University of Groningen

Non-Covalently Stabilized Alginate Hydrogels as Functional Cell Scaffold Material

Kuhn, Philipp T.; Meijer, Thomas L.; Schiavon, Irene; van Poll, Mathijs; van Aken, Joris; Groen, Swen; Kuijer, Roelof; van Kooten, Theo G.; van Rijn, Patrick

Published in:
Macromolecular Bioscience

DOI:
[10.1002/mabi.201600214](https://doi.org/10.1002/mabi.201600214)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Kuhn, P. T., Meijer, T. L., Schiavon, I., van Poll, M., van Aken, J., Groen, S., Kuijer, R., van Kooten, T. G., & van Rijn, P. (2016). Non-Covalently Stabilized Alginate Hydrogels as Functional Cell Scaffold Material. *Macromolecular Bioscience*, 16(11), 1693-1702. <https://doi.org/10.1002/mabi.201600214>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Non-Covalently Stabilized Alginate Hydrogels as Functional Cell Scaffold Material

Philipp T. Kühn, Thomas L. Meijer, Irene Schiavon, Mathijs van Poll, Joris van Aken, Swen Groen, Roel Kuijer, Theo G. van Kooten, Patrick van Rijn*

Biopolymers are an attractive class of compounds for being used in biomedical applications as they are widely available from biomass. Their drawback is the lack of mechanical stability and the ability to tune this properly. Covalent chemical cross-linking is an often used approach but it limits usability due to legislation as well as the need of advanced and specialized knowledge by end users such as clinicians. Here, increased and tunable mechanical properties are achieved of alginate-based hydrogels with non-covalent approaches using linear polyethyleneimine (LPEI) as a polyelectrolyte rather than only multivalent metal ions (Ca^{2+}). Gel stiffness increases with increasing LPEI content. Gel morphology changes from a thin fibrous mesh for alginate- Ca^{2+} to thicker fibrous networks when LPEI is introduced. The gels are able to efficiently release encapsulated small molecular dyes and the gels are able to host cells. For the cell encapsulation human skin fibroblasts (HSkF) and human bone marrow-derived mesenchymal stem cells (hBM-MSC) are used. HSkF can be successfully incorporated without diminished viability while the matrix components and gel preparation method are not compatible with hBM-MSC. The newly developed alginate-based system is regarded as a potential candidate for wound dressing materials.

1. Introduction

Hydrogels are an important class of materials in biomedical applications such as tissue engineering/regenerative

medicine, drug delivery, and wound healing.^[1–6] The soft and fibrous nature together with a high water content and the possibility for diffusion of small components make this class of materials an ideal mimic of the extracellular matrix (ECM). Therefore, hydrogels are regarded as excellent materials for encapsulating cells.^[7–10]

Many approaches have been taken to design new hydrogel biomaterials for cell encapsulation ranging from synthetic structures, biological based structures, or chemically derivatives thereof.^[11–19] Biologically derived structures generally offer more potential to be clinically relevant as these are less likely to be cytotoxic, are often already known by regulatory bodies, and trigger only minimal immune responses.^[9,14,20] Low molecular weight peptide-based hydrogels are often used as these can easily be integrated with functional peptide sequences to promote cell adhesion; biopolymer-based hydrogels offer other major advantages.^[21–23] Biopolymers, polypeptides

P. T. Kühn, T. L. Meijer, I. Schiavon, M. van Poll, J. van Aken, S. Groen, Dr. R. Kuijer, Dr. T. G. van Kooten, Dr. P. van Rijn
Department of Biomedical Engineering-FB40
W. J. Kolff Institute for Biomedical Engineering
and Materials Science-FB41
University of Groningen
University Medical Center Groningen
Groningen, A. Deusinglaan 1
9713 AV, Groningen, The Netherlands
E-mail: p.van.rijn@umcg.nl
Dr. P. van Rijn
Zernike Institute for Advanced Materials
University of Groningen
Nijenborgh 4, 9747 AG, Groningen, The Netherlands

such as collagen/gelatin, elastin, and silk but also polysaccharides like hyaluronic acid, chitosan, chondroitin sulfate, and alginate are used since these can be readily isolated from biomass.^[14,20,23–25] In addition, alginate systems have been used for the investigation of stress relaxation toward cells, since the ionic nature of their cross-linking allows stress relaxation, which is not the case for covalently cross-linked gels.^[26] Also, alginate shows a good biocompatibility and is cheap and hence cost-effective.^[27]

The polysaccharide-based hydrogels have the advantage that they are easy to work with and gel formation is generally done by simple mixing of the polysaccharide solution with a solution which initiates cross-linking, most often via electrostatic interactions using multivalent ions.^[9,28–31] This practical benefit also causes one of the most important drawbacks of these systems as cross-links induced by multivalent cations such as Ca^{2+} are easily broken, washed out, or replaced by other monovalent ions.^[20] This effect can be reduced by using cations such as Al^{3+} and Fe^{3+} which bind more efficiently but the effect cannot be prevented completely.^[32] Therefore increasing mechanical stability is a significant challenge especially without affecting the ease of use.

Many approaches have been taken to increase stability of polysaccharide hydrogels such as alginate, chondroitin sulfate, and hyaluronic acid which all face the problem of low stability. The most frequently taken route toward increased mechanical stability is chemical modification for the purpose of covalent cross-linking rather than using electrostatics.^[33] To this end, several routes have been explored including the introduction of disulfide bridges,^[34] using click-reactions to induce cross-linking,^[35] dopamine modification and cross-linking via polydopamine formation,^[36] or combination of oxidized structures with hydrazine derivatives able to form hydrazone cross-links.^[37] However, the most often used approach is introducing polymerizable units such as acrylates which offer the possibility to create a second polymer network based on free radical polymerization initiated either chemically or via UV-irradiation.^[38–42] This has also led to interesting highly stabilized structures for in vivo applications using chemical modifications and covalent cross-linking.^[13,24,40,43] Even though these systems possess higher stability, chemical modifications hamper efficient use as formation, purification, use by clinical experts, and legislation aspects all play important roles in successful biomaterials development.

Here, we devised a route which increases alginate gel stability both chemically and mechanically without synthetic modifications and with retention of ease of formation via simple two-component mixing. The system functions similar to the standard alginate- Ca^{2+} system with the important addition of linear polyethyleneimine

(LPEI) constituting as a polyelectrolyte at pH 7 and acting as a highly efficient cross-linker. Other systems involving the use of LPEI have also been investigated for the purpose of gene delivery and for modulating the matrix stiffness.^[44–47] For enhancing the matrix stiffness, two important aspects were overlooked in previous studies, preventing the matrix to be suitable for cell encapsulation. LPEI was pre-mixed with alginate which leads to pre-coordination and potential inhomogeneity inside the matrix. Additionally, such pre-mixing will make coordination for Ca^{2+} more difficult as exchanging a polyelectrolyte for many smaller ions is entropically unfavorable. Also for cell encapsulation, pre-mixing the LPEI with alginate will result in a high initial LPEI concentration which will affect cell viability as cells are generally suspended in the matrix-forming solution, the alginate. The route developed here uses a pre-mix of Ca^{2+} and LPEI which ensures more control of morphology but also of stiffness as the obtained stiffness is much lower as previously reported. Targeting the lower kPa-range as presented here can be important regarding cellular functions as cells respond very differently to moduli in the range of 1–20 kPa than toward the higher moduli (>50 kPa), as reported for the other alginate-LPEI systems.^[48] By partially replacing Ca^{2+} -ions for LPEI while keeping the overall available positive charge the same, stronger hydrogels were formed with tunable stiffness, controllable release profile of small molecules, and with the ability to entrap cells. The resulting cell laden alginate-LPEI hydrogels are envisioned to be potential candidates in wound dressing hydrogel applications, especially as the presented approach and altered mixing, using culture medium as a solvent rather than saline solution, displays the same low cytotoxicity toward fibroblasts compared to the regular alginate- Ca^{2+} system and only increased cytotoxicity for the mesenchymal stem cells at higher LPEI ratios.

2. Results and Discussion

2.1. Polymer-Alginate Hydrogel Formation

Polymer-alginate gels with improved and tunable mechanical properties were formed by starting from the original alginate- Ca^{2+} hydrogel system. Sodium alginate does not gelate while alginate- Ca^{2+} does due to formation of cross-links between two negatively charged carboxylic acid groups of the alginate and a divalent metal center, Ca^{2+} (Figure 1). Higher charged metal ions will only add stability in a limited way because the increased charge is very locally defined, namely, on a single atom^[32,49] and therefore the attention was focused on polyelectrolytes in the form of LPEI. LPEI is a polyelectrolyte at pH 7 as the pKa of the secondary amines in LPEI is about 10–11. Although,

Figure 1. Schematic representation of the gelation process for alginate- Ca^{2+} (left) and a mixture of Ca^{2+} and LPEI (right). LPEI shows multiple non-covalent bonds on a single LPEI molecule, while Ca^{2+} has only two.

LPEI can be considered toxic to cells due to the high amount of positive charges, when appropriately screened with polyanions, the toxicity diminishes drastically and hence it is also used as a gene-transfection agent.^[50,51] As alginate- Ca^{2+} hydrogels are known for a long time in biomedical applications, their preparation conditions have been optimized in order to facilitate efficient formation of gels.^[28,52,53] Therefore, the relative amount of charge within the system was kept constant in order to make use of the formerly optimized systems. However, even though the number of charges is kept constant it was envisioned that the resulting gel would be more stable due to better distribution and limited mobility of charge. The coordination of charged species is a reversible process. When the coordination of one charged species is broken, it will coordinate to an opposite charge again in time. While it is likely that Ca^{2+} ions are relatively mobile in the gel due to the reversibility of the coordination, the LPEI is expected to have limited mobility, since the multiple coordination holds it in place. Generally, a $50 \times 10^{-3} \text{ M}$ CaCl_2 is mixed with a 2 wt% alginate solution in a 1:1 ratio. This results in a hydrogel which is very soft and deformable. Often alginate beads are produced for studying cellular behavior in a 3D hydrogel matrix however, for

translation toward applicable systems, the gels are too soft and deformable. Using LPEI, it was anticipated that due to the increased possibilities for cross-linking, the hydrogel would become more stable. However, keeping the same molar concentrations as used for CaCl_2 , the concentration of positive charges would be much higher, as the used LPEI (M_W : 1200 Da) provides ≈ 28 amino groups per polymer chain and hence has a much higher overall charge at neutral pH. For this reason, the theoretically available charge was kept constant stating that $50 \times 10^{-3} \text{ M}$ CaCl_2 provides $100 \times 10^{-3} \text{ M}$ of positive charge while $3.6 \times 10^{-3} \text{ M}$ LPEI solution also provides $100 \times 10^{-3} \text{ M}$ positive charge, assuming all amino groups are protonated. Mixing the CaCl_2 and LPEI solutions in different ratios allows for tuning the cross-linking capabilities within the gel system without affecting the overall available charge and results in different hydrogel morphologies (Figure 1).

It was found that CaCl_2 alone together with the alginate forms a soft deformable hydrogel while the alginate cross-linked with only LPEI solution (same concentration positive charge) forms a highly condensed rubber-like material. Locally better distributed cross-linking with higher efficiency results in a precipitated bulk material. By replacing the strongly coordinating polyelectrolyte for Ca^{2+} with “weaker” cross-linking capabilities, intermediate stages were accomplished (Figure 2). This was initially investigated by macroscopic observations of a broad range of combinations between 0 and 2 wt% alginate, $0\text{--}50 \times 10^{-3} \text{ M}$ CaCl_2 , and $0\text{--}3.6 \times 10^{-3} \text{ M}$ LPEI. This approach allowed to quickly identify useable ranges of concentrations for improving mechanical properties and select compositions which would serve as potential cell carrier matrices useable for wound coverage applications in the future.

The macroscopic observations were classified according to the behavior of the formed gel materials. Visual observations along with applied mechanical forces using

Figure 2. Alginate-based hydrogels formed from various combinations of alginate concentration, CaCl_2 , and LPEI were investigated to assess efficiently the system behaviors and the conditions most likely to be suitable for cell scaffolds.

the inverted test-tube assessment indicate whether the formed system is a solution (completely soluble, viscous, or turbid) or a gel (weak, stable, self-supporting, or highly condensed). It was found that too low concentrations of CaCl_2 and LPEI resulted in solutions while a relatively high concentration of LPEI with respect to the alginate resulted in rubber-like material, which was not identified as a gel anymore. Two possible conditions which displayed the most promising macroscopic behavior were alginate (2 wt%) mixed in a 1:1 volume ratio with CaCl_2 /LPEI solutions of either $25 \times 10^{-3} \text{ M}/1.8 \times 10^{-3} \text{ M}$ or $50 \times 10^{-3} \text{ M}/3.6 \times 10^{-3} \text{ M}$. The $50 \times 10^{-3} \text{ M}/3.6 \times 10^{-3} \text{ M}$ displayed a condensed hydrogel-like structure which could be handled easily without damaging the material and which was not as dense as the rubber-like material obtained with higher LPEI content. Therefore, this was regarded as the best hydrogel material as it resembled a material which could directly be used as a potential wound coverage material since it can easily be handled without braking, but still contained a high amount of water for moisturizing wounds. The 1:1 ($50 \times 10^{-3} \text{ M}/3.6 \times 10^{-3} \text{ M}$) CaCl_2 /LPEI ratio provides $100 \times 10^{-3} \text{ M}$ positive charge concentration, the CaCl_2 /LPEI ratio was altered by lowering the LPEI content and increasing the CaCl_2 content, while keeping the overall charge concentration the same. Lowering the LPEI content would affect stability and stiffness which was investigated using low load-compression testing (LLCT).^[54]

2.2. Mechanical Properties of Polymer-Alginate Hydrogels

In alginate gels formed by Ca^{2+} cross-linking, the Ca^{2+} ions have a high mobility compared to LPEI molecules. This results from the fact, that the high number of reversible bonds formed by charge interactions are very likely of holding the LPEI molecule in place, while the Ca^{2+} ion with only two bonds can travel more freely through the gel. This mobility of the ions reduces stability and stiffness of the resulting gels. Replacing Ca^{2+} with LPEI should therefore increase stability as well as stiffness of the resulting gel. This was investigated using LLCT which determines the stiffness of the hydrogels. All hydrogels were in their completely hydrated state and were measured after 24 h equilibration.

For preparation the charge percentage (%) originating from LPEI was increased while keeping the overall amount of charge versus alginate content constant. The more charge originates from the LPEI, the higher the Young's modulus (Figure 3). The alginate- Ca^{2+} standard hydrogel amounts to 3.6 kPa in Young's modulus which increases continuously to 16.8 kPa for alginate- Ca^{2+} /LPEI (50/50 charge contribution), indicating a direct correlation between Young's modulus and the ratio of LPEI to Ca^{2+} ions. Apparently, the multi-charged polymer is

Figure 3. Young's modulus in kPa for alginate hydrogels formed by mixing 2 wt% alginate solution with polycation solution (1:1 volume) of varying composition between CaCl_2 and LPEI. The final alginate-charge ratio is equal in all cases, only the origin of the charge differs.

better capable of cross-linking the alginate. The stiffness is in this case also associated to mechanical stability. The higher percentage of charge originating from LPEI, the more mechanically robust the hydrogel. In case of the 50/50 charge contribution, the hydrogel is stable enough to be easily taken up with tweezers without being damaged (Figure 4) which was not possible for the pure alginate- Ca^{2+} hydrogel. This stability was also observed when investigating the stability concerning degradation. An additional problem of the conventional alginate hydrogels, also shared with other biopolymer hydrogels such as hyaluronic acid-based systems, is that the cross-links can be easily broken by high concentrations of monovalent

Figure 4. Photographs of the alginate hydrogels which display higher mechanical stability than the alginate- Ca^{2+} standard. The left image shows the Ca^{2+} /LPEI (90/10) (left) and Ca^{2+} /LPEI (50/50) (right) charge originating from LPEI. The right image shows how the most stable gel can be handled without inflicting mechanical damage.

Figure 5. Atomic force microscopy images of alginate hydrogels based on A,B) only Ca^{2+} (enlarged image of (A)) as a counter ion, C,D) Ca^{2+} /LPEI (90/10) (enlarged image of (C)), and E,F) Ca^{2+} /LPEI (50/50) (enlarged image of (E)). With increasing LPEI charge contribution, the network becomes more open and the fibers thicker.

cations or via strong chelating agents toward Ca^{2+} such as citrate.^[43,55] The addition of sodium citrate to alginate- Ca^{2+} resulted in rapid complete dissolution of the gel while the alginate- Ca^{2+} /LPEI (50/50) did not show any signs of dissolution. The alginate- Ca^{2+} /LPEI (50/50) hydrogel could only be destroyed by lowering the pH below the pKa (4.5) of the alginate resulting in loss of coordination due to protonation of carboxylic acids on the alginate. Alternatively, the gel could also be broken by increasing the pH above the pKa of the LPEI (10–11) which resulted in deprotonation of the LPEI and hence the loss of coordination/cross-links between LPEI and alginate.

The exchange of Ca^{2+} for LPEI clearly results in increased mechanical stability, higher stiffness, and lower susceptibility to chemical degradation. As mentioned above, this higher stability is mostly due to the increased cross-linking capability of LPEI, which should also affect the overall morphology as well as the release of molecular components. Particularly the latter is important when soluble factors from cells need to be transported through the network into the wound area in case of wound coverage materials.

2.3. Morphology and Permeability of Polymer-Alginate Hydrogels

In order to test, if the altered gel compositions change the gels morphology, namely, matrix density, porosity, and fiber thickness, and thereby diffusion capability, the gels were investigated using atomic force microscopy (AFM)

under wet conditions (Figure 5) by measuring in PBS (phosphate buffer saline) to have a hydrated network under similar conditions as for the final intended application as wound coverage material.

From the AFM analysis it was observed that the mesh-size changes drastically with increasing LPEI content. While the regular alginate- Ca^{2+} gel has thin fibers with a dense mesh with small pores, the higher LPEI content displays thicker fibers. The thicker fibers result in a more open network as the total mass content of the alginate remains the same, condensing fibers by using LPEI will be at the cost of network distribution. A closer analysis of the network with AFM shows that the thicker fibers of the Ca^{2+} /LPEI (50/50) are very globular and irregular in diameters while the alginate- Ca^{2+} is very homogeneously distributed.

AFM analysis displayed a more open structure for the alginate hydrogels with higher LPEI content and this was also reflected in the release of a small molecular dye from the alginate network. For the release study the dye, 4-amino-1,1'-azobenzene-3,4'-disulfonic acid sodium salt, was used. This is a relatively complex molecule resembling functional groups also found in molecular and peptide-based soluble factors released by cells, with negative ($-\text{SO}_3\text{H}$) and positive charge ($-\text{NH}_2$) at pH 7 as well as the capability to interact via hydrogen bonding ($-\text{N}=\text{N}-$). By determining the release from the gel using the molecular dye, it was found that the more open structures with larger pores as formed by the alginate- Ca^{2+} /LPEI release their content more rapidly than the denser structure with

Figure 6. Release profiles of 4-amino-1,1'-azobenzene-3,4'-disulfonic acid sodium salt from alginate- Ca^{2+} , alginate- Ca^{2+} /LPEI (90/10), and alginate- Ca^{2+} /LPEI (50/50) are shown. The more open network with larger pores as shown by AFM (Figure 5) is reflected in the increasingly higher and more rapid release of the dye from the hydrogel network.

smaller pores as formed by the alginate- Ca^{2+} hydrogel (Figure 6). The release becomes increasingly more efficient going from alginate- Ca^{2+} to alginate- Ca^{2+} /LPEI (90/10) and finally alginate- Ca^{2+} /LPEI (50/50). The final release in the surrounding solution for the Ca^{2+} /LPEI (90/10) and Ca^{2+} /LPEI (50/50) amounts to full release of 100% while for Ca^{2+} it was only 68%. In all cases an initial burst release between 10% and 12% of the total release was found. The release profile shows that small molecules with diverse chemical attributes are able to pass the gel matrix without difficulty. Although the Ca^{2+} /LPEI behaves differently than the alginate standard, the release profile is still in the same order of magnitude. An increased release rate would be regarded as beneficial. Cell nutrients and the potential therapeutic soluble factors secreted by cells should diffuse as freely and uninhibited as possible through the network enabling most efficient and long-term effects. Most likely the lowered cumulative release from the alginate- Ca^{2+} standard can be attributed to the small mesh-size. It should be noted that the soluble factors released by cells are more diverse in size and possible interactions and hence the used molecular dye provides insights in diffusion differences between the gels but not exactly reflects the final diffusing profile of the soluble cell factors.

In addition to the release profile, cells which ultimately should secrete the small soluble factors need to remain viable. Both the encapsulation procedure and the final hydrogel matrix should not direct cells into an apoptotic state. This was investigated for human skin fibroblasts (HScF) and bone marrow-derived mesenchymal stem cells (hBM-MSC), both very promising candidates for the purpose of wound repair.

2.4. Cell Encapsulation and Toxicity

Hydrogels are envisioned to be excellent environments for cells as it mimics the extracellular matrix to a certain degree. The nature of the hydrogel, open porous matrix, high water content, and possibility for diffusion are highly important for the cell function as nutrients and cell exudates need to be transported to and away from the cells. For encapsulation in the gels, two cell types were chosen, namely, HScF and hBM-MSC. Both these cell types are able to secrete soluble factor such as fibroblast growth factors (FGFs), transforming growth factor (TGFs) derivatives, and vascular endothelial growth factor (VEGF). These growth factors as well as many others play eminent roles in delegating wound repair.^[56,57]

In order to assess the microenvironment within the gel, the response of the cells toward the two solutions needed for creating the gels was determined, namely, alginate and CaCl_2 /LPEI. A metabolic activity assay (XTT assay) was performed using HScFs in combination with an alginate solution and polyelectrolyte solution compared to a negative control (Figure S1, Supporting Information). It was found that the polyelectrolyte solution containing LPEI negatively influenced the cell viability as expected while the alginate solution did not influence the cell viability.

It was not possible to perform a viability assay directly on the encapsulated cells as the gels inhibit proper absorbance measurements needed for the XTT assay, possibly due to binding of XTT to the matrix components. An indirect approach was used where freshly prepared gels were incubated in growth medium for 24 h. This growth medium was subsequently used for incubation of the cells for the assay. It was found that there is a significant difference in behavior comparing HScFs and hBM-MSCs (Figure 7). While the HScFs did not display any altered metabolic activity compared to the negative control which was not exposed to gel incubated medium, the hBM-MSCs were not able to tolerate the gel components left in solution. This clearly shows that the fibroblasts are more robust toward LPEI than the hBM-MSCs. For future applications as potential wound dressing material, hBM-MSCs are not the appropriate cell type to be used whereas fibroblasts are.

In order to obtain additional information concerning the survival of cells within the gels, live-dead analysis was performed using confocal laser scanning microscopy (CLSM) and fluorescence microscopy. HScF cells were entrapped inside the gels via mixing of the alginate solution (2 wt%) with one volume of polyelectrolyte solution. To entrap the cells inside the gel matrix, they were dispersed in the alginate solution. Gel preparation was performed as previously stated. Analysis of cells within the alginate hydrogels was cumbersome due to the

Figure 7. Metabolic activity assay (XTT) for human skin fibroblasts (cell line, CCD112SK) and human bone marrow mesenchymal stem cells. Cells were exposed to gel-exposed medium for 24 h before metabolic activity determination. The bar diagram displays the normalized metabolic activity with the activity corrected for the cell density and subsequently normalized in accordance to the control sample. The asterisk indicates significance ($p \leq 0.05$).

interference of the gel network with the CLSM analysis and hence only cells rather close to the surface of the hydrogel could be analyzed (Figure 8). It was observed that for a short-term culture, the LPEI showed no negative effect. After 5 d of culture, the HSKF were negatively affected in the gel with the highest concentration of LPEI (50:50). The Ca²⁺/LPEI (90:10) gel performed similar to the original alginate gel. Also cell proliferation seemed not to be affected by the polymer. This indicates that LPEI can be used to improve the alginates' stability, as long as the concentration is not too high.

In order to have an additional idea of cellular response toward the hydrogel, cells were seeded in well-plates and after sufficient adhesion times, the gels were prepared directly on top of the cells. After incubation for 3–4 d, the gel was removed uneventfully and the cells visualized via live-dead staining. From the live-dead staining, it is observed that the gels with higher LPEI content inhibit cell proliferation (Figure S2, Supporting Information). There are fewer cells visible when they are exposed to the alginate-Ca²⁺/LPEI hydrogel than when exposed to the conventional alginate hydrogel. Even though there are fewer cells visible, the live-dead staining only shows a minor fraction of dead cells which are also observed for the conventional alginate hydrogel. This shows a difference toward the encapsulated cells, where LPEI induced killing. The morphology of the cells in contact with LPEI-containing gels is aberrant, indicating that they are under stress. The observed difference in spreading and coverage is most likely due to the initial exposure to a too high

concentration of non-balanced LPEI. It is not due to the lack of nutrients as the alginate-Ca²⁺ hydrogel displays lower diffusion than the alginate-Ca²⁺/LPEI hydrogels as shown above.

LPEI by itself is toxic to cells however, by offering structures which are able to screen excess positive charges by coordination, if the concentration is not too high. This shows that the stability of alginate gels can be improved using LPEI, but that a low concentration should be chosen to do so.

3. Conclusion

Developing new hydrogel scaffolds for cell encapsulation is an ongoing endeavor and while improving one property, other properties will be affected. One major issue is hydrogel stability especially, of those which are already used clinically. Rather than developing new hydrogel systems, improving FDA-approved ones seems a more cost-efficient approach although not a reason for not pursuing new synthetic systems. Alginate-based hydrogels are already found in numerous biomedical products but as identified, still lack the mechanical stability hence the reason for chemical cross-linking as it poses as the more stable alternative. However, this at the expense of the ease of gel preparation will hamper true clinical use. Here we have defined an approach which allows for retention of the easy preparation method while enhancing the mechanical properties as well as tuning the stiffness. Both alginate and polyethyleneimine are FDA-approved materials for various biomedical applications and together with the ease of formation it offers a more versatile material formation than the covalent cross-linking approach. Both human skin fibroblast and human bone marrow mesenchymal stem cells have been included of which fibroblasts are able to endure the gel formation. This also displays still an eminent drawback of the system which will be subject to further investigation, which should aim at making the environment truly cell friendly. Although fibroblasts do survive the preparation procedure, still proliferation is inhibited. Future endeavors will encompass other preparation methods in which cells are more effectively shielded from the positive charges of the LPEI or alternatives will be identified, keeping in mind the cost effectiveness of LPEI against much costlier alternatives such as polylysine.

4. Experimental Section

All chemicals were obtained from Sigma Aldrich, USA, unless stated otherwise. Dissolved linear polyethyleneimine (50 wt%) 1200 M_w was used as the polyelectrolyte. Absorbance measurements were performed in microplate reader from FLUOstar Optima (BMG Labtech). Cell culture flasks and well plates

Figure 8. Dead/Live staining of HSKF seeded inside of gels made from a,d) alginate-Ca²⁺ (live staining and dead staining, respectively), b,e) alginate-Ca²⁺/LPEI (90/10) (live staining and dead staining, respectively), and c,f) alginate-Ca²⁺/LPEI (50/50) (live staining and dead staining, respectively) after 5 d of culturing. The asterisk indicates significance ($p \leq 0.05$). For live staining calcein AM (green) was used and ethidium homodimer-1 (red) for dead staining. Scale bar in all images represents 100 μm. Graphs displayed show the g) cell density and h) amount of dead cell (dead percentage) inside of the gels after 1 and 5 d.

were purchased from Greiner Bio-one. Human skin fibroblasts (HSKF-CCD-1112Sk, ATCC CRL2429) and human bone marrow-derived mesenchymal stem cells (Lonza) were used for the cell experiments. Cells were observed using a LEICA TCS SP2 CLSM equipped with 40 × NA 0.80 and 63 × NA 0.90 water immersion objectives. Cell viability was analyzed using an XTT assay (Applchem A8088).

Gel Formation: Gels were formed by mixing one volume of a 2 wt% alginate solution with one volume of polyelectrolyte solution. This results in a gel containing 1 wt% alginate. For the polyelectrolyte solution, the overall amount of charge was kept constant. It was calculated that a stock solution of 50×10^{-3} M CaCl₂ solution and a stock solution of 3.6×10^{-3} M LPEI solution formally contain the same amount of positive charge (under the assumption that all amine groups are protonated). The different gels were obtained by mixing the CaCl₂ and LPEI solution varying

the overall charge contribution of LPEI between 0% and 50 %, and to reach the same final volume as the desired volume of alginate solution. This was then mixed to obtain the final gel.

Cell Encapsulation: For the cell encapsulation, cells were suspended in the alginate solution before gel preparation. Alginate solution was prepared using DMEM (Dulbecco's modified Eagle medium, Thermo Fischer Scientific, USA) rather than water which ensured higher viability of the cells. This solution was then used similar as described above for the regular gel preparation. No significant differences in gel properties were found using DMEM instead of water. Cells were analyzed 3–4 d after incubation.

Determining Young's Modulus: The stiffness (Young's modulus) of the gels was measured using LLCT. A uniaxial compression was performed measuring strain and stress, in a non-destructive way. Gel samples were applied on filter paper resting on a glass slide in order to avoid sample displacement during compression.

A few microliters of water were added on top of the sample ensuring contact of the sample with the plunger before commencing compression. All measurements were performed with a fixed strain rate of $5\% \text{ s}^{-1}$ and a maximum deformation of 20%. For each gel, five samples were measured. Obtained values for stress and strain were extrapolated using Hooke's Law. For determining the stiffness, only the initial 10%–15% of the values of the Young's modulus curve were taken into account. Values of five individual experiments per gel were averaged.

Controlled Release from Hydrogels: 10 mmol of 4-amino-1,1'-azobenzene-3,4'-disulfonic acid sodium salt was dissolved in 2 wt% alginate solution. This solution was dropwise added to the polyelectrolyte solution of desired composition, forming beads rather than homogenous gels. For the release, the approach of bead formation was chosen as it is easier to homogenize the solution via gentle shaking, ensuring that surface bound dye is transferred to the bulk solution. After addition, 100 μL samples were taken and analyzed using photon-absorption spectroscopy. The initial sample was taken directly after bead formation and designated as time-point "0" and subtracted from the subsequent measurements to account for initial burst release. Thereafter, samples were taken until 27 h after initial bead formation, after which the beads were completely destroyed. The measurement of this solution was taken as 100%.

Cell Culture HSKf and hMSCs (HSKf-CCD-112SK, ATCC CRL2429, and hMSCs): Human skin fibroblast (CCD112SK) and hBM-MSC were used. Cells were incubated at 37°C , 5% CO_2 at maximum humidity. Cell culture stock was kept in liquid nitrogen. Cells were counted with hemocytometer. DMEM HG Complete (10% FBS, 1% L-glutamax with 1% (100 units) penicillin and (0.01 mg) streptomycin in DMEM) was used as growth medium for fibroblast cells. Alpha-MEM complete (10% FBS and 0.1% AA2P in Alpha-MEM). Cells were harvested from culture flasks by trypsin for 3–5 min at 37°C . Cells were cultured at 80%–90% confluence and 20% was seeded in daughter flasks.

Cytotoxicity Assay (XTT): Human fibroblast CCD112SK and mesenchymal stem cells were used for determining cytotoxicity. An indirect cytotoxicity assay was performed because it was not possible to isolate the cells from the non-standard alginate gels via mild approaches due to enhanced stability. The respective gels were prepared and submerged in growth medium for 24 h prior to use. Cells were grown and harvested and seeded in a 96-well plate with a density of 1.0×10^4 cells per well. Cells were incubated overnight to ensure sufficient adherence and were washed twice with PBS prior to exposure to gel exposed medium. Cells were incubated for 24 h in exposed medium. XTT and PMS were added to the cells and incubated for 6 h prior to analysis using absorbance measurements at 485 and 690 nm. Wavelength at 480 nm was used for quantifying metabolic activity observed as the reduction of XTT while monitoring at 690 nm provides the nonspecific absorbance. The metabolic activity was normalized for the number of cells present to account for any deviations in cell seeding density. The absorbance of the negative control, i.e., cells not (indirectly) exposed to gel material, was normalized to 1 and all others accordingly using the same factor.

Live-Dead Staining: Dead/Live staining was performed using PBS containing calcein AM ($2 \times 10^{-6} \text{ M}$, Life Technology) and ethidium homodimer-1 ($4 \times 10^{-6} \text{ M}$, Invitrogen molecular probes) in 30 mL of PBS. Cells were observed using CLSM.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements: P.T.K. and P.v.R. kindly thank the Graduate School Medical Sciences for financial support.

Received: May 23, 2016; Revised: July 13, 2016;
Published online: August 5, 2016; DOI: 10.1002/mabi.201600214

Keywords: cell scaffold; controlled release; encapsulation; hydrogel; polyelectrolyte

- [1] J. Malda, J. Visser, F. P. Melchels, T. Jüngst, W. E. Hennink, W. J. A. Dhert, J. Groll, D. W. Huttmacher, *Adv. Mater.* **2013**, *25*, 5011.
- [2] O. Z. Fisher, A. Khademhosseini, R. Langer, N. A. Peppas, *Acc. Chem. Res.* **2010**, *43*, 419.
- [3] R. Langer, D. A. Tirrell, *Nature* **2004**, *428*, 487.
- [4] J. Yang, *Polym. Int.* **2007**, *56*, 1078.
- [5] B. V. Slaughter, S. S. Khurshid, O. Z. Fisher, A. Khademhosseini, N. A. Peppas, *Adv. Mater.* **2009**, *21*, 3307.
- [6] B. Balakrishnan, M. Mohanty, P. Umashankar, A. Jayakrishnan, *Biomaterials* **2005**, *26*, 6335.
- [7] G. Orive, E. Santos, J. L. Pedraz, R. M. Hernández, *Adv. Drug Delivery Rev.* **2014**, *67*, 3.
- [8] D. Velasco, E. Tumarkin, E. Kumacheva, *Small* **2012**, *8*, 1633.
- [9] J. A. Rowley, G. Madhambayan, D. J. Mooney, *Biomaterials* **1999**, *20*, 45.
- [10] O. Chaudhuri, L. Gu, D. Klumpers, M. Darnell, S. A. Bencherif, J. C. Weaver, N. Huebsch, H.-P. Lee, E. Lippens, G. N. Duda, D. J. Mooney, *Nat. Mater.* **2016**, *15*, 326.
- [11] D. Horák, K. Matulka, H. Hlídková, M. Lapčiková, M. J. Beneš, J. Jaroš, A. Hampl, P. Dvořák, *J. Biomed. Mater. Res. B* **2011**, *98*, 54.
- [12] C. Xu, V. Breedveld, J. Kopecek, *Biomacromolecules* **2005**, *6*, 1739.
- [13] M. Prabakaran, J. F. Mano, *Macromol. Biosci.* **2006**, *6*, 991.
- [14] S. Van Vlierberghe, P. Dubruel, E. Schacht, *Biomacromolecules* **2011**, *12*, 1387.
- [15] P. a. Parmar, L. W. Chow, J.-P. St-Pierre, C.-M. Horejs, Y. Y. Peng, J. A. Werkmeister, J. A. M. Ramshaw, M. M. Stevens, *Biomaterials* **2015**, *54*, 213.
- [16] Y. Liu, T. Wang, M. Liu, *Chem. Eur. J.* **2012**, *18*, 14650.
- [17] E. A. Appel, J. Del Barrio, X. J. Loh, O. A. Scherman, *Chem. Soc. Rev.* **2012**, *41*, 6195.
- [18] J. Kopecek, *Biomaterials* **2007**, *28*, 5185.
- [19] J. Sun, H. Tan, *Materials* **2013**, *6*, 1285.
- [20] B. Balakrishnan, R. Banerjee, *Chem. Rev.* **2011**, *111*, 4453.
- [21] A. M. Jonker, D. W. P. M. Löwik, J. C. M. Van Hest, *Chem. Mater.* **2012**, *24*, 759.
- [22] J. B. Matson, S. I. Stupp, *Chem. Commun.* **2012**, *48*, 26.
- [23] J. Whittaker, R. Balu, N. R. Choudhury, N. K. Dutta, *Polym. Int.* **2014**, *63*, 1545.
- [24] J. L. Drury, D. J. Mooney, *Biomaterials* **2003**, *24*, 4337.
- [25] B. P. Partlow, C. W. Hanna, J. Rnjak-Kovacina, J. E. Moreau, M. B. Applegate, K. A. Burke, B. Marelli, A. N. Mitropoulos, F. G. Omenetto, D. L. Kaplan, *Adv. Funct. Mater.* **2014**, *24*, 4615.

- [26] O. Chaudhuri, L. Gu, M. Darnell, D. Klumpers, S. a. Bencherif, J. C. Weaver, N. Huebsch, D. J. Mooney, *Nat. Commun.* **2015**, 6, 6364.
- [27] G. Orive, S. Ponce, R. M. Hernández, A. R. Gascón, M. Igartua, J. L. Pedraz, *Biomaterials* **2002**, 23, 3825.
- [28] L. Wang, R. M. Shelton, P. R. Cooper, M. Lawson, J. T. Triffitt, J. E. Barralet, *Biomaterials* **2003**, 24, 3475.
- [29] X.-W. Shi, C.-Y. Tsao, X. Yang, Y. Liu, P. Dykstra, G. W. Rubloff, R. Ghodssi, W. E. Bentley, G. F. Payne, *Adv. Funct. Mater.* **2009**, 19, 2074.
- [30] R. R. Costa, A. M. S. Costa, S. G. Caridade, J. F. Mano, *Chem. Mater.* **2015**, 27, 7490.
- [31] A. S. Sergeeva, D. A. Gorin, D. V. Volodkin, *Langmuir* **2015**, 31, 7418.
- [32] C. H. Yang, M. X. Wang, H. Haider, H. Y. Yang, J.-Y. Sun, Y. M. Chen, J. Zhou, Z. Suo, *ACS Appl. Mater. Interfaces* **2015**, 7, 19546.
- [33] S. N. Pawar, K. J. Edgar, *Biomaterials* **2012**, 33, 3279.
- [34] X. Z. Shu, Y. Liu, Y. Luo, M. C. Roberts, G. D. Prestwich, *Biomacromolecules* **2002**, 3, 1304.
- [35] R. M. Desai, S. T. Koshy, S. A. Hilderbrand, D. J. Mooney, N. S. Joshi, *Biomaterials* **2015**, 50, 30.
- [36] C. Lee, J. Shin, J. S. Lee, E. Byun, J. H. Ryu, S. H. Um, D. I. Kim, H. Lee, S. W. Cho, *Biomacromolecules* **2013**, 14, 2004.
- [37] J. Dahlmann, A. Krause, L. Möller, G. Kensah, M. Möwes, A. Diekmann, U. Martin, A. Kirschning, I. Gruh, G. Dräger, *Biomaterials* **2013**, 34, 940.
- [38] D.-A. Wang, S. Varghese, B. Sharma, I. Strehin, S. Fermanian, J. Gorham, D. H. Fairbrother, B. Cascio, J. H. Elisseeff, *Nat. Mater.* **2007**, 6, 385.
- [39] O. Jeon, K. H. Bouhadir, J. M. Mansour, E. Alsberg, *Biomaterials* **2009**, 30, 2724.
- [40] S. I. Jeong, O. Jeon, M. D. Krebs, M. C. Hill, E. Alsberg, *Eur. Cell. Mater.* **2012**, 24, 331.
- [41] S. Khetan, M. Guvendiren, W. R. Legant, D. M. Cohen, C. S. Chen, J. A. Burdick, *Nat. Mater.* **2013**, 12, 458.
- [42] T. Billiet, E. Gevaert, T. De Schryver, M. Cornelissen, P. Dubruiel, *Biomaterials* **2014**, 35, 49.
- [43] A. T. Hillel, S. Unterman, Z. Nahas, B. Reid, J. M. Coburn, J. Axelman, J. J. Chae, Q. Guo, R. Trow, A. Thomas, Z. Hou, S. Lichtsteiner, D. Sutton, C. Matheson, P. Walker, N. David, S. Mori, J. M. Taube, J. H. Elisseeff, *Sci. Transl. Med.* **2011**, 3, 93ra67.
- [44] S. Patnaik, M. Arif, A. Pathak, N. Singh, K. C. Gupta, *Int. J. Pharm.* **2010**, 385, 194.
- [45] S. Patnaik, A. Aggarwal, S. Nimesh, A. Goel, M. Ganguli, N. Saini, Y. Singh, K. C. Gupta, *J. Controlled Release* **2006**, 114, 398.
- [46] S. Mohamed, A. B. Salleh, *Biotechnol. Lett.* **1982**, 4, 611.
- [47] H. J. Kong, D. J. Mooney, *Cell Transplant.* **2003**, 12, 779.
- [48] A. J. Engler, S. Sen, H. L. Sweeney, D. E. Discher, *Cell* **2006**, 126, 677.
- [49] M. X. Wang, C. H. Yang, Z. Q. Liu, J. Zhou, F. Xu, Z. Suo, J. H. Yang, Y. M. Chen, *Macromol. Rapid Commun.* **2015**, 36, 465.
- [50] O. Boussif, F. Lezoualc'h, M. a. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, J. P. Behr, *Proc. Natl. Acad. Sci. USA* **1995**, 92, 7297.
- [51] A. C. Hunter, *Adv. Drug Delivery Rev.* **2006**, 58, 1523.
- [52] J. L. Drury, R. G. Dennis, D. J. Mooney, *Biomaterials* **2004**, 25, 3187.
- [53] S. J. Bidarra, C. C. Barrias, P. L. Granja, *Acta Biomater.* **2014**, 10, 1646.
- [54] E. Paramonova, E. D. DeJong, B. P. Krom, H. C. Van Der Mei, H. J. Busscher, P. K. Sharma, *Appl. Environ. Microbiol.* **2007**, 73, 7023.
- [55] K. Y. Lee, D. J. Mooney, *Prog. Polym. Sci.* **2012**, 37, 106.
- [56] L. Formigli, F. Paternostro, A. Tani, C. Mirabella, A. Quattrini Li, D. Nosi, F. D'Asta, R. Saccardi, B. Mazzanti, G. Lo Russo, S. Zecchi-Orlandini, *Wound Repair Regener.* **2015**, 23, 115.
- [57] J. M. Reinke, H. Sorg, *Eur. Surg. Res.* **2012**, 49, 35.