Cell Encapsulation

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Noncovalent Hydrogel Beads as Microcarriers for Cell Culture**

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Abstract: *Hydrogel beads as microcarriers could have many applications in biotechnology. However, bead formation by noncovalent cross-linking to achieve high cell compatibility by avoiding chemical reactions remains challenging because of rapid gelation rates and/or low stability. Here we report the preparation of homogeneous, tunable, and robust hydrogel beads from peptide–polyethylene glycol conjugates and oligosaccharides under mild, cell-compatible conditions using a noncovalent crosslinking mechanism. Large proteins can be released from beads easily. Further noncovalent modification allows for bead labeling and functionalization with various compounds. High survival rates of embedded cells were achieved under standard cell culture conditions and after freezing the beads, demonstrating its suitability for encapsulating and conserving cells. Hydrogel beads as functional system have been realized by generating protein-producing microcarriers with embedded eGFP-secreting insect cells.*

Noncovalent polymer structures that possess simple components with basic biochemical properties similar to the extracellular matrix (ECM) are considered highly promising biomaterials. They can be used to culture cells in nature-like environments with chemically defined materials, for applications ranging from tissue engineering to drug screening.[1] Tailor-made biomaterials with functional complexities can be produced by conjugating different bioactive molecules to simple scaffolds.[1f,2] To engineer a physical hydrogel into a cell-encapsulating macrocarrier system can address a number of key challenges for creating living functional tissue, including control over the morphological and dimensional properties as well as cost-effective processes.[3] Hydrogel beads can also be formed using thermal-sensitive polymers or photo-resistive materials, whereas temperature changes or chemical cross-linking reactions can cause undesired effects on cells. To produce hydrogel beads through specific noncovalent molecular recognition is difficult because the gelation time of physical hydrogels are often too fast or too slow for the microfluidic process.[4] Cross-linking alginate with calcium is the major method to generate noncovalent hydrogel beads by using microfluidics.[4b,5] However, calcium is a pivotal second messenger for most types of cells and can cause adverse effects. Moreover, it is difficult to conjugate bioactive compounds to this system as chemically defined modifications, and a noncovalent network cross-linked through chelating metal ions is very different from the ECM structures. Here we report the synthesis of hydrogel beads using a noncovalent assembly system composed of conjugates of peptides with four-arm poly(ethylene glycol) (starPEG) polymers and oligosaccharides (Figure 1 a).[6] The

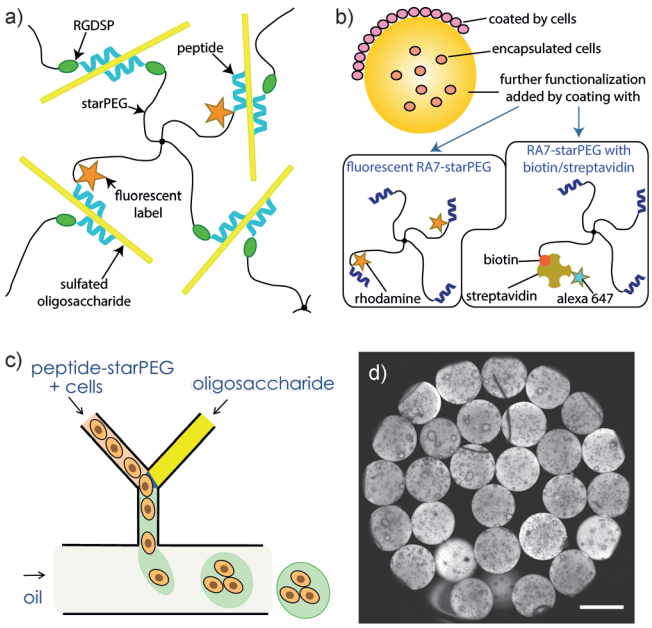

Figure 1. Monodisperse hydrogel beads synthesized using microfluidics. a) Scheme showing the structure of the noncovalent matrix. b) Applications and modifications of the hydrogel microcarriers. c) Scheme of the microfluidic system used for bead production. Components were injected simultaneously into the mixing device (green color indicates good mixing). d) Confocal laser scanning microscope image of rhodamine-labeled hydrogel beads using **KA7–Rho–starPEG** and dextran sulfate. Beads freshly separated from oil phase. The black dots can be removed after intensive washing steps (as shown later). Scale bar: 700 μm.

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matrix could be modified either covalently or noncovalently, without losing its properties as a physical hydrogel and the chemical simplicity (Figure 1 b).

In a previous study, we designed and screened simple heparin-interacting peptide motifs. When these peptides are conjugated with starPEG, they can form physical hydrogels with heparin (Figure 1 a).[6] The study revealed a simple structure–function relationship of $(BA)_n$ (in which B is the basic residue lysine or arginine, A is alanine, and n is the number of repetitions of the BA motif), in which both basic residues and heparin-induced α-helix formation of the peptides are important for the assembly process. The hydrogel properties and the gelation time can be tuned by changing the number of repetitions (n) and selecting lysine or arginine as basic residues.

The design of a microfluidic system to generate hydrogel beads is shown in Figure 1 c. A solution of sulfated oligosaccharides and a solution of peptide–starPEG conjugate were injected through two different channels. The flow rates of the oil phase and two aqueous phases have been adjusted to optimize droplet formation. $(RA)_7$ peptide–starPEG conjugate (RA7–starPEG) gelation with heparin is very fast and produced high backpressure, which blocked the microfluidic channels and produced inhomogeneous products. Gelation time for the $(KA)_7$ peptide–starPEG (KA7–starPEG) conjugate with heparin is more than one hour. Consequently, mixing KA7–starPEG with heparin does not produce high backpressure and homogeneous droplets can be produced. Unfortunately, the slow gelation causes some droplets to fuse with each other. Therefore, we investigated the gelation by mixing KA7–starPEG and various negatively charged oligosaccharides, to analyze their gelation rates using a centrifugation-based method[6] as well as to test the formation of hydrogel beads using the microfluidic device. We found that replacing heparin with dextran sulfate allowed an optimum gelation time of five minutes with KA7–starPEG. This results in the production of homogenous beads as shown in Figure 1 d. While both dextran sulfate and heparin are FDA-approved compounds, dextran sulfate is chemically synthesized, thus avoiding the potential risks associated with substances from biological sources, for example, heparin.[7] The KA7–starPEG hydrogels are very stable, with no visible degradation of the biomatrices observed for experiments lasting several months.[8] The synthesis of peptide–polymer conjugates was carried out according to the method described in our previous study (see the Supporting Information).[6]

Newly formed beads often stick to each other in the collection chamber, but the addition of surfactants to the aqueous phases can prevent the beads from clustering. However, for cell encapsulation experiments the amount of chemical additives should be minimized. We allowed the beads to flow for a long distance (10 min through a five meter long tube; movie S1). By extending the time before the beads met each other in the collection chamber, clustering was completely avoided without the addition of surfactants. Hydrogel beads were extracted from the oil with buffer or cell culture medium. The resulting beads had a narrow size distribution (755 ± 28 μm, Figure 1 d). An interesting feature of this system is that changing the buffer, for example, PBS and cell culture medium with 10% serum, does not cause swelling/shrinking of the hydrogel beads. This feature is very important for cell experiments because no mechanical force is applied to the cells when beads are transferred between solutions of different ionic strength. Furthermore, similar to the bulky heparin hydrogel reported previously, the resulting biopolymer is very stable in various buffer conditions and in cell culture media (Figure 3 a and b). No degradation of beads was observed over a period of months.

To investigate why 5 kDa dextran sulfate is more suitable for generating hydrogel beads than 14 kDa heparin, we measured the gelation rates (Figure 2 a), and the results are in good agreement with our previous observation. Although it took more than an hour for the hydrogel to reach final stiffness, the mixture of dextran sulfate and KA7–starPEG started to show clear gel properties after 5 min. No hydrogel formation could be observed for heparin and KA7–starPEG

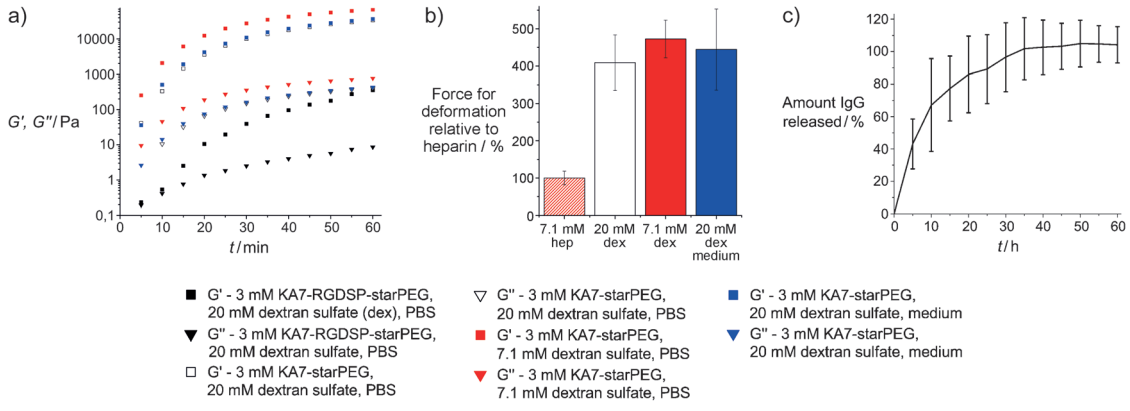

Figure 2. Gelation time, bulk rheology, and pore size analysis of peptide–starPEG conjugates with oligosaccharides. Hydrogel was formed by mixing 3 mM **KA7–starPEG** (or **KA7–RGDSP–starPEG**) with 7.1 mM 14 kDa heparin (hep), 7.1 mM or 20 mM 5 kDa dextran sulfate (dex). Solvent was physiological phosphate buffer (PBS, pH 7.4) or CO2-independent medium with 2% serum (medium). a) Gelation time analysis of hydrogels using rheometer with conical geometry. Stiffness was measured every 5 min. b) Final hydrogel stiffness (using only KA7–starPEG) was tested by measuring resistance to deformation using high-throughput centrifugation method after overnight incubation. c) Release of fluorescence-labeled IgG antibodies from hydrogel beads (10 mg mL^{-1}).

during the first hour. The gelation time of KA7–starPEG and dextran sulfate is in between that of RA7–starPEG and heparin, and KA7–starPEG and heparin.[6] After 1 h of incubation, KA7–starPEG with 7.1 mM ($G' \approx 70$ kPa, $G'' \approx 1\%$) and 20 mM dextran sulfate ($G' \approx 35$ kPa, $G'' \approx 1\%$) resulted in hydrogels with strong and stable network properties (Figure S3 a,b). Neither gelation rate nor stiffness is affected when PBS buffer was replaced by cell culture media (Figure 2 a,b). Although the KA7–RGDSP–starPEG and dextran sulfate form hydrogels that are much softer ($G' \approx 0.36$ kPa, $G'' \approx 2.5\%$; Figure S3 a,b), the gelation rate is comparable to those using KA7–starPEG and dextran sulfate (Figure 2 a). Using the microfluidic system, homogeneous hydrogel beads with the cell adhesion peptide sequence RGDSP can also be produced. The hydrogel beads produced by using either KA7–starPEG or KA7–RGDSP–starPEG are mechanically stable during the various handling processes, including separation from the collection chamber, pipetting, and continuous shaking (up to 250 rpm). Rheological measurements after overnight incubation confirmed that the final stiffness of dextran sulfate hydrogels is higher than that of heparin hydrogels (Figure 2 b).[6] This clearly shows that stiffness and gelation time are inversely proportional. With the same weight concentration, 7.1 mM heparin possesses a viscosity of 0.00731 ± 0.000212 Pa s, whereas the viscosity of 20 mM dextran sulfate is lower (0.00169 ± 0.000068 Pa s). Though, dextran sulfate and heparin have different structure and molecular weight, the low viscosity could also contribute to the formation of homogeneous hydrogel beads with the microfluidic system (Figure 1 d).

Compared to other 3D biomatrix formats, the spherical shape is ideal for studying the effect of a gradient of signaling molecules on embedded cells, because a sphere is completely symmetric. We investigated whether the porosity of the hydrogel network would allow large proteins to penetrate through the bead matrix. Hydrogel beads were formed with fluorescently labeled antibody premixed in KA7–starPEG solution. Figure 2 c shows that the encapsulated 150 kDa IgG can be released from the beads into the medium within 30 min. These results indicated that the hydrogel beads could be used to create niches of cells, physically segregated from other cells, whereas communication through biochemical signaling would not be hindered by the matrix.

We have previously shown that the KA7–starPEG/heparin hydrogel can be used to encapsulate mammalian cells and support cell survival. While encapsulating cells, the backpressure in the microfluidic system can affect the cells, and cells can also influence the gel formation. We investigated whether the procession through the microfluidic system and the substitution of dextran sulfate for heparin affected the fate of embedded cells. We mixed human neonatal dermal fibroblasts (HDFn) with KA7–starPEG (final concentration of 3 mM) in full cell culture medium. The resulting suspension of cells (with KA7–starPEG) and a solution of dextran sulfate (final concentration of 20 mM) in full cell culture medium were injected into the two channels of the microfluidic device, and hydrogel beads containing cells were produced. Cells were distributed evenly in the beads. As shown in Figure 3 a and b 98 % of the cells were alive after seven days of culture in the beads. Interestingly, using lower levels of dextran sulfate lead to suboptimal bead formation when encapsulated cells were unevenly distributed in the matrix (data not shown). Although the rheological properties and gelation rates are similar between the two concentrations (7.1 mM and 20 mM dextran sulfate), the mixing of different components in the conjunction and the noncovalent cross-linking around the cells could be affected by the concentration. This high survival rate demonstrated that the bead formation process was not detrimental to the cells.

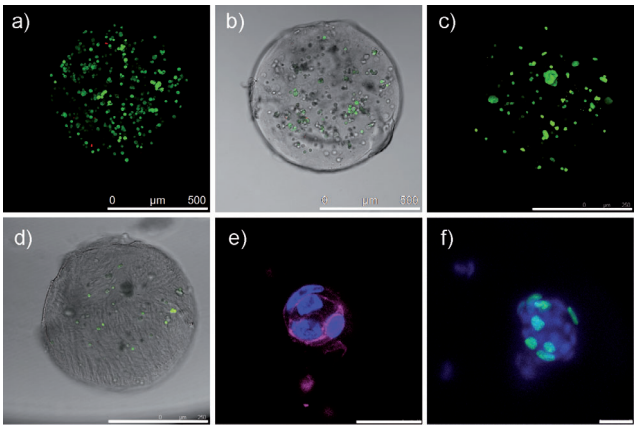

Figure 3. Cell survival of human neonatal dermal fibroblasts (HDFn) in hydrogel beads. a,b) Cell survival of HDFn seven days directly after embedding into hydrogel beads ($98 \pm 1\%$ survival; live cells, green (fluorescein); dead cells, red (propidium iodide)). c–f) HDFn seven days after recovering beads with cells from −196 °C. c–d) Cell survival of HDFn ($98 \pm 4\%$ survival). a,c) Confocal fluorescent image. b,d) Confocal fluorescent image and wide field image overlayed. e) DAPI phalloidin staining (for nucleus and actin). f) EdU proliferation staining (proliferating cells, green; nucleus, blue). a–f) Pictures taken using confocal laser scanning microscope. a–d) Scale bar: 500 µm. e–f) Scale bar: 25 µm.

Novel materials and methods that can improve survival rates of frozen cells would be useful for both biological research and the clinical use of cells.[10] We investigated whether hydrogel beads could be used as microcarriers for cell storage and transportation at low temperature. We froze and stored HDFn in hydrogel beads at −196 °C in liquid nitrogen for six weeks and analyzed cell survival (Figure 3 c and d). An amount of $86 \pm 3\%$ of the cells survived the freezing and storage procedure in hydrogel beads. About 3/4 of cells survived the normal freezing and storage procedure (Figure S7). In addition, the proliferation of cells embedded in the hydrogel beads was not disturbed (Figure 3 e and f). After culturing the encapsulated cells for seven days, the live/dead assay showed that 98 % of cells were alive in the microcarriers.

The cell compatibility of bead production, together with the fast release of large proteins from the beads, would allow us to design functional microcarriers, for example, for protein production by encapsulated cells.[9] SF9-ET insect cells infected with baculovirus expressing an eGFP fusion protein with the size of 88.7 kDa were embedded in the hydrogel beads. As control, a portion of the transfected cells were cultured in suspension. The amount of secreted protein was determined by measuring the fluorescence intensity in the

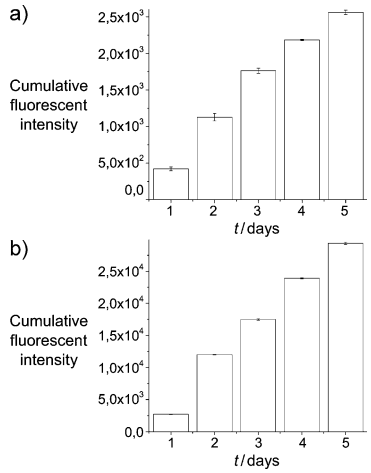

Figure 4. Protein expressed and secreted by insect cells embedded in hydrogel beads. SF9-ET insect cells were infected with baculovirus directing the secretion of an enhanced green fluorescent protein (eGFP) fusion protein of 88.7 kDa size. Cells were embedded in hydrogel beads prepared from KA7–starPEG and dextran sulfate in cell culture medium. Shown is the cumulative fluorescent intensity in the cell culture medium. a) Cumulative fluorescence intensity of eGFP produced by SF9-ET cells encapsulated in hydrogel beads. b) Cumulative fluorescence intensity of eGFP produced by SF9-ET cells in suspension culture.

supernatant. As shown in Figures 4 and S6, the expression profile of cells in suspension is similar to that of cells embedded in hydrogel beads. It is important to note that the amount of protein secreted per cell in the beads only varies by a factor of two compared to the cells in suspension. This shows that the 3D hydrogel environment does not influence the production and diffusion of the secreted protein.

Next we investigated whether the hydrogel bead system could support cell adhesion and growth on the bead surfaces, in order to create cell culture systems in the future mimicking the heterogeneity in vivo combined with embedded cells.[11,12] Hydrogel beads formed by KA7–starPEG and dextran sulfate showed poor attachment of HeLa cells. Addition of a cell attachment sequence RGDSP to the C-terminal of $(KA)_7$ peptide in KA7–starPEG, did not affect the gelation and bead production. HeLa cells expressing a GFP-actin fusion protein were added to the beads and cultured in full cell culture medium. As shown in Figure 5 a–e, addition of a cell attachment sequence resulted in good cell attachment on hydrogel beads.

Chemical postmodification of hydrogels that enables the tuning of the physical and biochemical environment of embedded cells to simulate natural conditions has received a large amount of interest recently.[5a,d,11,13] Noncovalent modifications have a number of advantages over covalent chemical reactions. Noncovalent binding is often not harmful to cells and can be used to create a gradient in a 3D matrix, resembling gradient-mediated biological events in vivo. We investigated whether analogues of the hydrogel components can be used to achieve noncovalent modification of the beads. We synthesized a TAMRA-labeled $(RA)_7$ peptide–starPEG conjugate (RA7–Rho–starPEG), because arginine-containing peptides have been shown to interact more strongly with

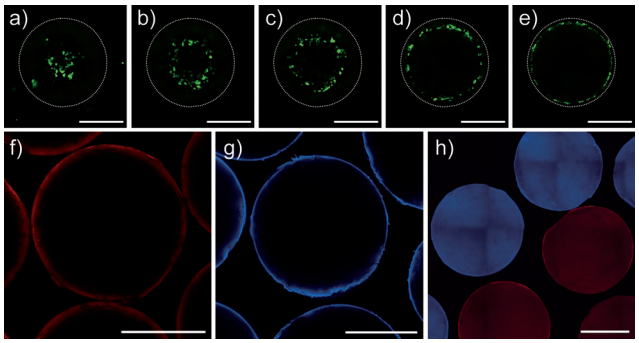

Figure 5. Cell attachment and noncovalent modifications of beads. Images were taken using confocal laser scanning microscope. a–e) GFP-HeLa culture on RGDSP-functionalized beads. Images from left to right show different focal height. f) Beads labeled with RA7–Rho–starPEG or g) modified with RA7–Bio–starPEG with Alexa 647 labeled streptavidin. h) Beads from (f) and (g) mixed in full cell culture medium for seven days. Scale bars: 400 μm.

sulfated oligosaccharides than their lysine-containing counterparts. As shown in Figure 5 f, the fluorescently labeled polymer formed a coating around the hydrogel beads instantly. Although no release of the labeled polymer was observed, slow diffusion of RA7–Rho–starPEG through the entire beads was observed over a time of two hours (Figure 5 f–h). The capture and release of biotinylated bioactive molecules has recently been developed as a versatile method to functionalize 3D matrices.[14] To demonstrate that the postmodification method can be used to introduce functional molecules, we synthesized a biotinylated $(RA)_7$ peptide–starPEG conjugate (RA7–Bio–starPEG). As shown in Figure 5 g, the RA7–Bio–starPEG-mediated coating and diffusion of Cy5-labeled streptavidin resembled those of RA7–Rho–starPEG. Moreover, no exchange of dyes between RA7–Rho–starPEG-treated beads and RA7–Bio–starPEG/streptavidin-treated beads was observed after 7 days. The postmodification method can lead to applications such as labeling a mixture of different microcarriers to mimic the heterogeneity and segregation of cells in vivo.

Here we report a hydrogel bead system based on noncovalent matrix formation. The high survival rate of cells encapsulated in the beads, even after freezing, demonstrates that the resulting macrocarrier is highly compatible for 3D cell culture. Large proteins, for example, antibodies, can diffuse through and be released from the beads. The simple and chemically defined matrix system can be functionalized through covalent conjugation with bioactive compounds, for example, a cell attachment sequence. Moreover, the noncovalent matrix can also be further modified noncovalently to introduce additional functionalities. The resulting materials are very stable and enable segregation of different cell populations, by either encapsulating cells in different beads or culturing cells on the sphere surface. It can be used for applications ranging from studying cells under natural and complex environment to protein production by microcarriers and cell replacement therapy.

Keywords: beads · cell encapsulation · gels · noncovalent interactions · protein release

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[1] a) D. Seliktar, *Science* **2012**, *336*, 1124 – 1128; b) C. A. DeForest, K. S. Anseth, *Annu. Rev. Chem. Biomol. Eng.* **2012**, *3*, 421 – 444; c) M. P. Lutolf, J. A. Hubbell, *Nat. Biotechnol.* **2005**, *23*, 47 – 55; d) A. Abbaspourrad, N. J. Carroll, S.-H. Kim, D. A. Weitz, *J. Am. Chem. Soc.* **2013**, *135*, 7744 – 7750; e) M. Windbergs, Y. Zhao, J. Heyman, D. A. Weitz, *J. Am. Chem. Soc.* **2013**, *135*, 7933 – 7937; f) S. W. Cranford, J. de Boer, C. van Blitterswijk, M. J. Buehler, *Adv. Mater.* **2013**, *25*, 802 – 824.

[2] M. Prewitz, F. P. Seib, T. Pompe, C. Werner, *Macromol. Rapid Commun.* **2012**, *33*, 1420 – 1431.

[3] a) G. Y. Huang, L. H. Zhou, Q. C. Zhang, Y. M. Chen, W. Sun, F. Xu, T. J. Lu, *Biofabrication* **2011**, *3*, 012001; b) R. Dangla, S. C. Kayi, C. N. Baroud, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 853 – 858; c) R. Gauvin, R. Parenteau-Bareil, M. R. Dokmeci, W. D. Merryman, A. Khademhosseini, *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2012**, *4*, 235 – 246; d) D. Velasco, E. Tumarkin, E. Kumacheva, *Small* **2012**, *8*, 1633 – 1642.

[4] a) A. C. Mendes, E. T. Baran, P. Lisboa, R. L. Reis, H. Azevedo, *Biomacromolecules* **2012**, *13*, 4039 – 4048; b) A. M. Smith, N. C. Hunt, R. M. Shelton, G. Birdi, L. M. Grover, *Biomacromolecules* **2012**, *13*, 4032 – 4038; c) A. R. Patel, C. Remijn, A. M. Cabero, P. C. M. Heussen, J. W. M. Seijen ten Hoorn, K. P. Velikov, *Adv. Funct. Mater.* **2013**, *23*, 4710 – 4718.

[5] a) A. Acarregui, J. L. Pedraz, F. J. Blanco, R. M. Hernández, G. Orive, *Biomacromolecules* **2012**, *14*, 322 – 330; b) J. F. Guo, G. W. Jourdian, D. K. MacCallum, *Connect. Tissue Res.* **1989**, *19*, 277 – 297; c) G. Palmieri, G. Cassani, G. Fassina, *J. Chromatogr. B* **1995**, *664*, 127 – 135; d) W. H. Tan, S. Takeuchi, *Adv. Mater.* **2007**, *19*, 2696 – 2701.

[6] R. Wieduwild, M. V. Tsurkan, K. Chwalek, P. Murawala, M. Nowak, U. Freudenberg, C. Neinhuis, C. Werner, Y. Zhang, *J. Am. Chem. Soc.* **2013**, *135*, 2919 – 2922.

[7] C. R. Ricketts, K. W. Walton, *Br. J. Pharmacol.* **1953**, *8*, 476 – 477.

[8] R. Wieduwild, W. Lin, A. Boden, K. Kretschmer, Y. Zhang, *Biomacromolecules* **2014**, *15*, 2058 – 2066.

[9] L. Chu, D. K. Robinson, *Curr. Opin. Biotechnol.* **2001**, *12*, 180 – 187.

[10] a) E. Jain, A. Kumar, *Nat. Protoc.* **2013**, *8*, 821 – 835; b) P. F. Costa, A. F. Dias, R. L. Reis, M. E. Gomes, *Tissue Eng. Part C* **2012**, *18*, 852 – 858; c) P. B. Welzel, M. Grimmer, C. Renneberg, L. Naujox, S. Zschoche, U. Freudenberg, C. Werner, *Biomacromolecules* **2012**, *13*, 2349 – 2358.

[11] a) Y. Morimoto, R. Tanaka, S. Takeuchi, *Adv. Healthcare Mater.* **2013**, *2*, 261 – 265; b) M. Serra, C. Brito, C. Correia, P. M. Alves, *Trends Biotechnol.* **2012**, *30*, 350 – 359.

[12] a) S. Allazetta, T. C. Hausherr, M. P. Lutolf, *Biomacromolecules* **2013**, *14*, 1122; b) J. A. King, W. M. Miller, *Curr. Opin. Chem. Biol.* **2007**, *11*, 394 – 398; c) S. G. Kupal, B. Cerroni, S. V. Ghugare, E. Chiessi, G. Paradossi, *Biomacromolecules* **2012**, *13*, 3592 – 3601.

[13] a) Y. Du, E. Lo, S. Ali, A. Khademhosseini, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 9522 – 9527; b) C. Y. Li, D. K. Wood, C. M. Hsu, S. N. Bhatia, *Lab Chip* **2011**, *11*, 2967 – 2975; c) T. Rossow, J. A. Heyman, A. J. Ehrlicher, A. Langhoff, D. A. Weitz, R. Haag, S. Seiffert, *J. Am. Chem. Soc.* **2012**, *134*, 4983 – 4989.

[14] R. G. Wylie, S. Ahsan, Y. Aizawa, K. L. Maxwell, C. M. Morshead, M. S. Shoichet, *Nat. Mater.* **2011**, *10*, 799 – 806.

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