, C., Bernstein, I. D., Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability. *Blood* 2003, *101*, 1784–1789.
- [14] Fox, R. A., Sigman, M., Boekelheide, K. I. M., Transmembrane versus soluble stem cell factor expression. *Lab. Med.* 2000, *21*, 579–585.
- [15] Huang, E. J., Nocka, K. J., Buck, J., Besmer, P., Differential expression and processing of two cell associated forms of the kit-ligand: KL-1 and KL-2. *Mol. Biol. Cell* 1992, *3*, 349–362.
- [16] Delaney, C., Varnum-Finney, B., Aoyama, K., Brashem-Stein, C. et al., Dose-dependent effects of the Notch ligand Delta1 on ex vivo differentiation and in vivo marrow repopulating ability of cord blood cells. *Blood* 2008, *106*, 2693–2699.
- [17] Ohishi, K., Varnum-Finney, J., Bernstein, I. D., Delta-1 enhances marrow and thymus repopulating ability of human CD34(+)CD38(-) cord blood cells. *J. Clin. Invest.* 2002, *110*, 1165–1174.
- [18] Ehrbar, M., Djonov, V. G., Schnell, C., Tschanz, S. A. et al., Cell-demanded liberation of VEGF121 from fibrin implants induces local and controlled blood vessel growth. *Circ. Res.* 2004, *94*, 1124–1132.
- [19] Ito, Y., Zheng, J., Imanishi, Y., Yonezawa, K. et al., Protein-free cell culture on an artificial substrate with covalently immobilized insulin. *Proc. Natl. Acad. Sci. USA* 1996, *93*, 3598–3601.
- [20] Aratsu, F., Harada, I., Yoshimura, S., Cho, C. S. et al., Dynamic chemotactic response of fibroblasts to local stimulation using EGF-immobilized microbeads. *Biomaterials.* 2014, *35*, 2471–2476.
- [21] Guex, A. G., Hegemann, D., Giraud, M. N., Tevaearai, H. T. et al., Covalent immobilization of VEGF on plasma-coated electrospun scaffolds for tissue engineering applications. *Colloids Surf., B* 2014, *123*, 724–733.
- [22] Hermanson, G., *Bioconjugation Techniques*, Elsevier Inc., 2008.
- [23] Rowley, J., Abraham, E., Campbell, A., Brandwein, H. et al., Meeting lot-size challenges of manufacturing adherent cells for therapy. *Bio-process Int.* 2012, *10*, 16–22.
- [24] Kirouac, D. C., Zandstra, P. W., The systematic production of cells for cell therapies. *Cell Stem Cell.* 2008, *3*, 369–381.
- [25] Kitamura, T., Tange, T., Terasawa, T., Chiba, S. et al., Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. *J. Cell. Physiol.* 1989, *140*, 323–334.
- [26] Wong, S. S., *Chemistry of Protein Conjugation and Cross-Linking*, CRC Press LLC, 1991.
- [27] Shankaran, H., Wiley, H. S., Resat, H., Receptor downregulation and desensitization enhance the information processing ability of signalling receptors. *BMC Syst. Biol.* 2007, *1*, 48–62.
- [28] Yee, N. S., Langen, H., Besmer, P., Mechanism of kit Ligand , Phorbol Ester , and Calcium-induced Down-regulation of c-kit Receptors in Mast Cells. *J. Biol. Chem.* 1993, *268*, 14189–14201.
- [29] Wilson, A., Trumpp, A., Bone-marrow haematopoietic-stem-cell niches. *Nat. Rev. Immunol.* 2006, *6*, 93–106.
- [30] Macri, L., Silverstein, D., Clark, R. A., Growth factor binding to the pericellular matrix and its importance in tissue engineering. *Adv. Drug Deliv. Rev.* 2007, *59*, 1366–1381.
- [31] Larochelle, A., Gillette, J. M., Desmond, R., Ichwan, B. et al., Bone marrow homing and engraftment of human hematopoietic stem and progenitor cells is mediated by a polarized membrane domain. *Blood* 2012, *119*, 1848–1855.
- [32] Hynes, R. O., Extracellular matrix: Not just pretty fibrils. *Science* 2009, *326*, 1216–1219.
- [33] Tummino, P. J., Copeland, R. A., Residence Time of Receptor-Ligand Complexes and Its Effect on Biological Function. *Biochemistry* 2008, *47*, 5481–5492.



Cover illustration

The cover of this regular issue of BTJ shows fluorescence microscopy images of bacteria along with their processed counterparts after CellShape analysis. While the former are simple raw images, the latter reveal important quantitative information e.g. intensity contours and spots. Altogether, they form a complete dataset from which we can accurately interpret cellular fluorescent signals. The cover is prepared by Ángel Goñi-Moreno, Juhyun Kim and Víctor de Lorenzo authors of the article "CellShape: A user-friendly image analysis tool for quantitative visualization of bacterial cell factories inside". (<http://dx.doi.org/10.1002/biot.201600323>).

Biotechnology Journal – list of articles published in the February 2017 issue.

Review

Bringing 3D tumor models to the clinic – predictive value for personalized medicine

Kathrin Halfter and Barbara Mayer

<http://dx.doi.org/10.1002/biot.201600295>

Research Article

Metabolic engineering of *Mannheimia succiniciproducens* for succinic acid production based on elementary mode analysis with clustering

Won Jun Kim, Jung Ho Ahn, Hyun Uk Kim, Tae Yong Kim and Sang Yup Lee

<http://dx.doi.org/10.1002/biot.201600701>

Research Article

Genome analysis of a hyper acetone-butanol-ethanol (ABE) producing *Clostridium acetobutylicum* BKM19

Changhee Cho, Donghui Choe, Yu-Sin Jang, Kyung-Jin Kim, Won Jun Kim, Byung-Kwan Cho, E. Terry Papoutsakis, George N. Bennett, Do Young Seung and Sang Yup Lee

<http://dx.doi.org/10.1002/biot.201600457>

Research Article

Camelid V_H affinity ligands enable separation of closely related biopharmaceuticals

Timothy M. Pabst, Michaela Wendeler, Xiangyang Wang, Sandra Bezemer, Pim Hermans and Alan K. Hunter

<http://dx.doi.org/10.1002/biot.201600357>

Research Article

Combination of two epitope identification techniques enables the rational design of soy allergen Gly m 4 mutants

Heide Havenith, Karolin Kern, Paul Rautenberger, Holger Spiegel, Michael Szardenings, Elke Ueberham, Jörg Lehmann, Matthias Buntru, Simon Vogel, Regina Treudler, Rainer Fischer and Stefan Schillberg

<http://dx.doi.org/10.1002/biot.201600441>

Research Article

A versatile modular bioreactor platform for Tissue Engineering

Sebastian Schürlein, Thomas Schwarz, Steffan Krzimirski, Sabine Gätzner, Anke Hoppensack, Ivo Schwedhelm, Matthias Schweinlin, Heike Walles, Jan Hansmann

<http://dx.doi.org/10.1002/biot.201600326>

Research Article

Immobilized hematopoietic growth factors onto magnetic particles offer a scalable strategy for cell therapy manufacturing in suspension cultures

Matthew J Worrallo, Rebecca LL Moore, Katie E Glen and Robert J Thomas

<http://dx.doi.org/10.1002/biot.201600493>

Research Article

Integrated genome and protein editing swaps α -2,6 sialylation for α -2,3 sialic acid on recombinant antibodies from CHO

Cheng-yu Chung, Qiong Wang, Shuang Yang, Bojiao Yin, Hui Zhang and Michael Betenbaugh

<http://dx.doi.org/10.1002/biot.201600502>

Research Article

Smartphone-based portable wireless optical system for the detection of target analytes

Shreedhar Gautam, Bhagwan S Batule, Hyo Yong Kim, Ki Soo Park and Hyun Gyu Park

<http://dx.doi.org/10.1002/biot.201600581>

Research Article

High-throughput downstream process development for cell-based products using aqueous two-phase systems (ATPS): A case study

Sarah Zimmermann, Christian Scheeder, Philipp K Zimmermann, Are Bogsnes, Mattias Hansson, Arne Staby and Jürgen Hubbuch

<http://dx.doi.org/10.1002/biot.201600587>

Research Article

Improved production of propionic acid using genome shuffling

*Carlos H Luna-Flores, Robin W Palfreyman, Jens O Krömer,
Lars K Nielsen and Esteban Marcellin*

<http://dx.doi.org/10.1002/biot.201600120>

Biotech Method

CellShape: A user-friendly image analysis tool for quantitative visualization of bacterial cell factories inside

Ángel Goñi-Moreno, Juhyun Kim and Víctor de Lorenzo

<http://dx.doi.org/10.1002/biot.201600323>

Biotech Method

A simplified procedure for antibody engineering by yeast surface display: Coupling display levels and target binding by ribosomal skipping

Julius Grzeschik, Steffen C. Hinz, Doreen Könning, Thomas Pirzer, Stefan Becker, Stefan Zielonka and Harald Kolmar

<http://dx.doi.org/10.1002/biot.201600454>

Biotech Method

Predictive glycoengineering of biosimilars using a Markov chain glycosylation model

*Philipp N. Spahn, Anders H. Hansen, Stefan Kol,
Bjørn G. Voldborg and Nathan E. Lewis*

<http://dx.doi.org/10.1002/biot.201600489>