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Three-Dimensional Microgel Platform for the Production of Cell Factories Tailored for the Nucleus Pulposus

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Supporting Information

ABSTRACT: Intradiscal injection of growth factors or cells has been shown to attenuate symptoms of intervertebral disc degeneration. However, different approaches are needed to overcome limitations such as short-term efficacy and leakage of the injected solutions. The current study aims at creating a platform for the realization of functional cell factories by using in parallel cell delivery and gene therapy approaches. Superfect, a transfecting agent, was used as nonviral gene vector because of its ability to form complexes with plasmid DNA (polyplexes). Polyplexes were loaded into collagen hollow microsphere reservoirs, and their ability to transfect cells was ascertained in vitro. Adipose-derived stem cells were then embedded in three-dimensional (3D) microgels composed of type II collagen/hyaluronan, which mimics the environmental cues typical of the healthy nucleus pulposus. These were functionalized with polyplex-loaded collagen hollow spheres and the secretion of the target protein was assessed quantitatively. Delivery of polyplexes from a reservoir system lowered their toxicity significantly while maintaining high levels of transfection in a monolayer culture. In 3D microgels, lower levels of transfection were observed, however; increasing levels of luciferase were secreted from the microgels over 7 days of culture. These results indicate that 3D microgels, functionalized with polyplex-loaded reservoirs offer a reliable platform for the production of cell factories that are able to manufacture targeted therapeutic proteins for regenerative therapies that have applications in nucleus pulposus repair.

INTRODUCTION

Intervertebral discs (IVD) are shock-absorber fibro-cartilaginous tissues interposed between the vertebral bodies of the spine.[1−4] IVD degeneration is a cause of discomfort especially in the elderly population[5−8] and is also a factor which can reduce workforce productivity with a cost to our society in billions of dollars every year.[9] The IVD is an avascular tissue with very low cell density.[6,10] Consequently, the diffusion of nutrients, and therefore cell survival, is dependent upon the ability of the tissue to retain water. Because of the high hydrophilicity of its core, the nucleus pulposus (NP) creates the internal pressure necessary for the IVD to maintain its homeostasis. For this reason, the integrity of the NP and in particular its extracellular matrix (ECM) composition is crucial for the survival of the IVD.[1,11] So far, various unimodal strategies have been investigated to promote regeneration in the NP, and these include stem cell therapy,[12,13] implantation of scaffolds,[14−16] or injection of growth factors.[17−23] The implantation of NP and adipose-derived stem cells (ASCs)[24−28] cells for repopulation of NP increases the likelihood for regeneration, and this has been reported in a number of studies.[15,29] However, degenerated discs are characterized by lower levels of extracellular signals such as growth factors.[17,18] Intradiscal delivery of growth factors has shown promising results by priming NP cells to synthesize ECM and overall mitigating disc degeneration.[19,20,30] Even so, because of the short half-life of growth factors,[31,32] these approaches serve only as a short-term solution and require multiple injections of growth factors to retain long-term efficacy.[33] In addition, multiple injections can lead to irreparable tissue damage, and thereby weigh negatively from a risk/benefit perspective. These considerations highlight the need for a multimodal approach where cells are delivered to the NP but, on the other hand, are engineered to secrete growth factors or other proteins of interest.[34] This can be achieved by combining cell therapy and gene therapy approaches. The introduction of the short sequences of DNA encoding for therapeutic proteins into the target cells allows the conversion of transfected cells into protein-producing factories.[35] Hence, desired proteins can be produced by cells for an extended period. In gene therapy, there is growing interest toward the development of nonviral, polymeric-based gene carriers. These are generally cationic polymers with high affinity for pDNA to form complexes also known as polyplexes.[36] The use of polyplexes overcomes some of the limitations of viral vectors such as the size of DNA that can be packaged, immunogenic responses, reproducibility, and scale-up.[37,38] However, a primary concern that limits their use is

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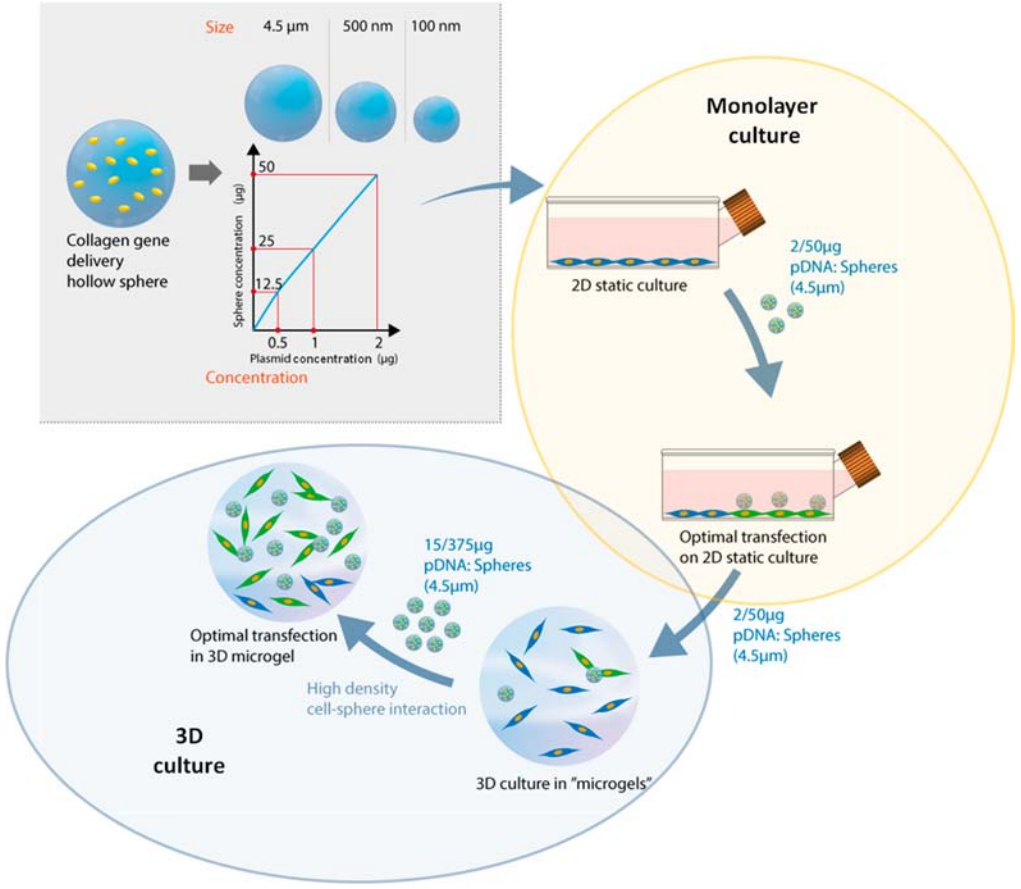

Figure 1. Schematic representation of the study design. Type II collagen hollow spheres were prepared by using the template method and loaded with polyplexes with a ratio of 1/25 complexed pDNA/spheres, respectively. The effect of sphere size and concentration was ascertained in monolayer culture of ASCs. Reservoirs with the largest size allowed the highest level of transfection in monolayer, and were therefore embedded in 3D microgel systems and optimized to obtain functional cell factories.

their cytotoxicity.[39−41] In a tissue with a sparse cell population such as the NP, this is seen as a major obstacle. Nonetheless, it has been shown that such a drawback can be overcome simply by modulating polyplex delivery rate, via loading into a reservoir.[42] It has been shown that the reservoir system loaded with polyplexes preserved their ability to transfect cells over a long period while significantly lowering their cytotoxicity,[42,43] thereby exerting a protective role on both cells and polyplexes.[42,44] However, another problem often encountered in tissue engineering approaches of the IVD is the leakage of liquid systems due to the internal pressure of the tissue.[45] This implies that delivering cells and growth factors in biomaterial systems is a preferred choice as it allows higher retention at the desired site.[1,27,46] Furthermore, the use of hydrogel systems for cell therapy in the disc is promising because they are not only a vehicle for the delivery of cells, but also serve as a pliable scaffold, which acts as a template for remodeling, synthesis, and deposition of new matrix. Moreover, 3D hydrogel systems have been shown to be more suitable environments than the traditional monolayer culture for the maintenance of NP cells phenotype.[47−49] These promising results have resulted in the adoption of beads and spheroid systems for the culture of NP-like cell lines.[50] Hydrogel systems that mimic the composition of the disc matrix were shown to provide a suitable environment for the synthesis and deposition of new ECM.[6,51−53] In particular, hydrogels based on type II collagen and HA seem to be an ideal platform for the cell therapy of the IVD.[27,52,54−57] The objective of the current study is to design a 3D cell delivery platform, tailored for the NP, in which cells can be programmed to secrete specific proteins of interest. It is hypothesized that a type II collagen/HA microgel system functionalized with a gene delivery reservoir system will allow for in situ engineering of cells to manufacture target proteins, thereby offering a reliable platform for the production of functional cell factories.

■ RESULTS AND DISCUSSION

Unimodal strategies for disc regeneration such as intradiscal injection of growth factors or cells have shown encouraging results.[13−23] However, the limitations of these approaches such as short-term efficacy and leakage of the injected solution need to be overcome. Delivery of cells in a controlled environment in parallel with system functionalization with nonviral gene vectors constitute a multimodal approach that may provide synergistic improvements of such therapies.[58] To provide cells with instructive cues able to guide them during the process of proliferation, differentiation, and synthesis of new matrix, a microgel system which mimics the NP matrix was developed. Here, type II collagen and HA, two important biomacromolecules abundant in the healthy NP matrix, were used as building blocks for the fabrication of the microgel system. Since cell therapy alone was found to only moderately stimulate NP regeneration,[13−16] the microgel system was functionalized with a reservoir system for the delivery of nonviral gene vectors as

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illustrated in Figures 1 and 6. The gene delivery reservoir consisted of type II collagen hollow spheres fabricated by using the template method. Superfect was used as a nonviral gene vector. This is a partially degraded dendrimer able to complex plasmid DNA forming polyplexes. As a proof-of-concept, the plasmids used in this study were pCMV-GFP (expressing for GFP, used for qualitative analysis) and pCMV-Gluc (expressing for luciferase, used to quantify the ability of the cells to secrete eventual therapeutic proteins). Using an in situ transfection approach has some advantages over the pre-engineering of the cells. First of all, the pre-engineering of cells would require extensive stem cells expansion in vitro[59] which could severely impact the stem cell's ability to differentiate,[60] while no pretreatments are required with transfection in situ. Moreover, transfection in situ does not present the risks associated with the random integration of the transgene in the genome typical of pre-engineered cells; therefore, it is a safer approach. The main advantage of using the template method for the fabrication of type II collagen reservoirs is that by using monodisperse commercial beads it is possible to obtain spheres of defined size and shape as shown in Figures 2A and S1

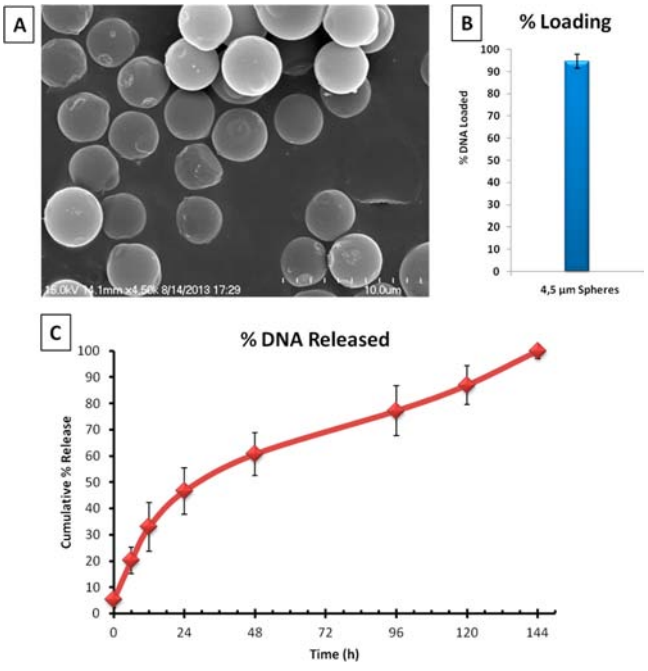

Figure 2. High loading efficiency and sustained release of polyplexes from collagen hollow spheres. (A) SEM image of type II collagen hollow spheres homogeneous in size and shape and fabricated by using the template method. (B) Loading study assessed by incubating 50 μg of 4.5 μm spheres and 2 μg of complexed pDNA (ratio of 1/25 pDNA/spheres), the graph shows a loading efficiency of 95% (n = 3), (C) followed by sustained release where 100% of the loaded pDNA was released over 144 h (n = 3).

(Supporting Information). Moreover, adopting type II collagen as a building block for the fabrication of reservoir systems not only mimics the cues and composition of the nucleus pulposus ECM, but also offers good performance in terms of loading and release of polyplexes (Figure 2). This is because type II collagen physiologically exerts the role of reservoir for a number of macromolecules.[13,54] In fact, the loading efficiency for polyplexes was above 90% (2 μg of complexed pDNA per 50 μg of type II collagen). Furthermore, as shown in Figure 2, the polyplexes were released gradually and in a sustained manner. To narrow the conditions to be tested in 3D microgels, a variety of conditions were tested in monolayer such as the effects of sphere size and concentration on cell viability and transfection efficiency (Figure 1). As shown in Figure 3, type II collagen hollow spheres did not possess intrinsic cytotoxicity and cell viability was not affected even by large amounts of spheres (from 12.5 μg to 50 μg). On the other hand, this reservoir system offered a protective environment for the cells from the toxicity associated with polyplexes (Figure 3). In turn, this allowed the use of high amounts of complexed plasmids (from 0.5 μg up to 2 μg) without affecting cell viability but with higher transfection efficiency (Figure 4). When the same amount of complexed plasmids was used with or without reservoir system, the samples with the reservoir system showed higher transfection (Figure 4) due to the fact that there were more viable cells to be transfected (Figure 3). Surprisingly, the size of the spheres seemed to play a role, with higher transfection being obtained with bigger spheres (4.5 μm) (Figure 4). This can be explained by the fact that there are two transfection mechanisms occurring: the release of polyplexes from the reservoirs and the internalization of the reservoirs by the cells (Figure 5). Because bigger reservoirs have the tendency to precipitate to the bottom of the wells sooner than the smaller ones, the size of the reservoir clearly determines their exposure to the cells. However, because of their higher transfection efficiency, 4.5 μm reservoirs were used to functionalize 3D microgels. Microgel functionalization with loaded spheres allows the delivered cells to act as a protein-producing factory. This enables the programming of delivered cells to express desired proteins for a prolonged period, eliminating the need for multiple injections. Even so, 3D microenvironments are also known to influence the outcome of gene therapies,[35,58,61−64] often affecting their efficacy.[58] In fact, when functionalizing 3D microgels with spheres containing 2 μg of complexed plasmids, only limited transfection was observed. For this reason, it was decided to functionalize the microgels with higher amounts of polyplex-loaded spheres (5, 10, and 15 μg of complexed pDNA in 125, 250, and 375 μg of spheres, respectively). Not only does the cell viability in the microgels remain unaffected by high concentrations of loaded reservoirs, but as shown in Figure 7, the cell metabolic activity increased in the microgels containing high amounts of loaded spheres. A possible explanation for this is that the polyplexes have buffering abilities,[36,64,65] and thereby might neutralize microacidic environments resulting from cellular metabolic activity within the microgels. Furthermore, embedding nano and microparticles within hydrogels was shown to alter the mechanical properties of the system.[66] Such a combination of particles and hydrogels can be considered a composite biomaterial and it often results in improved mechanical properties.[66,67] Hence, the functionalization with the hollow spheres may alter the mechanical properties of the microgels, probably making them stiffer. Therefore, because mechanical properties have been shown to exert influence on cell proliferation rate,[68] the improved stiffness of the microgels can be another cause of the greater metabolic activity detected in microgels functionalized with large amounts of microspheres. Moreover, the matrix of the microgels further shields the ASCs from direct contact with polyplexes (but also lowers the transfection efficiency). Additional support for this claim comes from the measurements of microgel diameter over a period of 7 days. Microgels containing higher amounts of loaded spheres

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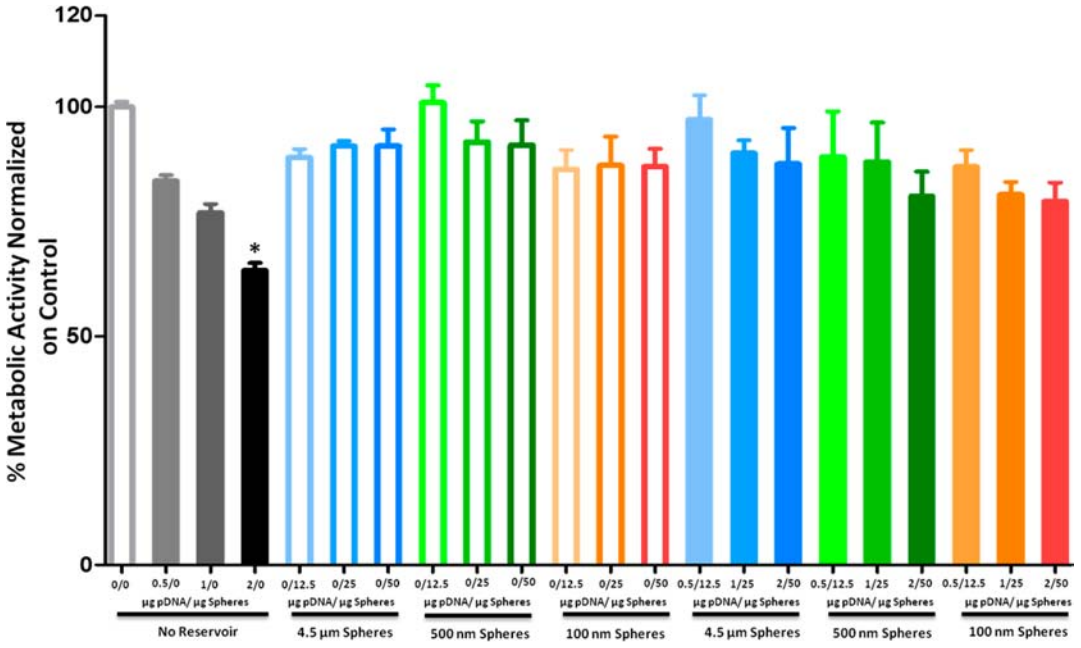

Figure 3. Type II collagen hollow spheres lower the toxicity of polyplexes. Percentage of metabolic activity of monolayer ASCs (measured by the alamarBlue assay) exposed to different concentrations of loaded spheres and compared to cells alone and cells exposed to polyplexes without reservoir (n = 3, one way ANOVA, Tukey test $p > 0.05$).

showed higher shrinkage due to increased cell metabolic activity and ability to remodel the microgels (Supporting Information Figure S4). However, increasing the amount of polyplex-loaded spheres in the microgels successfully increased the transfection efficiency as shown in Figure 8, resulting in significantly higher amounts of active luciferase being released into the media. Moreover, in all the samples tested the transfection rate increased after 7 days, suggesting a prolonged effect (Figure 8). The aim of this study was to design a functionalized cell delivery system able to activate paracrine loops for the synthesis of targeted proteins. Although this was a proof-of-concept study, and only plasmids coding for GFP or luciferase were used, the results obtained are encouraging as the embedded cells were able to express and secrete functional proteins for a prolonged period. Future studies will be designed toward functionalizing microgels with polyplexes encoding genes for a variety of growth factors such as TGF-β1, IGF-1, and BMP-2. This combined nucleic acid transfer has been reported to have greater anabolic effects than the use of a single gene.[69] Nevertheless, since there are profound differences between the human spine and those of other species,[70] finding appropriate in vivo models will be challenging. To overcome the unsuitability of existing animal models, organ-culture systems have been developed. Culturing IVD tissues in confined cultures and the possibility of subjecting them to mechanical stresses comparable to those on human discs allows for a greater control over the disc environment. Because of this advantage, organ culture systems have been used as a tool to elucidate the effects of potential disc therapies.[71] Hence, future studies will use organ-culture models to assess the beneficial effects of the functionalized microgel platform developed in the current study. However, another challenge that needs to be overcome for this platform to become clinically relevant is the retention and survival of cells at the injected site, avoiding leakages. However, despite a recent in vivo study has revealed that generally microgels help in retaining cells in an implanted site,[46] the inherent high pressure of the IVD will probably require the additional use of sealants to help closing the defect and avoid leakages.

CONCLUSIONS

The use of microsphere reservoirs for the delivery of polyplexes shielded the cells from the toxicity of the gene vector, while allowing high levels of transfection in monolayer. Although lower levels of transfection were observed in 3D microgels, the use of reservoir system allowed us to increase the concentration of the delivered polyplexes without compromising cell metabolic activity and significantly increasing the transfection rate. Both qualitative and quantitative analysis revealed that ASCs embedded in 3D microgels could be transfected for a prolonged period, and as a result, functional transgenic proteins were released from the 3D microgel system. Therefore, the platform developed not only provides cells with instructive cues typical of ECM-derived biomacromolecules, but the functionalization of the microgels with a reservoir system for polyplexes was found to be able to stimulate targeted protein production. This allows the production of cell factories to manufacture targeted therapeutic proteins for regenerative therapies of a number of tissues. However, in the current study the platform was tailored for the NP by using type II collagen and HA as building blocks.

EXPERIMENTAL PROCEDURES

Materials. If not specified, all materials were purchased from Sigma-Aldrich (Ireland). Type II calf articular joint atelocollagen (Affymetrix (UK)) and high molecular weight (200−750 kDa) hyaluronic acid (Contipro group, (Czech Republic)) were used. Four-arm polyethylene glycol succinimidyl glutarate MW 10 000 Da (4S-StarPEG) was purchased from JenKem Technology (USA). ASCs were extracted from rabbit adipose tissue as previously described[27] and used strictly at passage three for every experiment and were cultured in

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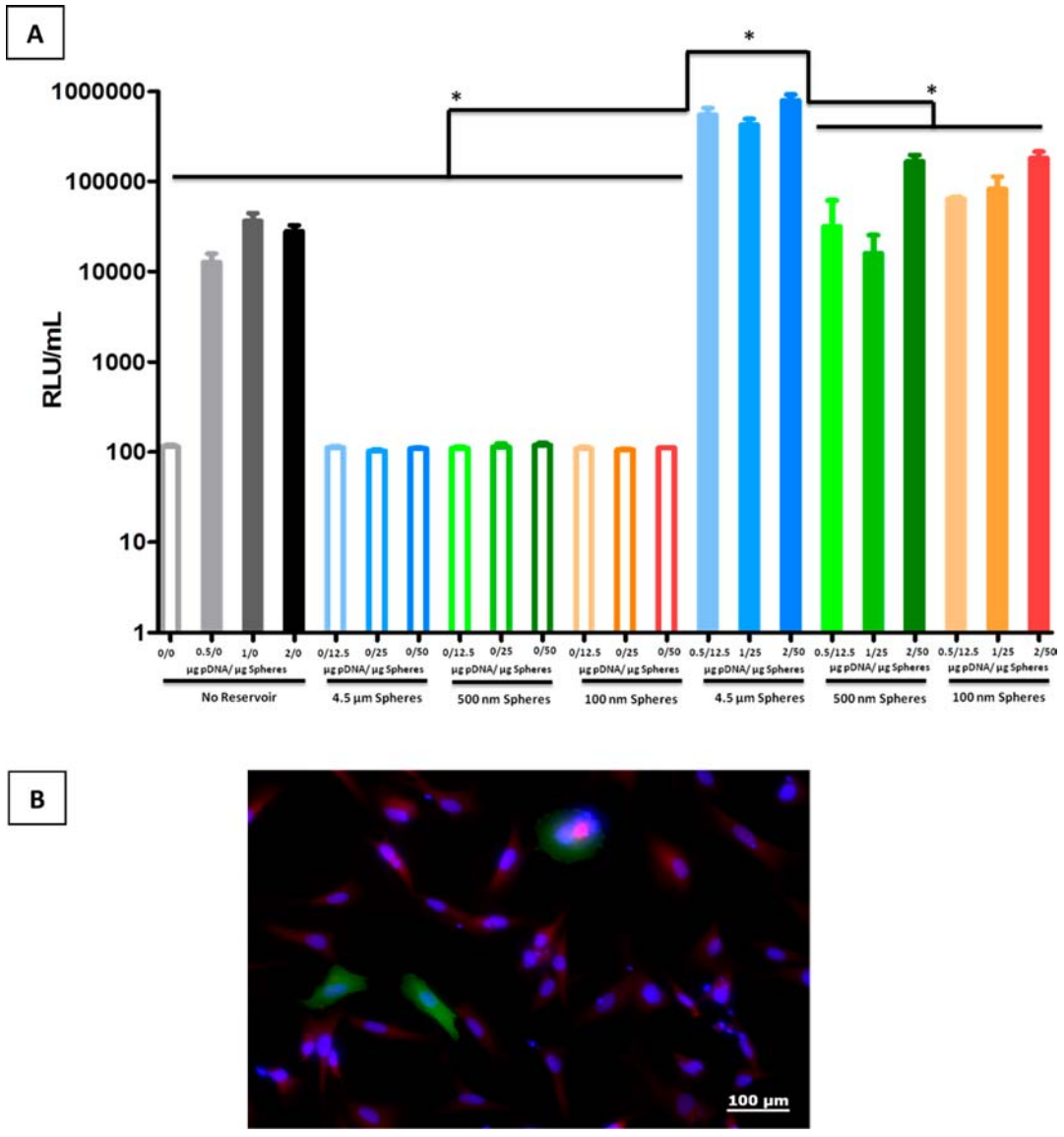

Figure 4. Type II collagen hollow spheres enhances transfection efficiency of polyplexes. (A) Gaussian luciferase assay to assess the ability of sphere reservoir to release active polyplexes capable of transfecting cells in monolayer culture; a significantly higher level of transfection was obtained by using 4.5 μm spheres compared to 500 and 100 nm or polyplexes alone (n = 3, one way ANOVA, Tukey test $p > 0.05$). (B) ASCs expressing GFP following treatment with polyplex-loaded spheres (green for GFP, blue for DAPI, and red for rhodamine-phalloidin).

Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS 10%) and penicillin–streptomycin (P/S 1%). Polystyrene beads of defined sizes were purchased from Gentaur (USA). Gaussia princeps luciferase plasmids (pCMV-Gluc; New England Biolabs, USA) were propagated and isolated using standard techniques.[72]

Fabrication of Type II Collagen Hollow Spheres. Type II collagen hollow spheres were fabricated by using the template based method as described in previous reports.[42,43] Briefly, polystyrene beads of defined sized (4.5 μm, 500 nm, and 100 nm) were sulfonated to increase their negative charge. Following sulfonation, the beads were resuspended in 0.5 M acetic acid and stirred at a moderate speed at room temperature. Type II collagen was added dropwise to the bead suspension at a ratio of 1:4 (collagen:beads), while maintaining the final collagen concentration of 2 mg/mL. Although the electrostatic interaction between type II collagen and beads is immediate, the mixture was stirred for 4 h at room temperature to allow a uniform coating. To eliminate the excess of collagen, the coated beads were washed in acetic acid and collected by centrifugation at 4500 rpm. To create the most favorable conditions for the cross-linking to occur, the coated beads were then resuspended in PBS 1× and the pH adjusted to 7.4 by using NaOH 1 M. The cross-linking was performed using 4S-StarPEG at a ratio of 1:2 w/w (collagen/4S-StarPEG). The solution was stirred gently for 1 h at 37 °C. Washing in THF dissolved the polystyrene core of the spheres. The suspension of microspheres was diluted at a ratio of 1:1 with THF and agitated for 1 h. The washing step was repeated twice to ensure complete removal of polystyrene.

Analysis of Charge on Microspheres. To characterize the surface coating of microspheres, zeta potential was analyzed using a Zetasizer (Malvern, Nano-ZS90). Microspheres (4.5 μm) at various stages of fabrication were analyzed to determine the charge of the spheres and to confirm the coating process.

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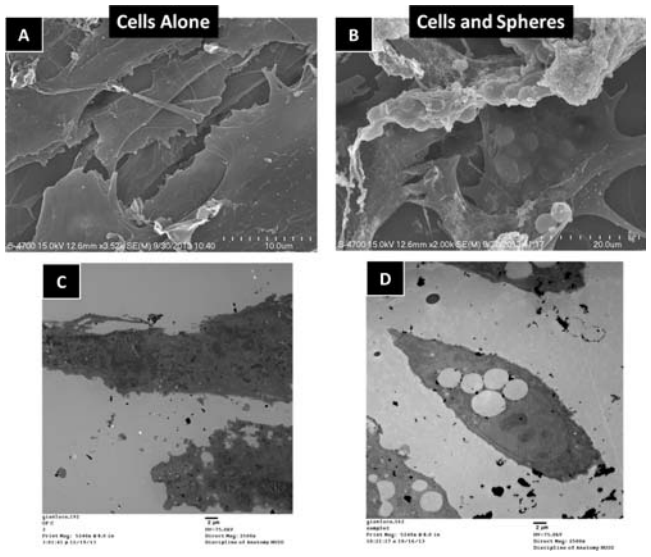

Figure 5. ASCs can internalize collagen hollow spheres in monolayer. (A,B) SEM images of ASCs alone (A) and incubated with collagen hollow spheres (B); (C,D) TEM images of cross sections through ASCs alone (C) and incubated with collagen hollow spheres (D). B and D clearly show the process of internalization that collagen hollow spheres undergo when incubated with ASCs in monolayer.

The stages at which microspheres were assessed for charge analysis were as follows: polystyrene beads precoating, and polystyrene beads following collagen coating (two ratios collagen:beads tested: 1:1 and 1:4) and after cross-linking step.

Size Analysis of Microspheres. The size of microspheres was determined by using Image J software (National Institute of Health, USA) and the diameter of 25 spheres was measured and averaged from SEM images. To achieve high accuracy, the analysis was repeated for four different batches of fabricated spheres.

Formation of Polyplexes. A partially degraded PAMAM dendrimer (Superfect, Qiagen) (SF) was used as a complexing agent. In brief, the plasmid of interest was resuspended in PBS 1× at a final concentration of 15 μg/mL. SF was added to the plasmid solution at a ratio of 9:1 w/w (SF/plasmid) and the solution incubated for 15 min at room temperature prior to use.

Labeling of Plasmid. Plasmids were labeled by using a Cy5 labeling kit Mirus (USA). Briefly, the dye was incubated with the plasmid in the provided buffers for 1 h. Following the incubation, the plasmid was eluted through a microspin column to remove any unbound dye. The labeled plasmid was then stored in the dark at −20 °C until use.

Loading of Microsphere Reservoirs with Polyplexes. 50 μg of collagen microspheres were resuspended in 500 μL of phosphate buffered saline (PBS). To this solution, 2 μg of Cy5 labeled complexed pDNA was added. This mixture of microspheres and labeled complexes was agitated on a mechanical shaker for 4 h at room temperature. The microspheres were spun down and the supernatant removed. The supernatant was then measured on fluorescent spectroscopy (Varioskan Flask plate reader, Thermo Scientific, Ireland) at excitation/emission 649/670 nm. The supernatant was compared with a standard curve to determine the amount of pDNA remaining, which enabled quantification of loading efficiency.

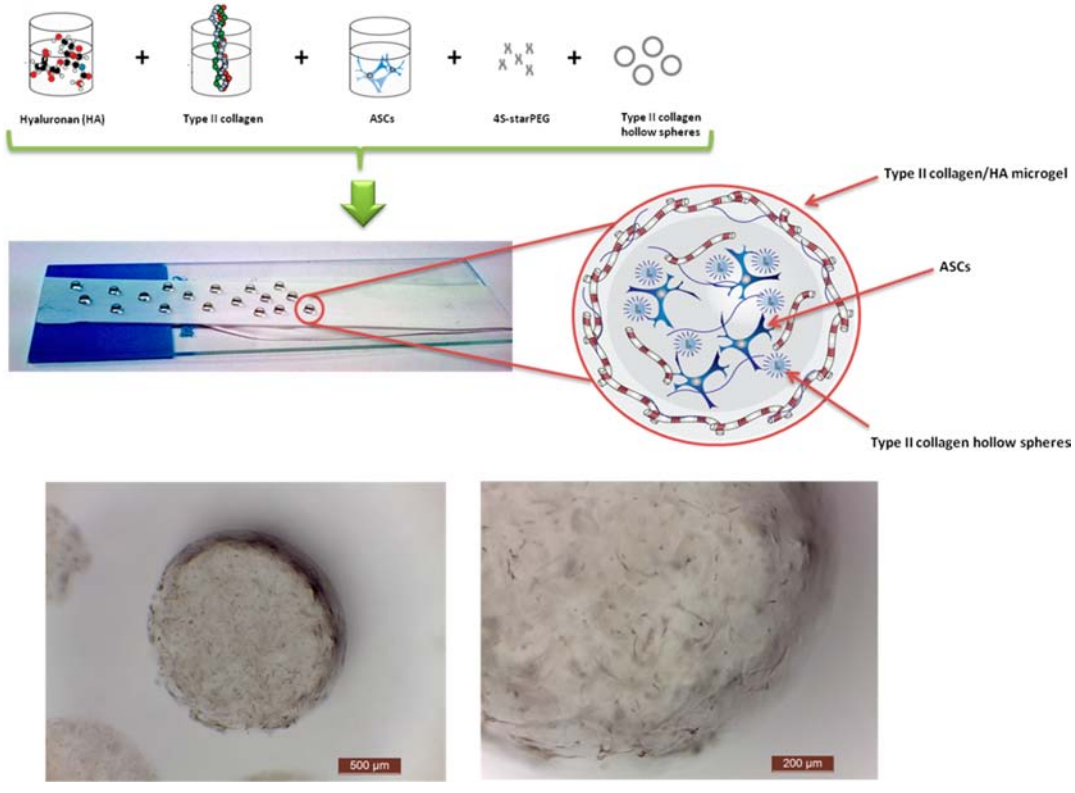

Figure 6. Schematic representation of 3D microgel preparation. The forming gel solution is formed by mixing hyaluronic acid, type II collagen, ASCs, and 4S-StarPEG. The gelling solution is then mixed with sphere reservoir and deposited in the form of 2 μL droplets on a hydrophobic surface and allowed to gel for 1 h at 37 °C.

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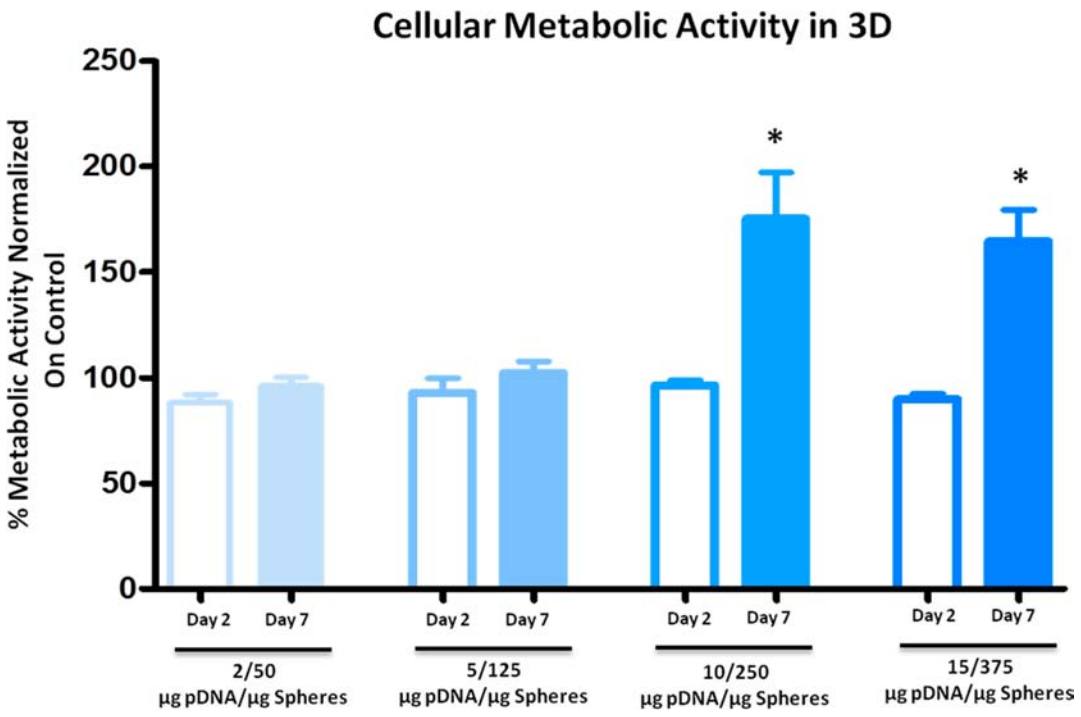

Figure 7. High concentrations of polyplexes do not affect cell metabolic activity in 3D. (A) Percentage of metabolic activity (measured by the alamarBlue assay) of ASCs embedded in 3D microgels and exposed to different concentrations of loaded spheres and compared to cells alone; even elevated concentrations of polyplexes do not affect cell metabolic activity (n = 3, one way ANOVA, Tukey test $p > 0.05$).

Release Study. The release profile of Cy5 labeled polyplexes was characterized in PBS at 37 °C. Loaded microspheres were spun down; the supernatant was collected and replaced at various time intervals. The collected supernatant was assayed for polyplex content spectroscopically as described in the loading study.

Sample Preparation for Electron Microscopy (SEM and TEM). Preparation of samples for SEM analysis consisted of fixation with 3% glutaraldehyde in freshly prepared 0.2 M sodium cacodylate buffer for 1 h. After washing the samples in sodium cacodylate buffer, the samples were resuspended in osmium tetroxide for 2 h under the fumehood. After a series of washings in the buffer, samples were dehydrated by immersion in a graduated series of ethanol in H_2O and hexamethyldisilazane (HDMS) in ethanol solutions of 30%, 50%, 80%, 95% and 33%, 50%, 66%, 100%, respectively. The samples were allowed to vacuum-dry on the sample holder.

Sample Embedding in Epoxy Resin (TEM). TEM samples were embedded in an epoxy-based resin (Agar Scientific, Low Viscosity Resin kit). The embedding process consisted of incubations of samples in a mixture of resin/propylene oxide 50:50 for 4 h, then replaced with a mixture 75:25 overnight, and finally in pure resin for 6 h. The final step consisted of thermo-cross-linking at 65 °C for 48 h which allows the resin to acquire the hardness needed to cut cross sections of the embedded samples.

ASC Encapsulation in 3D Microgels. Type II atelocollagen was neutralized (pH 7.4) in 1 M NaOH and 10× phosphate buffered saline (PBS) in volumes adjusted to have a final concentration of collagen of 5 mg/mL. The gel-forming solution was then enriched with HA (9 to 1 weight ratio collagen to HA). ASCs (10^6 cells/mL final) and polyplex-loaded microspheres were added to the mixture. After addition of 4S-StarPEG (2 mM), the gel-forming solution was deposited as 2 μL droplets on a hydrophobic surface (Teflon tape, Fisher Scientific) to create a spherical microgel and incubated for 1 h at 37 °C.

Cell Viability. The ability of the microsphere reservoirs to preserve the metabolic activity of ASC was quantified using the alamarBlue cell metabolic activity assay. The same assay was conducted in monolayer and in 3D microgels but over different incubation times.

Monolayer. Twenty-four hours prior to the experiment, ASCs were seeded in a 96 well plate (10^4 cells/well) and allowed to grow in complete media. Cells were then washed twice in Hank's balanced salt solution (Hanks) and incubated with 10% alamarBlue/Hanks for 2 h at 37 °C. The supernatant was collected (100 μL) in a clear 96 well plate and the absorbance measured at 550 and 595 nm (0.5 s per well). The absorbance values were then blanked and a correction factor was then calculated following the "simplified method of calculating percent reduction" available in the alamarBlue handbook. The absorbance at higher wavelength was then multiplied by the correction factor and subtracted from the absorbance at lower wavelength. The percent of reduced alamarBlue was then obtained by multiplying the final number by 100. The value for each sample was then normalized on the control.

3D Microgels. Twenty-four hours post-cell encapsulation, microgels were washed in Hanks twice and collected via centrifugation at 1000 rpm for 5 min. Subsequently, these cells were resuspended in 10% alamarBlue/Hanks and incubated for 8 h at 37 °C. The supernatant was collected (100 μL) into a clear 96 well plate and the absorbance measured at 550 and 595 nm (0.5 s per well).

Size of Microgels. The size of microgels functionalized with different amounts of loaded microspheres was monitored by analyzing images of microgels after 2 and 7 days of culture.

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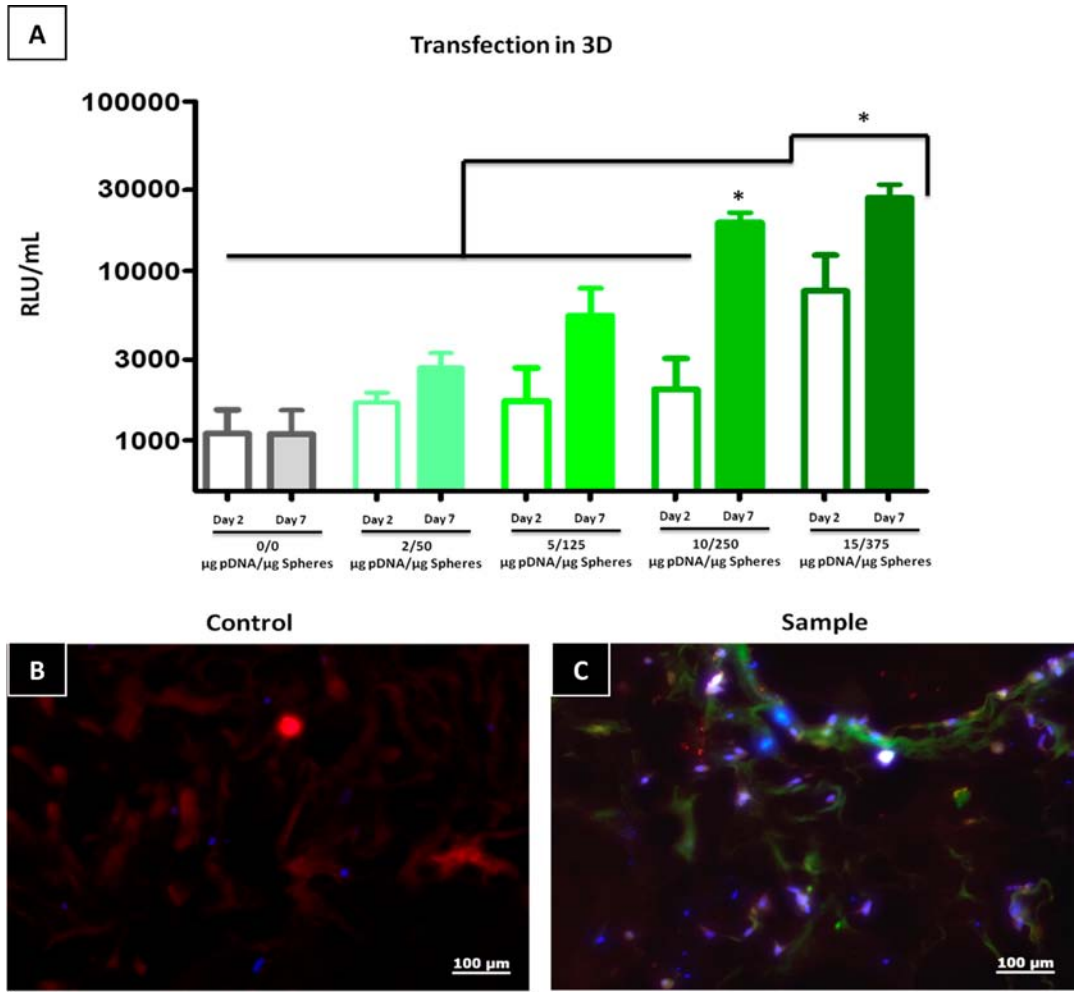

Figure 8. Transfection efficiency in 3D is dependent on polyplex concentration. (A) Gaussia luciferase assay to assess the ability of loaded reservoirs to transfect cells in 3D microgels. Embedding higher concentrations of reservoirs into 3D microgels leads to higher level of transfection over 7 days (n = 3, one way ANOVA, Tukey test $p > 0.05$). (B,C) Sections through cell-embedded 3D microgels without reservoir (B) and with reservoir (C) (15/375 μg pDNA/spheres). The sections were incubated with FITC-labeled anti-GFP primary antibody to ascertain the presence of transfected cells (green); cell nuclei and cytoplasm were stained with DAPI (blue) and rhodamine-phalloidin (red), respectively.

ImageJ software was used to assess the diameter of the microgels and the percent shrinkage was obtained by normalizing the diameter of microgels at day two and seven.

Transfection. The ability of polyplex-loaded microspheres to transfect ASC was investigated both qualitatively and quantitatively in monolayer and in 3D microgels.

Transfection—Quantitative Analysis. To determine the best conditions for transfection, microspheres of different sizes were loaded with polyplexes bearing a plasmid coding for G-luciferase (pCMV-GLuc). ASCs were seeded in a 96 well plate (10^4 cells/well) and incubated for 24 h in complete media. Different concentrations of microspheres were then added on them and incubated for 48 h in complete media. After 48 h at 37 °C, the supernatant (50 μL) was transferred into a new 96 well plate and analyzed for G-luciferase activity.

Transfection—qualitative Analysis on Monolayer. ASCs were seeded in a 96 well plate (10^4 cells/well) and incubated for 24 h in complete media. 50 μg of microspheres loaded with polyplexes bearing a plasmid coding for green fluorescent protein (pCMV-GFP) were added to the cells and incubated for 48 h in complete media. ASCs were then fixed in 4% paraformaldehyde for 30 min at room temperature. To stain the cytoskeleton of the cells, samples were incubated for 30 min in rhodamine−phalloidin (Invitrogen) according to the supplier's protocol and 10 min in 4,6-diamidino-2-phenylindole (DAPI) to stain the nuclei. Images were acquired by using a fluorescence microscope (Olympus BX51).

Qualitative Analysis in 3D Microgels. ASCs were embedded in microgels as described previously. After 7 days of culture, the samples were fixed in 4% paraformaldehyde for 1 h. Due to the loss of fluorescence intensity over time, it was not possible to image the microgels directly; therefore, GFP-positive cells were located via immunolabeling. The samples were dehydrated in an ascending series of ethanol baths and cleared in xylene. Following embedding in paraffin, the samples were sectioned at 10 μm thickness using a microtome and incubated 2 h with FITC-labeled primary anti-GFP antibody (dilution 1:200). Cell nuclei and cytoplasm were stained as described above.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional data on characterization of the spheres reservoir system and a TEM image of ASCs embedded in 3D microgels showing absence of microsphere internalization. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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