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Developing reservoir systems for therapeutic delivery of biomolecules to a degenerated disc

A thesis submitted to the National University of Ireland for the degree of Doctor of Philosophy

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Table of Contents

Table of Figures ........................................................................................................... IX
Table of Tables ............................................................................................................... XXIV
Acknowledgment ........................................................................................................... XXVII
List of Abbreviations .................................................................................................... XXIX

Chapter 1 ......................................................................................................................... 1
  1.1 Overview on the importance of treating disc degeneration ....................... 2
  1.2 Overview on Disc Degenerative Disease ..................................................... 2
  1.3 The Intervertebral Disc ................................................................................. 3
    1.3.1 The Nucleus Pulposus .......................................................................... 4
    1.3.2 The Annulus Fibrosus .......................................................................... 4
    1.3.3 The End Plates ....................................................................................... 5
    1.3.4 The Cellular Component of the Disc ..................................................... 7
  1.4 Intervertebral Disc Degeneration ................................................................. 7
    1.4.1 Vascularisation and Inflammation Increase Catabolism in the Disc .......... 9
    1.4.2 Discogenic Pain .................................................................................... 9
  1.5 Current Treatments ......................................................................................... 12
    1.5.1 Interbody Fusion .................................................................................... 12
    1.5.2 Discectomy ........................................................................................... 12
    1.5.3 Disc Replacement .................................................................................. 13
    1.5.4 Tissue Engineering Approaches to Restore Disc Anabolism ............... 13
      1.5.4.1 Protein Therapy ............................................................................. 13
      1.5.4.2 Optimizations in the use of Growth Factors and their Limitations .... 15
      1.5.4.3 Gene Therapy ............................................................................... 19
      1.5.4.4 Viral Vectors ................................................................................ 19
      1.5.4.5 Optimizations in the Use of Viral Vectors .................................... 20
      1.5.4.6 Non-Viral Vectors ....................................................................... 21
      1.5.4.7 Gene Therapy in Three Dimensional Scaffolds ............................ 22
      1.5.4.8 The Synergistic use of 3D Scaffolds and Gene Vectors ............... 24
      1.5.4.9 Cell Therapy of the Nucleus Pulposus ......................................... 27
      1.5.4.10 The Importance of Microenvironments in Cell Therapy .......... 28
      1.5.4.11 ECM-based Scaffolds for Cell Delivery to the Nucleus Pulposus ... 28
4.2 Materials and methods .......................................................... 136
  4.2.1 Materials and cells .......................................................... 136
  4.2.2 Fabrication of type II collagen hollow spheres ..................... 136
  4.2.3 Analysis of charge on microspheres .................................. 137
  4.2.4 Size analysis of microspheres ......................................... 137
  4.2.5 Formation of polyplexes ................................................. 137
  4.2.6 Labeling of plasmid ....................................................... 137
  4.2.7 Loading of microsphere reservoirs with polyplexes .............. 138
  4.2.8 Release study ............................................................... 138
  4.2.9 Sample preparation for electron microscopy (SEM and TEM) .... 138
  4.2.10 Sample embedding in epoxy resin (TEM) .......................... 139
  4.2.11 ASC encapsulation in 3D microgels ................................. 139
  4.2.12 Cell viability .............................................................. 139
  4.2.13 Size of microgels ......................................................... 140
  4.2.14 Transfection ............................................................... 140
    4.2.14.1 Quantitative analysis in a monolayer system .................. 140
    4.2.14.2 Quantitative analysis in a monolayer system .................. 140
    4.2.14.3 Qualitative analysis in a monolayer system ................. 141
    4.2.14.4 Qualitative analysis in 3D microgels ......................... 141
  4.3 Results .............................................................................. 141
    4.3.1 Fabrication and characterization of type II collagen hollow spheres ............................................. 141
    4.3.2 Polyplexes loading into type II collagen spheres reservoirs and release kinetics ........................................ 142
    4.3.3 Cell viability in monolayer ............................................ 142
    4.3.4 Transfection in monolayer ............................................ 142
    4.3.5 Cell internalization of reservoirs ................................... 143
    4.3.6 Cell behavior in functionalized 3D microgels ................... 143
    4.3.7 Transfection in 3D microgels ........................................ 144
  4.4 Discussion ........................................................................... 154
    4.4.1 Fabrication of microspheres reservoir with the template method ......................................................... 154
    4.4.2 Transfection in 3D microgels ........................................ 155
    4.4.4 Lower transfection in 3D microgels requires higher doses of polyplex-loaded microspheres ...................... 156
F. Type II collagen/Hyaluronan microgels fabrication .............................................200
   F.1 Preparation of reagents ................................................................................200

G. Amine quantification by TNBS assay .................................................................202

H. Microgel stability assay .....................................................................................202

I. Bromodeoxyuridine (BrdU) proliferation analysis .............................................203
   I.1 Pulsing, harvesting and fixing cells from microgels ....................................203
   I.2 BrdU-PI staining ..........................................................................................204

J. Samples preparation for electronic microscopy (TEM and SEM) .................205
   J.1 Reagents ......................................................................................................205
   J.2 Samples fixation .........................................................................................205
   J.3 Samples dehydration for SEM .....................................................................206
   J.4 Embedding samples in epoxy resin for TEM .............................................206
   J.5 Resin preparation ..........................................................................................206

K. Isolation of Total RNA ......................................................................................207

L. Quantification of mRNA ...................................................................................208

M. Synthesis of cDNA ..........................................................................................209
   M.1 Preparation before reverse transcription ..................................................209
   M.2 Preparation of reaction mix for reverse transcription ............................209

N. Polymerase Chain Reaction (PCR) ................................................................211

O. Electrophoresis in 1% agarose gel ................................................................212

P. Sulfonation of polystyrene beads ......................................................................213

Q. Fabrication of collagen hollow spheres ............................................................213

R. Polyplex formation ............................................................................................214

S. Loading of collagen hollow spheres with polyplexes .....................................214

T. Plasmid labeling ..................................................................................................214

U. Protocols for transfection *in-vitro* .................................................................215
   U.1 Transfection in monolayer .........................................................................215
   U.2 Transfection in 3D collagen microgels .......................................................215
   U.3 Quantification of transfection via G-luciferase assay ...............................216

V. AlamarBlue™ assay ..........................................................................................216

W. Emulsion method for the fabrication of collagen microspheres .................217

X. Microphase separation method for the fabrication of collagen microspheres .................................................................................................217

Y. Synthesis of pH-sensitive crosslinker .............................................................218
   Y.1 Crystallization of toluene sulfoonic acid (catalyst) ....................................218
   Y.2 Reaction setup ..............................................................................................219
Y.3 Extraction of the organic phase ........................................... 219
Y.4 Purification ........................................................................ 220
Z. Supplementary information for Chapter 2 ......................... 222
AA. Supplementary information of chapter 3* ..................... 234
AA.1 Introduction .................................................................. 234
AA.2 Methods ....................................................................... 235
AA.2.1 Surface analysis of spheres ........................................... 235
AA.2.2 Analysis of polystyrene removal ................................. 236
AA.2.3 Labelling of plasmid ..................................................... 236
AA.2.4 Polypeptide formation ................................................. 237
AA.2.5 Loading of microsphere reservoirs with polypeptides ...... 237
AA.2.6 Release study .............................................................. 237
AA.2.7 Transfection .............................................................. 237
AA.2.8 Cell proliferation ........................................................ 238
AA.2.9 Toxicity of polypeptides .............................................. 238
AA.3 Results ........................................................................ 238
AA.3.1 Collagen spheres characterization .............................. 238
AA.3.2 Characteristics of loading and release ........................ 239
AA.3.3 Protein expression ...................................................... 239
AA.3.4 Reduction in polypeptide toxicity ............................... 240
AA.3.5 Maintenance of cell proliferation ............................... 240
AA.4 Discussion .................................................................... 247
AA.4.1 Collagen microspheres showed high loading of polypeptides
and sustained release .......................................................... 247
AA.4.2 Microsphere reservoirs lowered the cytotoxicity associated
with polypeptides while maintaining high transfection efficiency ...... 248
AA.5 Conclusions ................................................................. 249
BB. Supplementary information for Chapter 4 ....................... 250
CC. References ..................................................................... 253
DD. Research outputs ............................................................. 256
DD.1 Manuscripts ................................................................. 256
DD.2 Book chapters .............................................................. 257
DD.3 Research poster and oral presentations .......................... 257
DD.4 Research awards and prizes ........................................... 260
Table of Figures

**Chapter 1**

**Figure 1.1** Schematic representation of the intervertebral disc. The nucleus pulposus, is the highly hydrated core of the intervertebral disc. It is surrounded by the annulus fibrosus and enclosed by the cartilage end-plates. (Page 6)

**Figure 1.2** Schematic highlighting the effects of disc catabolism on back pain. Prolonged disruptions of the equilibrium anabolism/catabolism in the IVD lead to disc degeneration. This environment is characterized by a decrease in ECM production concurrently with an increase in secretion of pro-inflammatory cytokines. The developing inflammation is a self-perpetuating process that enhances the catabolism in the disc while favoring hyperalgesia and nerve ingrowth. With the progression of disc degeneration disc compression occurs which can lead to back pain. (Page 11)

**Figure 1.3** Schematic representation of strategies to restore disc anabolism. Disc degeneration coincides with the disruption of the equilibrium between anabolism and catabolism of the disc’s matrix. By delivering cells or growth factors, current strategies aim at restoring disc’s matrix anabolism. However, drawbacks of these strategies lead to the adoption of multimodal approaches in which cell and gene therapy are combined in controlled microenvironments (biomaterials) which synergistically contribute to the restoration of disc anabolism. (Page 31)

**Chapter 2**

**Figure 2.1** Schematic representation of the cross-talk between integrins and cytoskeleton. The following schematic depicts how the composition of the ECM can influence cellular cytoskeletal organization. When integrin α10β1 binds type II collagen it triggers a cascade of phosphorilations and activations of a number of intracellular proteins, in particular RhoA and its downstream effector ROCK which are responsible for
cytoskeletal organization. Therefore, allowing cells responding to the composition of the ECM by changing their shape. (Page 65)

**Figure 2.2** The microgels’ preparation. The forming-hydrogel solution is formed by mixing hyaluronic acid, type II collagen, ASCs and 4S-StarPEG. The gelling solution is then deposited in form of 2 μL droplets on a hydrophobic surface and allowed to gel for 1 h at 37°C. (Page 78)

**Figure 2.3** The effects of ASCs’ incorporation in the microgel on their viability. A–C) Effects of collagen concentration, cross-linker concentration and cell density on ASCs viability assessed by live/dead assay, no significant difference was found between the groups (n = 9, one way ANOVA, Tukey test p > 0.05). (Page 79)

**Figure 2.4** The effects of ASCs’ incorporation in the microgel on their proliferation. A–C) ASCs-loaded microgels after two days of culture and composed respectively of 2, 4 and 5 mg/mL type II collagen enriched with HA; D) Effects of collagen concentration on cells’ proliferation assessed by the BrdU assay, illustrating that cells maintain their ability to proliferate in every condition although the rate of proliferation drops after 14 days of culture. (Page 80)

**Figure 2.5** Enzymatic degradation of microgels and their stability over 21 days of culture. A) Degree of cross-linking of microgels (composed of 5 mg/mL type II collagen enriched with HA) assessed by the TNBS assay; the results are shown as the percentage of free amine groups normalized to non-cross-linked gels, a concentration of 2mM of 4S-starPEG is sufficient to obtain a similar degree of crosslinking of glutaraldehyde (n = 4, one way ANOVA, Tukey test p > 0.05); B) The effect of collagen concentration on stability of the microgels was quantified colorimetrically by Coomassie Blue Brilliant assay, results are shown as percentage of dye released over time, microgels composed of higher collagen
concentration had a higher resistance to enzymatic degradation \((n = 4, \text{ one way ANOVA, Tukey test } p > 0.05)\); C) Variation of diameter of cell seeded microgels was measured over 2, 7, 14 and 21 days of culture, high collagen concentration seems to increase the stability of the microgels as it significantly reduces the shrinkage of microgels. (Page 81)

**Figure 2.6** Calcein staining of embedded ASCs shows different cell morphologies in different concentrations of collagen. A, B, C) Bright field image of cells embedded in respectively 2, 4 and 5 mg/mL collagen II microgels for two days; D, E, F) Fluorescent image of calcein-stained cells embedded in respectively 2, 4 and 5 mg/mL collagen II microgels for two days. (Page 82)

**Figure 2.7** Effect of collagen concentration on cell morphology. A) Cell volume of ASCs embedded in microgels with different collagen concentration; live cells were stained with calcein and z-stacks analyzed by using an image analysis software; cells retain a higher volume when embedded in high collagen concentrations \((n = 4, \text{ one way ANOVA, Tukey test } p > 0.05)\); B) Measure of the longest axis of cells embedded in microgels, after two days. Cells in lower concentrations of collagen are more stretched but these differences decrease after 21 days of culture \((n = 4, \text{ one way ANOVA, Tukey test } p > 0.05)\); C) Shape factor index of cells embedded in microgels for 2 and 21 days. This index quantifies the differences in morphology of cells; over time cells showed a tendency to spread but maintained a more rounded morphology after 21 days of culture when embedded in high collagen concentrations \((n = 4, \text{ one way ANOVA, Tukey test } p > 0.05)\). (Page 83)

**Figure 2.8** High collagen concentrations hamper the formation of stress fibers in the cytoskeleton of the cells. A–D) Fluorescent images of cells cultured respectively on monolayer and in 2,
4, and 5 mg/mL microgels for 14 days (scale bars correspond to 50 µm); the nucleus was stained blue with DAPI and the cell cytoskeleton was stained red with Rhodamine/Phalloidin; cells in monolayer and 2 mg/mL microgels showed formation of stress fibers within their cytoskeleton, whereas in higher collagen concentrations the cell cytoskeleton was less organized and moreover showed a cortical actin organization, a hallmark of chondrogenic-like cells. (Page 84)

**Figure 2.9** Embedded ASCs possess a cytoplasm rich in glycogen deposits. A–C) TEM images of cross-sections of cells embedded in 2, 4 and 5 mg/mL collagen microgels respectively; the cytoplasm of these cells is rich in vesicles and glycogen deposits (indicated by arrows), hallmark of NP and chondrogenic-like cells. (Page 85)

**Figure 2.10** Embedding cells in high collagen concentration favours the expression of integrin α10 while delaying expression of ROCK 1. A) mRNA expression of the gene ROCK 1 in plain and differentiation media relative to monolayer control, type II collagen seems to delay the expression of ROCK 1 but this increases after 21 days of culture (n = 3, one way ANOVA, Tukey test p > 0.05); B) Relative mRNA expression of integrin alpha 10 in plain and in differentiation media; in differentiation media the expression of integrin alpha 10 is significantly higher in 5 mg/mL collagen, highlighting a stronger chondrogenic differentiation (n = 3, one way ANOVA, Tukey test p > 0.05). (Page 86)

**Figure 2.11** Embedding cells in high collagen concentration favours the expression of SOX9 and CD44. A) Relative mRNA expression of SOX9 in plain and in differentiation media; although there is an increase of SOX9 expression in all the samples, 5 mg/mL collagen microgels showed the highest increase (n = 3, one way ANOVA, Tukey test p > 0.05); B) Relative mRNA expression of CD44 in plain and in differentiation media; in presence of differentiation media
5mg/mL collagen microgels showed the highest increase of expression of this receptor \((n = 3,\) one way ANOVA, Tukey test \(p > 0.05\)). (Page 87)

**Figure 2.12** Embedding cells in high collagen concentration favours the expression of aggrecan and type II collagen. A) Relative mRNA expression of aggrecan in plain and in differentiation media; in differentiation media the expression of aggrecan increases in every sample, showing the highest increase in 5 mg/mL microgels \((n = 3,\) one way ANOVA, Tukey test \(p > 0.05\)); B) Relative mRNA expression of type II collagen in plain and in differentiation media, cells in higher collagen concentration started expressing type II collagen already after two days of culture and after 21 days the expression increased in a similar manner in all the conditions \((n = 3,\) one way ANOVA, Tukey test \(p > 0.05\)). (Page 88)

**Figure 2.13** Embedding cells in high collagen concentration lowers the expression of type I collagen. Relative mRNA expression of type I collagen in plain and in differentiation media, when in plain media the expression of type I collagen increased significantly in cells embedded in high collagen concentrations, but this trend was reversed in presence of differentiation media, highlighting a stronger chondrogenic differentiation \((n = 3,\) one way ANOVA, Tukey test \(p > 0.05\)). (Page 89)

**Figure 2.14** Microgels in plain media do not trigger ADSC differentiation. A) Correlation analysis of the mRNA expression of Integrin alpha 10 and other chondrogenic markers in plain media; in high collagen concentrations integrin expression negatively correlated with the expression of ROCK 1; correlation was calculated by using the Pearson coefficient and Prism® software \((n = 6,\) \(p > 0.05\)); B) Correlation analysis of the mRNA expression of CD44 and other chondrogenic markers in plain media; in low collagen concentration CD44 expression was correlated with the
expression of type I collagen while in high collagen concentration CD44 expression was correlated with the expression of type II collagen; C) Correlation analysis of the expression of Rock I and other chondrogenic markers also including cell morphology in plain media; as expected, there is a negative correlation between Rock I expression shape factor and integrin alpha 10 expression; D) Correlation analysis of the expression of SOX9 and other chondrogenic markers in plain media; note that Rock I did not downregulate the expression of SOX9, in addition; the expression of SOX9 did not correlate with the expression of other chondrogenic markers due to the absence of differentiation stimuli. (Page 90)

**Figure 2.15** 5 mg/mL collagen microgels offer an optimal microenvironment for ASCs differentiation in presence of differentiation media. A) Correlation analysis of the expression of Integrin alpha 10 and other chondrogenic markers in differentiation media; in 5 mg/mL collagen microgels the expression of integrin alpha 10 is positively correlated with the expression of other chondrogenic markers, showing a stronger differentiation; B) Correlation analysis of the expression of CD44 and other chondrogenic markers in differentiation media; in 5 mg/mL collagen microgels the expression of CD44 had the highest correlation with the expression of other chondrogenic markers, showing a stronger differentiation; C) Correlation analysis of the expression of ROCK 1 and other chondrogenic markers in differentiation media; ROCK 1 expression did not result in downregulation of chondrogenic markers; D) Correlation analysis of the expression of SOX9 and other chondrogenic markers in differentiation media; in 5 mg/mL collagen microgels the expression of SOX9 had the highest correlation with the expression of other chondrogenic markers; the higher SOX9 expression could explain the stronger
chondrogenic differentiation in higher concentrations of collagen II. (Page 92)

**Figure 2.16** 5 mg/mL collagen microgels offer the optimal conditions for ASCs delivery. Schematic representation of the results obtained in the current study. Overall 5 mg/mL microgels have been shown to possess superior stability and ability to prime ASCs’ differentiation towards an NP-like phenotype. (Page 96)

**Chapter 3**

**Figure 3.1** Schematic representation of the emulsion method. Water phase containing the neutralized collagen is mixed with the oil phase. Collagen spheres are obtained following stirring at 37°C for 2 hours. (Page 111)

**Figure 3.2** Schematic representation of the microphase separation method. By fast freezing a collagen solution there is formation of collagen compartments surrounding ice crystals. Following a gradual increase in temperature, the collagen will tend to acquire a globular structure because it is energetically more favourable. In this process water is removed by freeze-drying. (Page 112)

**Figure 3.3** Graphical representation of the fabrication of hollow collagen microspheres. The process involves the sulfonation of a commercially available polystyrene bead of defined size (100 nm, 1 μm and 10 μm in this case), coating of these beads in a collagen solution, crosslinking of the coating and removal of the polystyrene bead core. (THF: Tetrahydrofuran). (Page 113)

**Figure 3.4** Collagen spheres fabricated by using the emulsion method. (A) TEM image of a collagen sphere filled with a dense network of collagen fibers; (B and C) SEM images of collagen spheres. (Page 118)

**Figure 3.5** Collagen particles obtained with the macrophase separation method. SEM images of collagen particles fabricated with the
microphase separation; although these particles were homogeneous in appearance, they showed very low stability in water environments and were therefore deemed not suitable for use as reservoir systems. (Page 119)

**Figure 3.6** Steps of sphere fabrication. SEM images of the coating process, showing (A) polystyrene beads, (B) collagen-coated beads and (C) hollow spheres following treatment with THF to dissolve the template. (Page 120)

**Figure 3.7** Characterization of the template coating. Charge analysis showing the change in zeta potential during the fabrication of hollow collagen spheres of two sizes (500 nm (A) and 4.5 µm (B)) (n=3). (Page 121)

**Figure 3.8** Dissolution of the polystyrene template leaves a hollow core. (A) TEM image through polystyrene beads coated with type I collagen. (B) TEM image through type I collagen hollow spheres obtained after the dissolution of the polystyrene template in THF. (C) Size distribution of 500 nm microspheres measured by image analysis on SEM images. While the mean size is around 440 nm, this reduction in size compared to the 500 nm template may be explained by the effect of drying on the sample (n=180). (Page 122)

**Figure 3.9** Cell-spheres interactions. (A)* Percentage viability compared with cell alone control as measured by the alamarBlue® assay, data represents mean ± SD (n=3, one way ANOVA, Tukey test p > 0.05), (B) SEM image of type I collagen spheres, (C) SEM image showing the interaction between 3T3 Fibroblasts and collagen spheres. *Data obtained in collaboration with Shane Browne. (Page 123)

**Figure 3.10** Spheres’ internalisation. (A) TEM image of a cross-section through a 3T3 cell. (B) TEM image of a cross-section through a 3T3 cell following incubation with 1 µm collagen spheres. A small number of spheres can be seen to be internalized, with many more remaining external to the cell. (Page 124)
Chapter 4

Figure 4.1 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 spheres size and concentration was ascertained in monolayer culture of ASCs. Reservoirs of the largest size allowed the highest level of transfection in monolayer, and were therefore embedded in 3D microgels systems and optimized to obtain functional cell factories. (Page 145)

Figure 4.2 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 50µg of 4.5µm 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 hours; (n=3). (Page 146)

Figure 4.3 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). (Page 147)

Figure 4.4 Type II collagen hollow spheres enhances transfection efficiency of polyplexes. Gauussia luciferase assay to assess the ability of spheres reservoir to release active polyplexes capable of transfecting cells in monolayer culture; significantly higher level of transfection was obtained by using 4.5 µm spheres compared to 500 nm and 100 nm or polyplexes alone; (n=3, one way ANOVA, Tukey test p > 0.05). (Page 148)
Figure 4.5  ASCs transfected in monolayer expressing GFP. ASCs expressing GFP following treatment with polyplex-loaded spheres (green for GFP, blue for DAPI and red for rhodamine-phalloidin). (Page 149)

Figure 4.6  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); Figures B and D show the process of internalization that collagen hollow spheres undergo when incubated with ASCs in monolayer. (Page 150)

Figure 4.7  Schematic representation of fabrication of 3D microgels. The forming gel solution is formed by mixing hyaluronic acid, type II collagen, ASCs and 4S-StarPEG. The gelling solution is then mixed with spheres reservoir and deposited in the form of 2 μL droplets on a hydrophobic surface and allowed to gel for 1 h at 37°C. (Page 151)

Figure 4.8  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); B) Percentage of shrinkage of cell-seeded microgels loaded with different concentration of reservoirs and normalized on day two. The shrinkage is related to cellular metabolic activity and increases with the increasing concentration of reservoir; (n=3, one way ANOVA, Tukey test p > 0.05). (Page 152)

Figure 4.9  Transfection efficiency in 3D is dependent on polyplexes 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 a higher level of transfection over seven
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. (Page 153)

Chapter 5

Figure 5.1 Summary of the main outcomes from each phase of this thesis. (Page 170)

Figure 5.2 Representation of different droplet generation portions generally used in microfluidic devices. Channel geometries can be designed with a T-junction (A) or cross-junction (B); C) Example of a cross-junction. (Page 175)

Figure 5.3 Schematic representation of the possible applications for pH-sensitive crosslinkers. A) Collagen microspheres can be crosslinked by using pH-sensitive crosslinkers, the resulting reservoir will have a high degradation rate in acidic environments, thereby the releasing rate of their cargo (for example polyplexes) will be proportional to the pH of the environment; B) pH-sensitive crosslinkers can be used to link proteins or peptides directly to microspheres reservoirs. The link will be degradable in acidic environments thereby causing release of the cargo in a proportional way to the pH of the environment. (Page 178)

Figure 5.4 Schematic depicting future directions of the project. (Page 182)

Appendix

Figure F.1 Type II collagen/HA microgels deposited on a hydrophobic surface. (Page 202)

Figure Y.1 Reaction setup. Anisoaldehyde, hydroxyethylacrylate and toluene solufonic acid are mixed after being dehydrated for at
least 24 hours. The reaction is carried out for a minimum of 15 hours in ice. (Page 20)

**Figure Y.2** Schematic representation of the column setup used for the purification. (Page 21)

**Figure Y.3** NMR spectra of the reaction reveal the presence of product. (Page 21)

**Figure Z.1** A) 5 mg/mL microgels after deposition on a commercial Teflon® tape; B) SEM image of 5 mg/mL microgel seeded with ASCs and cultured for 7 days. (Page 22)

**Figure Z.2** Cell volume of ASCs embedded in microgels with different collagen concentrations; live cells were stained with calcein and z-stacks analyzed by using image analysis software; cell volume was influenced by collagen concentration and time but no significant differences were observed in cell volume of cells at the surface or the center of the microgels (n = 4, one way ANOVA, Tukey test \( p > 0.05 \)). (Page 23)

**Figure Z.3** Length of ASCs embedded in microgels with different collagen concentration; cell longest axis was influenced by collagen concentration and time but no significant differences were observed at the surface or the center of the microgels (n = 4, one way ANOVA, Tukey test \( p > 0.05 \)). (Page 24)

**Figure Z.4** Shape factor was influenced by collagen concentration and time but no significant differences were observed in cell morphology at the surface or the center of the microgels (n = 4, one way ANOVA, Tukey test \( p > 0.05 \)). (Page 25)

**Figure Z.5** A, B, C) Brightfield image of cells embedded in, respectively, 2, 4 and 5 mg/mL collagen II microgels for 7 days; D, E, F) Fluorescent image of calcein-stained cells embedded in, respectively, 2, 4 and 5 mg/mL collagen II microgels for 7 days. (Page 26)

**Figure Z.6** Comparison of shape factor index after 21 days of culture in different types of microgels. The shape factor index was significantly higher in ASCs embedded in microgels composed of high concentrations of type II collagen (average...
number of cells analyzed = 750 from 4 independent experiments, one way ANOVA, Tukey test p > 0.05). (Page 227)

**Figure Z.7** mRNA expression of the gene RhoA in plain media relative to monolayer control. The culture of cells in different concentrations of collagen does not seem to significantly influence the RhoA expression. (Page 228)

**Figure Z.8** Cells in microgels produce ECM. A) H&E staining of ASCs cultured in a pellet for 21 days in presence of differentiation media; B) H&E staining of ASCs embedded in 5 mg/mL collagen microgels for 21 days in plain media; C) H&E staining of ASCs embedded in 5 mg/mL collagen microgels for 21 days in differentiation media; D–F Alcian Blue staining of ASCs cultured in a pellet for 21 days in presence of differentiation media (D), in 5 mg/mL collagen microgels for 21 days in plain media (E) and in 5 mg/mL collagen microgels for 21 days in differentiation media (F). The staining revealed a more substantial deposition of GAGs for cells cultured in differentiation media while a light deposition of GAGs is observed when the cells are cultured in plain medium. (Page 230)

**Figure Z.9** Flow cytometric analysis of microgel embedded ASCs pulsed with BrdU. Cells stained with (blue) isotype control (FITC) or (red) monoclonal antibody against BrdU (FITC). (Page 233)

**Figure AA.1** Characterization of spheres’s surface and template removal. AFM image of the (A) surface topography of a hollow collagen sphere. Amplitude modulation AFM (B) amplitude and (C) phase images of the same sphere, displaying the fibrous structure present on the surface of the spheres. (B, C) Images have been 2nd-order flattened to subtract the curvature of the sphere and reveal the surface structure; (D) FTIR spectrum showing the removal of the polystyrene core following treatment with THF. The characteristic peaks of
polystyrene (dotted lines) are removed from the sample following THF treatment. (THF: Tetrahydrofuran). *Data obtained in collaboration with Shane Browne. (Page 241)

**Figure AA.2** Collagen spheres have a negative charge when immersed in PBS. Zeta analysis of collagen microspheres in PBS; all three sizes were seen to be negative, with the largest size, 10 µm, being more negative than the others. (n=6). *Data obtained in collaboration with Shane Browne. (Page 242)

**Figure AA.3** Reservoir characterization. (A) Loading efficiency of polyplexes within three sizes of hollow collagen sheres shows no significant difference, while release profile in PBS over 6 days, (B), shows an ability to delay the release of the polyplexes over time. Data represents mean ± SD (n=3). *Data obtained in collaboration with Shane Browne. (Page 243)

**Figure AA.4** Characterization of spheres’ loading with polyplexes. (A) TEM image of a cross-section through collagen hollow spheres before incubation with polyplexes; (B) TEM image of a cross-section through collagen hollow spheres after incubation with polyplexes. (Page 244)

**Figure AA.5** Transfection and polyplex toxicity. (A) Gaussia Luciferase assay to assess the ability of the microspheres to release bioactive polyplexes capable of transfecting cells *in vitro*. In this case, the 1 µm and 10 µm spheres have displayed an ability to release polyplexes capable of transfecting 3T3 Fibroblasts with a similar ability to polyplexes alone. (B) 3T3 Fibroblasts expressing GFP following treatment with polyplex-loaded spheres (Green – GFP, Blue - DAPI). (C) Cell metabolic activity is dramatically reduced following incubation with cationic polymers. However, this effect is removed following loading of the polymers with collagen microspheres (1 µm). Data represents mean ± SD (n=3). (PEI: Polyethyleimine, SF: dPAMAM, Superfect™, Qiagen).
*Data obtained in collaboration with Shane Browne. (Page 245)

**Figure AA.6** Collagen hollow spheres do not affect cell proliferation. Cell proliferation as measured by DNA content using the PicoGreen™ assay. It can be seen that there is no significant effect on the proliferation of 3T3 fibroblasts over 48, 120 and 168 hours when compared to the control (n=3). *Data obtained in collaboration with Shane Browne. (Page 246)

**Figure BB.1** Collagen hollow spheres can be produced with uniform size. Graph showing the diameter of collagen hollow spheres measured by analyzing SEM images of 25 spheres fabricated using 4.5 µm templates. This analysis was repeated for four different batches of spheres, one way ANOVA, Tukey test p > 0.05. (Page 250)

**Figure BB.2** Differing ratios of beads/collagen do not affect the charge on the spheres. Charge analysis of 4.5 µm spheres was conducted during each step of their fabrication. Following collagen coating, the charge of the polystyrene beads used as template turn positive, independently of the ratio of beads/collagen used. The surface charge of the spheres decreases after crosslinking. (n=3). (Page 251)

**Figure BB.3** No internalization of the reservoirs was observed in three-dimensional (3D) microgels. TEM images of cross sections through ADSCs embedded in 3D microgels functionalized with a polyplex-loaded collagen reservoir system. (Page 252)
Table of Tables

Chapter 1

Table 1.1 Delivery of growth factors to restore disc anabolism. (Page 16)
Table 1.2 Gene therapy approaches to restore disc anabolism. (Page 25)
Table 1.3 Cell therapy approaches to restore disc anabolism. (Page 32)

Chapter 2

Table 2.1 Percentage of proliferating cells over 14 days of culture in microgels. (Page 80)

Chapter 3

Table 3.1 Charge measurements during each step of spheres’ fabrication. (Page 121)

Appendix

Table F.1 Preparation of 200 µL of type II collagen hydrogel. (Page 201)
Table M.1 Reagents and their volumes required for a single reverse transcriptase reaction. (Page 210)
Table M.2 Reverse transcription program. (Page 210)
Table N.1 Polymerase chain reaction components. (Page 211)
Table N.2 General PCR program. (Page 212)
Table U.1 Preparation of 200 µL of type II collagen hydrogel functionalized with polyplex-loaded microspheres. (Page 216)
Table Z.1 Nucleotide primers used for reverse transcription-polymerase chain reaction. (Page 229)
Table Z.2 Correlation analysis of the mRNA expression of key genes such as Integrin α10, CD44, Rock I and SOX9 and other chondrogenic markers in plain media; in the table are reported only significative correlations and their strength represented by gradients of colors; correlation was calculated by using the Pearson coefficient and Prism® software (n = 6, p > 0.05). (Page 231)
Table Z.3 Correlation analysis of the mRNA expression of key genes such as Integrin α10, CD44, Rock I and SOX9 and other
chondrogenic markers in differentiation media; in the table are reported only significant correlations and their strength represented by gradients of colors; correlation was calculated by using the Pearson coefficient and Prism® software ($n = 6$, $p > 0.05$). (Page 232)
I certify that the thesis is all my own work and I have not obtained a degree in this University, or elsewhere, on the basis of this work.
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List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4S-StarPEG</td>
<td>4-arm polyethylene glycol succinimidyl glutarate</td>
</tr>
<tr>
<td>AAVs</td>
<td>Adeno-associated viruses</td>
</tr>
<tr>
<td>AF</td>
<td>Annulus fibrosus</td>
</tr>
<tr>
<td>ASCs</td>
<td>Adipose-derived stem cells</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMP-2</td>
<td>Bone morphogenic protein-2</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CBB</td>
<td>Coomassie brilliant blue assay</td>
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<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DDD</td>
<td>Disc degeneration diseases</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle's medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Desoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EPs</td>
<td>End plates</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HDMS</td>
<td>Hexamethyldisilazane</td>
</tr>
<tr>
<td>IBMX</td>
<td>Isobutylmethylxanthine</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IVD</td>
<td>Intervertebral disc</td>
</tr>
<tr>
<td>LBP</td>
<td>Low back pain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>miRNA</td>
<td>micro RNA</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
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<td>Metalloproteinases</td>
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<td>MSCs</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleus pulposus</td>
</tr>
<tr>
<td>OP-1</td>
<td>Osteogenic protein-1</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Poly(amidoamine)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
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<td>Polydimethylsiloxane</td>
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<td>Plasmid DNA</td>
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<tr>
<td>PEI</td>
<td>Polyethyleimine</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
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<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
</tr>
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<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>siRNA</td>
<td>small-interfering RNA</td>
</tr>
<tr>
<td>SCB</td>
<td>Sodium cacodylate buffer</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electronic microscopy</td>
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<tr>
<td>SF</td>
<td>Superfect™</td>
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<tr>
<td>SOX9</td>
<td>Sry-related HMG box-9</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electronic microscopy</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor-β1</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TNBS</td>
<td>Trinitrobenzenesulfonic acid</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Abstract

Low back pain has severe consequences for the quality of life of the affected patients and strong evidence suggests that this condition is caused by degeneration of the intervertebral disc (IVD). Disc degeneration coincides with alterations in structure and composition of the extracellular matrix (ECM) and a loss of anabolic potential by the disc cells. Unfortunately, current therapies can only provide symptomatic relief without addressing the underlying biological problem. The failure in regenerating a degenerated disc not only aggravates the patient’s discomfort but also risks to impair the ability of the spine to absorb shocks, with consequent spreading of degeneration to the adjacent discs. The unique physiology of the IVD and the disruption of a vast array of intercellular signalling mechanisms in disc degeneration are posing serious challenges for the tissue engineering of the disc. In fact, unimodal strategies such as cell delivery or growth factor delivery alone had only moderate success. For this reason, this project synergistically combines cell and gene therapy approaches to trigger regeneration in a key region of the IVD, the nucleus pulposus (NP). The main objective is to develop an optimal microenvironment, tailored for the NP, in which stem cells can be programmed to secrete specific proteins of interest. Overall, the project has three phases. In the first phase, a three dimensional (3D) type II collagen/hyaluronan microgel platform was designed to deliver adipose-derived stem cells (ASCs) to the nucleus pulposus (NP). The macromolecular concentration of the microgel was tailored to prime desired cellular phenotype and ultimately to favour the process of stem cell differentiation towards a NP-like phenotype. The second phase consisted in the fabrication of a microsphere reservoir system for the delivery of non-viral gene vectors such as polyplexes. The key features of this reservoir system are high control over size and shape of the microspheres and also the use of type II collagen as a building block for their fabrication. In the last phase, the therapeutic potential of the platforms developed was characterized and 3D microgels were functionalized with polyplex-loaded microsphere reservoirs. These collagen reservoirs shielded the cells from the toxicity of the polyplexes, while allowing high levels of transfection. Although lower levels of transfection were observed in 3D...
microgels than in monolayer, 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. Hence, the functionalization of 3D microgels with polyplex-loaded microsphere reservoirs allows for the production of cell factories able to manufacture targeted therapeutic proteins. This offers great potential for regenerative therapies of the NP.
Chapter 1

Introduction

Sections of this chapter have been published:

G. Fontana, E. See, A. Pandit,

*Current Trends in Biologics Delivery to Restore Intervertebral Disc Anabolism*

Advanced Drug Delivery Reviews, 2014
1.1 Overview on the importance of treating disc degeneration

Together, the intervertebral discs and the vertebrae form the spinal canal which is where the spinal cord and the nerve roots are located. As a consequence of disc degeneration, structural changes occur to the disc that affect its height and also its integrity. The dehydration caused by disc degeneration provokes a decrease of the internal pressure needed to maintain disc height and it often coincides with disc compression. As a result, the space between the vertebrae decreases, reducing the stability of the spine and causing the growth of bone spurs or osteophytes. Bone spurs can apply pressure on the nearest spinal nerve roots or on the spinal cord, causing the resurgence of pain and affecting nerve function. Moreover, with aging, a tissue that contains the nucleus pulposus (NP) such as the annulus fibrosus (AF) wears and dehydrates. If as a consequence, tears or fissures disrupt the integrity of the AF, this may be the first stage of disc herniation. The second stage, in fact, consists of the prolapse of the NP that pushes through the layers of the AF but remains contained by the outermost layers. In the worst cases the NP can extrude from the AF and may project into the spinal canal compressing nerves and thereby cause pain. This process is also favoured by other anatomical processes such as the thinning of the posterior longitudinal ligament. Furthermore, depending on the type of herniation, the symptoms and the nature of pain can vary. For example, if a herniated disc is compressing the sciatic nerves, the patient is most likely to experience leg pain or sciatica. And, massive disc herniation or extrusion can also lead to disturbances in bowel or bladder function (sphincter incontinence). Therefore, the failure in regenerating a degenerated disc increases the patient’s discomfort and also risks impairing the ability of the spine to absorb shocks, with consequent spreading of degeneration to the adjacent discs. Considering that the self-healing capabilities of the intervertebral discs are very limited, regenerative therapies are needed.

1.2 Overview on Disc Degenerative Disease

Low back pain (LBP) is a cause of disability, with significant socio-economic consequences \(^1-^4\) and annual healthcare costs in the United States alone are estimated at $100 billion \(^5,^6\). Although LBP is prevalent among
20-50 year olds, it becomes more severe with aging, thereby adversely affecting the quality of life in older patients\(^7\).

LBP is generally attributed to degeneration of the intervertebral disc (IVD)\(^8\)-\(^11\). Disc degeneration is a multifactorial disease characterized by genetic and environmental factors\(^12\). In degenerative IVDs there are alterations in the matrix composition\(^13\),\(^14\) that coincide with the deterioration of biochemical properties\(^15\),\(^16\), a decrease in ability to withstand mechanical loading\(^17\) and reduced cellular activity\(^18\),\(^19\) caused by the aberration of the crosstalk between cells and the matrix of the IVD. Current therapies are focused on gaining symptomatic relief without addressing the underlying biological problem\(^20\). These therapies consist in medications and physical therapy or in surgical intervention such as spine fusion, discectomy or total disc replacement and offer only a temporary solution as they do not halt the deterioration of the IVD\(^11\),\(^21\). Because the nucleus pulposus (NP) is the tissue that undergoes the most dramatic changes with aging\(^22\), it has attracted much attention in the tissue engineering field. In particular, new strategies are being developed to deliver therapeutic agents such as cells, growth factors and gene vectors to the NP to inhibit further degeneration and restore its functionality.

### 1.3 The Intervertebral Disc

The intervertebral disc (IVD) is composed of different fibro-cartilaginous tissues that are interposed between the vertebral bodies in the spine. The IVD acts as a shock-absorber and while transmitting forces from one vertebral body to the next imparts, overall, mobility to the spine\(^23\)-\(^26\). The IVD is the largest avascular organ in the human body\(^27\), yet it is a highly hydrated tissue\(^23\),\(^26\). This is due to the composition and structure of its extracellular matrix (ECM). To resist compression imposed by gravity and to accommodate a number of other mechanical loads, the IVD has to encompass different physico-chemical microenvironments. It can be roughly divided into three regions: a highly hydrated core which is the nucleus pulposus (NP); a less hydrated, fibrous structure that surrounds the core and is known as the annulus fibrosus (AF); and the complete structure is enclosed by the end plates (EPs) at the interfaces with the vertebral
bodies. Each region is structurally very different, and in the healthy IVD, these differences are well demarcated 28.

1.3.1 The Nucleus Pulposus
The NP is the most hydrated region of the IVD and is constituted by a network of randomly oriented type II collagen fibrils in which is positioned an amorphous matrix of heteropolysaccharides, the glycosaminoglycans (GAGs) 27. GAGs can be linked to a core protein, forming macromolecules called proteoglycans or can be found as free chains also known as hyaluronan (HA) 29, 30. HA is one key components of the NP’s matrix as it fulfills a number of roles. From a biomechanical point of view, it helps to maintain intradiscal pressure because of its ability to retain water 23 but also because it plays a role in matrix assembly by facilitating the formation of aggregates 31. From a biological standpoint, because of its ability to bind to the membrane receptor CD44, HA plays an important role in favouring cell adhesion to ECM components and is implicated in the homeostasis of the disc by maintaining cell phenotypes, regulating cell functions such as migration and proliferation 32, 33 and in preventing disc cells from undergoing apoptosis 34. Proteoglycans, on the other hand, are responsible for the elevated level of hydration in the NP. This is possible because proteoglycans, and in particular aggrecan 12, possess the ability to bind HA forming large aggregates 23, 35. Because these aggregates are too large to diffuse out of the type II collagen network, they create osmotic imbalances attracting water into the tissue 36. The internal pressure of the NP is very important not only from a physiological point of view, but also because it enables the tissue to withstand compressive forces 37. Furthermore, the high concentration of aggrecan in the NP contributes to the maintenance of the disc homeostasis by inhibiting the calcification of the tissue 38 and by inhibiting nerve ingrowth 39.

1.3.2 The Annulus Fibrosus
While the NP attracts and retains water, the AF holds the NP 40 and, by containing its swelling pressure, allows the disc to maintain its height. This, in turn, allows the compression/decompression cycle needed for diffusion of
nutrients and metabolites in and out of the tissue. To resist tensile and shear stresses due to the internal pressure exerted by the NP, the AF needs to be highly organized in concentric lamellae reinforced with collagen fibers. Moreover, the AF is characterized by different sub-regions, also called the inner-AF and the outer-AF. While the inner-AF is a transitional zone between the AF and the NP, the outer-AF has an abundant presence of type I collagen in its matrix, which is more capable than type II collagen of resisting tensile loads as it possesses a greater ability to form fibrillated structures.

1.3.3 The End Plates
The doorway that selects and allows the entry of biomacromolecules into the IVD is constituted by the end plates. These are thin layers structurally similar to hyaline cartilage that enclose the disc, distribute pressures onto the adjacent vertebrae and prevent the NP from bulging in the adjacent vertebral bodies. To do this, the end plates must balance the conflicting requirements of being sufficiently strong to maintain stability while at the same time allowing for diffusion of nutrients to the disc. The maintenance of their hydration and structural integrity is vital for the homeostasis of the disc. The IVD, in fact, relies heavily on diffusion of solutes across the end plates for nutrition. However, with aging, the end plates undergo a process of calcification that alters their permeability. This impairs diffusion of nutrients to the disc, leading to degeneration.
Figure 1.1: Schematic representation of the intervertebral disc. The nucleus pulposus, is the highly hydrated core of the intervertebral disc. It is surrounded by the annulus fibrosus and enclosed by the cartilage end-plates.
1.3.4 The Cellular Component of the Disc

The IVD is a tissue with a low cell population. In the NP, cells occupy less than 1% of the tissue volume and the two main cell types are notochordal and NP cells \(^{23}\). The notochordal cells are large (30\(\mu\)m) and possess a cytoplasm rich in vacuoles \(^{54}\). These cells actively participate in the development of the disc through the synthesis of high amounts of GAGs and also in production of soluble factors that stimulate NP cells to produce more matrix \(^{54, 55}\). The NP cells, on the other hand, are morphologically similar to chondrocytes, with a rounded shape and an average diameter of 10 \(\mu\)m \(^{56}\).

Going towards the outer AF, there is a different cell population with fibroblastic appearance; the AF cells. When compared to the NP, the AF has a higher cell density. In fact, while in the NP there are on average 4,000 cells/mm\(^3\), in the AF there are 9000 cells/mm\(^3\) \(^{57}\). Cells in the AF and in the NP differ not only in number and shape but also in the ECM they produce. In adult discs, there is a decrease in the disc cell population. Aging, in fact, is correlated with the disappearance of notochordal cells from the disc \(^{58}\). This loss of notochordal cells coincides with the manifestation of the first signs of disc degeneration \(^{54}\). Furthermore, deprived of notochordal cells stimulation, the NP cells significantly lower the synthesis of ECM, which in turn profoundly impacts the disc anabolism \(^{23}\). However, recent studies suggest the presence of progenitor cells in the disc \(^{59-62}\). These cells have been shown to possess similar characteristics to those of MSCs \(^{62}\). But, since their presence markedly decreases with age and degeneration \(^{61}\), the regenerative potential of the disc is very limited.

1.4 Intervertebral Disc Degeneration

IVD is a dynamic structure because its ECM undergoes continuous remodeling, a process that involves IVD cells to synthesize both ECM and proteases \(^{25}\). However, the physiology of the IVD limits its regenerative potential. Due to its avascular nature and low cell density, the disc is prone to degeneration \(^{63}\). This is a multifactorial disease, caused by genetic and environmental factors. Although the impact of each individual environmental factor remains unclear, the accumulation of insults and injuries, obesity and psycho-social habits, seem to accelerate degeneration.
in the disc. Moreover, while studies highlighted genetic predisposition to this disease, it is still unclear whether there are specific genes that exert a relatively stronger effect. Even so, most of the genes identified to date encode for molecules that fulfill key roles in the homeostasis of disc matrix. Disc degeneration is a process that starts very early in life. Its severity increases with aging and results in alterations in composition and organization of disc matrix. This, in turn, disrupts the interactions between disc cells and the surrounding ECM causing a phenotypic switch that compromises the ability of IVD cells to maintain homeostasis. Moreover, it was found that in degenerative discs, endogenous cells can be responsible for the degradation of the ECM by expressing abnormal amounts of degradative proteases such as matrix metalloproteinases (MMPs) and aggrecanases. Changes in the phenotype of IVD cells also affect their ability to synthesize functional matrix. This was shown in a study by Gruber et al. where human AF tissues obtained from surgical procedures were analyzed and it was found that AF cells in degeneration and aging were not synthetically inactive but instead produced inappropriate matrix products. In the NP, disc degeneration coincides with a decrease in type II collagen and proteoglycans and an increase in type I collagen. Furthermore, because of the elevated activity of the degradative enzymes and the harsh conditions, many proteins of the disc matrix become denatured or altered. For example, collagen fibrils were found to possess a smaller degree of cross-linking, thus further weakening the structural integrity of the IVD. The loss of proteoglycans, and in particular of aggrecan, severely reduces the ability of the NP to retain water. The consequent decrease of the internal swelling pressure hampers the diffusion of nutrients in the disc and causes the pH of the tissue to drop because of the accumulation of metabolic waste. The acidic environment of a degenerated disc triggers a series of detrimental effects that exacerbate this situation. Nutrient deprivation and the acidic pH can affect cell metabolism and hinder further the ECM production. In these harsh conditions, disc cells accumulate irreparable damage to their DNA, becoming increasingly senescent and apoptotic. Overall, the hallmark
of disc degeneration is the disruption of the equilibrium between the anabolism and catabolism of the matrix.\textsuperscript{78, 79}

1.4.1 Vascularisation and Inflammation Increase Catabolism in the Disc

While the proteolytic activity of MMPs and aggrecanases plays an important role in breaking down the disc matrix,\textsuperscript{80} another constant feature of disc degeneration is angiogenesis.\textsuperscript{81} Neovascularization allows the infiltration of macrophages into the IVD, triggering inflammation.\textsuperscript{76} Hamamoto et al. showed that following interaction with macrophages, disc cells produce high amounts of prostaglandin (PG)E2 and IL-6.\textsuperscript{82} The ability of degenerative disc cells to produce pro-inflammatory cytokines such as IL-8, IL-6, IL-1β, IL-17 and TNF-α was also confirmed in a number of studies.\textsuperscript{82-85} Wang et al. showed that cytokines such as TNF-α and IL-1β promote aggrecan degradation by enhancing aggrecanases activity.\textsuperscript{83} On the other hand, IL-1β was also shown to induce further angiogenesis by promoting VEGF expression in the disc,\textsuperscript{86} thereby triggering a self-perpetuating process. Due to a lower intradiscal pressure, the AF is more exposed to mechanical stresses. The disorganization of AF matrix that occurs with aging, lowers the ability of the AF to withstand mechanical stresses and increases the likelihood of tear formation.\textsuperscript{88, 89} This allows formation of blood vessels that from the outer AF grow towards the center of the disc.\textsuperscript{74}

1.4.2 Discogenic Pain

There is a correlation between disc degeneration and low back pain (Figure 1.2).\textsuperscript{12, 63} With the progression of degeneration, innervation of the disc occurs.\textsuperscript{90-93} This process results in the growth within the disc of unmyelinated nerve fibers, which require a low level of chemical and mechanical stimuli to trigger pain.\textsuperscript{94} Increased innervations were observed to originate along the tears in the AF and, because of alterations in ECM composition and favored by a lower internal pressure, these nerve fibers were found to penetrate even into the NP.\textsuperscript{95} The matrix of the healthy IVD, in particular the aggrecan, is very effective in controlling and inhibiting nerve ingrowth within the disc.\textsuperscript{39} Even so, the loss of proteoglycans that
occurs during degeneration lowers the ability of disc matrix to maintain its aneural condition 39, 96. Furthermore, the complex array of glycoproteins, collagens and GAGs that constitutes the disc microenvironment and is crucial for the maintenance of disc cells phenotype 97, is markedly altered in degenerative discs. This change in disc composition is responsible for the phenotypical switch that occurs in degenerative disc cells, affecting their ability to control processes such as neural ingrowth. Healthy disc cells produce abundant amounts of semaphorin 3A, an axonal guidance molecule with the ability to hinder neural ingrowth. Tolofari et al. demonstrated that in degenerated painful IVD the expression of semaphorin 3A is significantly lower, resulting in increased innervation 98. Overall, despite the fact that mechanisms underlying neural ingrowth are still not well understood, they are favored by a weaker disc homeostasis. Another cause of discogenic pain is the decrease of disc height, which results in painful compression of the adjacent nerves 63. However, what little is known about discogenic pain derives from the comparison of healthy and degenerated discs in animal models or in cadavers, where measurement of pain is unfeasible 77. Moreover, because the spinal structures are multi-segmentally innervated, the propagation of pain overlaps with the adjacent areas, resulting in a phenomenon known as referred pain, making pain projections unreliable for determining the source 99. Nevertheless, in terms of likelihood, the most frequent sources of patient discomfort is usually the end plate and the outer region of the AF 90.
Figure 1.2: Schematic highlighting the effects of disc catabolism on back pain. Prolonged disruptions of the equilibrium anabolism/catabolism in the IVD lead to disc degeneration. This environment is characterized by a decrease in ECM production concurrently with an increase in secretion of pro-inflammatory cytokines. The developing inflammation is a self-perpetuating process that enhances the catabolism in the disc while favoring hyperalgesia and nerve ingrowth. With the progression of disc degeneration disc compression occurs which can lead to back pain.
However, because small end plate defects are difficult to observe radiographically \(^{100, 101}\), the clinical significance of end plates damage and its association with neo-innervation is generally underappreciated \(^{102}\). Increased innervation was found in damaged end plates \(^{103}\) with substantial presence of pain fibres \(^{104}\). Moreover, in damaged end plates, the communication between NP and vertebral marrow is facilitated \(^{51, 105}\). This can cause the adverse combination of nerve proliferation within the disc and also their chemical sensitization \(^{102}\). In fact, growing evidence suggests a link between the presence of end plate defects and back pain \(^{106-109}\).

1.5 Current Treatments
Conventional treatments of patients suffering from LBP aims at eliminating the painful motion that occurs in degenerated discs. The most widely used surgical interventions consist on interbody fusion, discectomy and disc replacement \(^{87, 110}\).

1.5.1 Interbody Fusion
This technique is used when instability of the spine segment is detected \(^{110}\). The damaged disc is partially removed and by using spacers the vertebrae are lifted, thus reducing the eventual pressure exerted on spine nerves. To stabilize the spine, a metal cage containing a bone graft is placed within the disc space \(^{111}\). This, results in bone growth and fusion of the vertebrae. Studies conducted in the US revealed that interbody fusion succeeds in eliminating motion through the joint and alleviating pain \(^{112-114}\). However, long-term follow-ups of patients that received spinal fusion showed pronounced degeneration occurring in the discs adjacent to the fused vertebrae, probably due to altered biomechanics and uneven transmission of loads through the spine \(^{115}\).

1.5.2 Discectomy
When disc herniation occurs, the bulging disc might compress the spinal nerves thereby inducing pain. Discectomy is used to remove this portion of the disc and was found to alleviate pain \(^{87}\). However, a limitation to this procedure is that the removal of the disc often results in destabilization of
the spine segment with consequent altered biomechanics and degeneration of the adjacent discs\textsuperscript{87}.

1.5.3 Disc Replacement

In an attempt to preserve the spine stability and flexibility, disc replacements have been developed\textsuperscript{116}. \textit{In vivo} studies in baboons and rabbits revealed that poly(vinyl)alcohol-based hydrogels succeeded in delaying significantly the degeneration process that usually affects the adjacent discs\textsuperscript{117, 118}. However, the weak adhesion of the hydrogel to the native tissue causes mechanical destabilization of the implant that in the long term fails to integrate\textsuperscript{119}.

1.5.4 Tissue Engineering Approaches to Restore Disc Anabolism

None of the above mentioned surgical approaches showed satisfactory long-term effects on treated patients\textsuperscript{87}. In fact, they can only attenuate pain in the short-term but do not address the underlying biological problems\textsuperscript{18}. Moreover, the removal of functional native disc tissue often results in the disruption of the spine biomechanics, with loss of flexibility and alterations in the transmission of mechanical loads which in turn, causes degeneration of adjacent discs. In recent years, research activities in tissue engineering of the IVD has been focused on restoring the lost anabolic potential of degenerative discs via delivery of biologics and engineering biomaterials\textsuperscript{23}. Moreover, targeting disc anabolism can disrupt the self perpetuating process that fuels and characterizes disc degeneration. If successful, pro-anabolic approaches can trigger disc regeneration while lowering pain-related symptoms\textsuperscript{120}.

1.5.4.1 Protein Therapy

Growth factors are the effectors of intercommunication between cells. They are polypeptides that, by targeting specific receptors on the surface of the cells, influence their behavior in terms of proliferation, differentiation and ability to synthesize ECM\textsuperscript{121}. Depending on their range of action, their effects can be defined as autocrine, endocrine and paracrine. Because of the avascularity of the IVD, disc cells are often regulated by autocrine and
paracrine mechanisms. However, both the phenotypical switch and the loss of cells during disc degeneration leave the IVD devoid of signals to fuel matrix anabolism. Therefore, it appears that approaches aimed at regenerating the IVD should also aim at restoring the disrupted cell signaling mechanisms. Studies that investigated the effects of growth factors on cell behavior underpinned this concept, showing increased ECM production following intradiscal injections of growth factors (Table 3). Kim et al. conducted an in vitro experiment using human IVD cells exposed to different concentrations of BMP-2. The anabolic effects on disc cells were evident and, moreover, the increase in proteoglycan synthesis was correlated to the dose of BMP-2. In fact, administration of 300 ng/mL BMP-2 corresponded to an increase in proteoglycans by 67%, whereas for 1500 ng/mL the increase was in the range of 200% \textsuperscript{122}. In another in vitro study, Li et al. demonstrated that administration of BMP-2 not only coincides with upregulation of collagen II and aggrecan expression but also increases the expression of other growth factors such as TGF-β1 and BMP-7 \textsuperscript{123}. However, the context in which cell signaling occurs can exert great influence over the cellular response \textsuperscript{124}. The simple interaction between cells and ECM ligands can enable cells to become more responsive to the effects of growth factors \textsuperscript{125}. For this reason, in the context of a degenerated disc, where the environment is not favorable, the ability of cells to respond to growth factors can be seriously compromised. Takegami et al. hypothesized that NP and AF cells respond positively to administration of osteogenic protein-1 (OP-1) even in a degenerative environment. The study was conducted in vitro, whereas NP and AF cells were cultured for two weeks in alginate beads and then exposed to chondroitinase ABC to digest the GAGs in their matrix, mimicking a degenerative environment. Despite the harsh conditions, both NP and AF cells responded positively to administration of OP-1 by producing higher amounts of proteoglycans, thus they repaired their matrix much more rapidly \textsuperscript{126}. Imai et al. tested the same hypothesis in vivo in a rabbit model of disc degeneration induced by injection of chondroitinase ABC. The injection of chondroitinase caused a dramatic decrease in disc height; however, discs that received a single injection of
recombinant human OP-1 displayed a more prominent recovery of disc height after 12 weeks $^{127}$.

1.5.4.2 Optimizations in the use of Growth Factors and their Limitations

Even though a single injection of growth factors can have some positive effects, this approach is far from mimicking the physiological cell signaling machinery. In fact, growth factors seem to act synergistically in enhancing cells’ anabolic activity. Thus clinically significant effects can potentially be obtained by using cocktails of growth factors. Cho et al. designed an *in vitro* model of AF degeneration where AF cells were cultured in monolayer and activated with IL-$1\beta$ and TNF-$\alpha$. In this study the synergistic effects of administration of TGF-$\beta1$ and BMP-2 were analyzed and compared with the administration of either growth factor alone. The combination of the two growth factors coincided with the optimal outcome, with lower levels of MMP-1 and increased expression of aggrecan suggesting that the use of cocktails of growth factors can give promising results $^{128}$. However, despite the therapeutic significance of growth factors, their instability and short half-life *in vivo* limits their use $^{129, 130}$. Therefore, to obtain long-term effects, it may be necessary to repeat multiple injections of growth factors $^{131}$. This, though, can have serious implications because each injection can lead to irreparable damage, and thereby weigh adversely on the risk/benefit ratio. Moreover, the injection of growth factors in a liquid state is not the ideal form of delivery because the internal pressure of the disc can cause leakage in the adjacent tissues provoking undesired effects $^{132}$. Dosage problems and limited half-life of growth factors can be overcome by accessing the cell’s secretory machinery through gene therapy approaches.
Table 1.1: Delivery of Growth Factors to Restore Disc Anabolism

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Approach</th>
<th>Environment</th>
<th>Outcomes</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-2 and TGF-β1</td>
<td>Exposure of activated AF cells to both BMP-2 and TGF-β1</td>
<td>In Vitro</td>
<td>Delivered BMP-2 and TGF-β1 acted synergistically in increasing aggrecan expression and in lowering MMP-1 expression</td>
<td>121</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>NP cells seeded into type I collagen/HA scaffold for 60 days in presence of TGF-β1</td>
<td>In Vitro</td>
<td>Proteoglycan accumulation was enhanced by addition of TGF-β1 but in all conditions the amount of proteoglycans that was not retained in the scaffold was greater than the amount that was deposited</td>
<td>195</td>
</tr>
<tr>
<td>Growth Factor</td>
<td>Approach</td>
<td>Environment</td>
<td>Outcomes</td>
<td>Ref.</td>
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<tr>
<td>IGF-1, EGF, FGF, and TGF-β1</td>
<td>In vitro culture of entire IVD explants in presence of serum and various growth factors</td>
<td>In Vitro</td>
<td>An increase in proteoglycan synthesis and cell proliferation was observed in presence of EGF, FGF and TGF-β1. Only a limited response was observed in presence of IGF-1</td>
<td>199</td>
</tr>
<tr>
<td>OP-1</td>
<td>A degenerative disc model received a single injection of OP-1 four weeks post-injury</td>
<td>In Vivo</td>
<td>A single injection of OP-1 was found to restore disc height six weeks post-treatment</td>
<td>200</td>
</tr>
<tr>
<td>GDF-5</td>
<td>IVD cells from GDF-5-deficient mice were isolated and cultured in 3D alginate beads in presence of GDF-5</td>
<td>In Vitro</td>
<td>Following administration of GDF-5, cells showed a dose-dependent increase in mRNA expression of type II collagen and aggrecan</td>
<td>201</td>
</tr>
</tbody>
</table>
Table 1.1 (Continued)

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Approach</th>
<th>Environment</th>
<th>Outcomes</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>OP-1</td>
<td>NP or AF cells were cultured in alginate beads and exposed to Chondroitinase and OP-1</td>
<td>In Vitro</td>
<td>NP and AF cells cultured in presence of OP-1 recovered their proteoglycan content more rapidly than their relative control did</td>
<td>119</td>
</tr>
<tr>
<td>BMP-2 and TGF-β3</td>
<td>IVD explants were cultured in presence of BMP-2 and TGF-β3</td>
<td>Ex Vivo</td>
<td>Exposure to BMP-2 and TGF-β3 induced ossification of the AF</td>
<td>125</td>
</tr>
<tr>
<td>BMP-2</td>
<td>Human IVD cells cultured in presence of BMP-2</td>
<td>In Vitro</td>
<td>BMP-2 increased the expression of aggregan and collagens in a dose-dependent manner</td>
<td>115</td>
</tr>
</tbody>
</table>
1.5.4.3 Gene Therapy
Gene therapy involves the delivery into a patient’s cells of short sequences of DNA encoding for therapeutic proteins. The advantages of such an approach are evident as it allows the conversion of the transfected cells into protein-producing factories. This results in the manufacture of the desired growth factors for an extended period. However, there are some hurdles that gene therapy must overcome in order to show efficacy. In fact, as a defence mechanism, cells generally do not take up genetic material; consequently, gene therapy needs vectors to transport, protect and deliver genetic material to target cells. In particular, the ability of the gene vector to overcome cells’ membranes and at the same time maintain the integrity of the delivered genetic material is crucial for the successful transgene expression. To date, viral vectors offer the highest efficiency and thus are the most widely used platform for the gene therapy of the intervertebral disc.

1.5.4.4 Viral Vectors
Adenoviruses are large viruses with a genome formed by double stranded linear DNA. Because of their ability to infect even non-dividing cells, they are a suitable vector for the gene therapy of cells with low proliferation rate such as the IVD cells. Adenoviruses have been modified to carry genes coding for therapeutic proteins and showed high efficiency in infecting disc cells. However, because NP and AF cells phenotypes are yet not fully understood, it remains unclear which genes possess the highest pro-anabolic potential. As described in Table 3, adenoviruses have been used as a vector for genes coding for a variety of molecules, from growth factors to transcription factors such as SOX-9. This transcription factor coordinates the synthesis as well as the post-translational modifications of several ECM proteins. SOX-9 expression is severely downregulated in degenerative discs and might affect not only the ability of IVD to synthesize ECM but also to maintain it. In fact, studies in SOX-9 depleted mice have shown that absence of SOX-9 coincides with higher expression of MMP-3 and MMP-9, suggesting a role in regulating ECM homeostasis. Paul et al. used a viral vector expressing SOX-9 and studied its effects in a rabbit model...
Chapter 1

model. The synthesis of both SOX-9 and its downstream target, type II collagen, was significantly increased in the transfected discs, which also had a better histological appearance than that of control discs \(^{137}\). The pro-anabolic effects of SOX-9 can be further enhanced by the presence of different growth factors. This was investigated *in vitro* by Zhang et al. where both AF and NP cells were transfected with adenoviruses expressing SOX-9 and a series of BMPs \(^{138, 139}\). In both cases, an increase in proteoglycans and collagen accumulation was reported; but while adenoviruses expressing BMP-2 and -7 were most effective in stimulating proteoglycan accumulation, the transfection with BMP-4 and -14 promoted higher collagen accumulation \(^{138, 139}\). The potential in using biologic “cocktails” was further investigated by Moon et al., with interesting results. Human IVD cells were cultured in 3D alginate beads and transfected with adenoviruses bearing the gene for TGF-β1 or IGF-1 or BMP-2, or transfected with a combination of all. The highest increase in proteoglycan synthesis was obtained with the combination of all three growth factors \(^{140}\).

### 1.5.4.5 Optimizations in the Use of Viral Vectors

However, there are some safety concerns regarding the use of adenoviruses which severely limit their adoption in clinic. In fact, they activate immune responses shortly after administration *in vivo* despite the removal of the majority of viral genes. This occurs because the innate immune response can be triggered by the viral capsid and therefore is transcription independent \(^{141}\). Nevertheless, because the IVD is an immune-privileged tissue, using adenoviral vectors for the gene therapy applications offers limited likelihood of triggering immune responses within the disc \(^{129}\). However, the chances of adenovirus leakage in adjacent tissues are elevated and the possible consequences cannot be understated. Wallach et al. conducted a study in a rabbit model to assess the safety of high doses or misdirected gene delivery to the spinal column. Animals receiving high doses of adenoviruses bearing the gene for TGF-β1 developed bilateral lower extremity paralysis, raising some concerns over the use of adenoviruses \(^{142}\). However, to overcome such dose issues, it is possible to use adenoviruses bearing tet-off inducible systems. Vadala et al. designed
an adenoviral vector expressing for fas ligand and green fluorescent protein (GFP) with a tet-off inducible system. Human NP cells were efficiently transduced and, by addition of tetracycline in the media, it was possible to transiently turn off the expression of the transduced genes\textsuperscript{143}. An alternative to adenoviruses is adeno-associated viruses (AAVs). These are single-stranded DNA viruses incapable of replication without assistance of a helper virus such as adenovirus. Moreover, AAVs have shown little or no immune reaction and allow for a prolonged transgene expression\textsuperscript{135}. Ren et al. used AAVs to co-transduce rabbit IVDs to over-express SOX-9 and OP-1. The dual gene co-transduction significantly ameliorated the height of the infected discs and induced higher expression of proteoglycans and type II collagen\textsuperscript{144}. As with the adenoviruses, AAVs were engineered to allow a higher degree of control over the expression of the transduced genes. Unlike the tet-off system, an AAV–RheoSwitch system only allows the expression of the transgene in the presence of an activator ligand. Sowa et al. designed an AAV–RheoSwitch GFP system and tested its efficacy in a rabbit in vivo model. After transfection, only the discs that received the ligand expressed GFP and this expression was dose and time-dependent. Moreover, 24 hours after removal of the ligand, the GFP expression decreased significantly\textsuperscript{145}. Although these results are quite encouraging, they do not address the shortcomings in the use of AAV. AAVs are difficult to produce and can carry only a small amount of DNA\textsuperscript{135}. So an alternative to viral vectors might be needed.

\textbf{1.5.4.6 Non-Viral Vectors}

The use of non-viral approaches for the gene therapy of the IVD is still quite limited. Nonetheless, there is growing interest in developing alternatives to viral vectors. For example, Nishida et al. tested in vivo a new gene transfer technique which involves the use of an ultrasonography contrast agent (microbubbles) to enhance the ultrasound gene transfer. As a proof of concept, two reporter plasmids were used: GFP and firefly luciferase. The ultrasound exposure combined with the presence of an ultrasound contrasting agent (microbubble) are able to temporarily increase the permeability of cells’ membrane to large molecules such as plasmid DNA.
This significantly enhanced the transfection of NP cells that maintained the transgene expression for at least 24 weeks. Morrey et al. investigated an alternative by using 17 lipid-based non-viral reagents and tested their transfection efficiency in vitro. Overall, the transfection efficiency was much lower than that of viral vectors; this is due to the fact that the matrix surrounding the cells was shielding them from the transfecting agents. In fact, the transfection rate was significantly higher when the agents were used in combination with hyaluronidase.

Another non-viral gene therapy approach attracting much attention is the use of cationic polymeric systems that are able to bind and form complexes with nucleic acids, also known as polyplexes. These complexes not only protect the nucleic acid but also facilitate its uptake and intracellular trafficking toward the nucleus. Moreover, the use of polyplexes overcomes some of the limitations of viral vectors such as the size of DNA that can be packaged as well as problems of immunogenicity, mutagenesis, reproducibility and scale-up. However, their transfection efficiency is dependent on cell proliferation rate because the delivered DNA can easily enter the nucleus in dividing cells. Considering that many IVD cells are senescent or have a low proliferative rate, the use of polyplexes for the gene therapy of the disc has been limited so far. Nevertheless, the ability of polyplexes to be internalized by the cells can be used for the cytoplasm delivery of siRNA. SiRNA are short, double-stranded RNA sequences used by the cells to regulate protein expression. SiRNA can trigger degradation of specific mRNA sequences, thereby silencing the expression of specific proteins. One of the major concerns over the use of polymeric systems is their toxic effect on cells. To date many platforms are available for the gene therapy of the disc, and there are continued efforts to improve these systems. Even so, these efforts need to be complemented with a better understanding of the effects of the microenvironment that hosts the targeted cells.

1.5.4.7 Gene Therapy in Three Dimensional Scaffolds

The outcome of gene therapy is also influenced by the microenvironments that surround the targeted cells. In fact, the ability of resident cells in a
diseased tissue to take up genetic material and activate the appropriate cell machinery is likely to differ substantially from the response of cells anchored to a normal ECM in a disease-free situation\textsuperscript{125}. Hence, the design of appropriate 3D scaffolds to be used for gene therapy can lead to improved outcomes. Similarly to what was noted for protein therapy, substantial differences were found in the outcomes of gene therapy conducted in 2D or in 3D\textsuperscript{125}. These differences are likely to be related to the decreased frequency of cell cycle progression in 3D\textsuperscript{156}. This is influential because during cell mitosis, cell and nuclear membranes undergo a temporal disruption which in turn allows genetic material to enter the cells’ nucleus and code for therapeutic proteins\textsuperscript{125}. A possible way to overcome this limitation is to increase cells’ proliferative rate within the 3D scaffold. Associating gene therapy and the co-delivery of basic fibroblast growth factor (bFGF) was found to enhance cell proliferation and indirectly, to increase the nucleotide uptake rate with a resulting increase in the levels and duration of transgene expression\textsuperscript{125}. Further modifications of the 3D scaffold environment were also reported to influence transgene expression rate, especially the introduction of cell adhesion molecules such as RGD (Arg-Gly-Asp) sequences\textsuperscript{157}. This was observed in alginate hydrogels, which normally are inert to cell adhesion and protein adsorption\textsuperscript{158}, that were functionalized with RGD peptides. The spatial distribution of these cell adhesion molecules influenced the rate of DNA uptake by the embedded cells and RGD-functionalkised alginate hydrogels showed the highest transgene expression\textsuperscript{157,156}. For this reason, the first scaffold that functioned as gene delivery vehicle was made of collagen\textsuperscript{159}, which contains RGD sequences\textsuperscript{160} and also the triple-helical GFOGER (Gly-Phe-Hyp-Gly-Glu-Arg) sequence recognized by the collagen-binding integrins\textsuperscript{161}. Such collagen scaffolds have been employed for the delivery of a range of gene vectors, both viral and non-viral. These scaffolds were found to be capable of inducing \textit{in vivo} transgene expression and have been reported to trigger regeneration in a wide range of pre-clinical models of bone regeneration\textsuperscript{159,162}, wound healing\textsuperscript{163-166}, muscle repair\textsuperscript{167}, and optic nerve repair\textsuperscript{168}. As for the delivery of growth factors, 3D scaffolds were found to prolong the half-life of viral vectors, and furthermore to shield them from the host immuno
response and limit their side effects $^{169}$. Moreover, direct delivery of the vector from the scaffold has the advantage of localizing the transgene expression to the implant site $^{170}$.

1.5.4.8 The Synergistic use of 3D Scaffolds and Gene Vectors

The modality of 3D scaffolds functionalization with gene vectors will determine their release profile and the length of their biological activity $^{170}$. Gene vectors were found to interact with scaffold polymeric backbone through non-specific mechanisms, including hydrophobic electrostatic, and van der Waals interactions that have been well characterized for adsorption and release from these 3D systems $^{171}$. In an attempt to mimic the natural process of viruses binding to ECM proteins, a number of techniques have been developed to immobilize gene vectors in biomaterials $^{22,23}$. Recently, scaffolds have been used for multimodal approaches that encompass cell and drug delivery within the same system. Overall, the functionalization of ECM-based 3D matrices with gene vectors present multiple advantages. These can function as substrate for cell infiltration, organization and differentiation, while gene vectors provide inducive factors to guide cells’ behaviour $^{170}$. Gene therapy is a powerful approach that can stimulate local production of proteins capable of activating autocrine and paracrine loops that influence tissue development and repair $^{172}$. The co-localization of adhesion cues and immobilized gene vectors within the scaffolds can favour a prolonged exposure of such vectors with the cells $^{125}$. Hence, engineered microenvironments in the form of scaffolds and gene vectors can provide synergistic improvements to the conventional therapies.
**Table 1.2: Gene Therapy Approaches to Restore Disc Anabolism**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Approach</th>
<th>Environment</th>
<th>Outcomes</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1, IGF-1, BMP-2</td>
<td>IGD cells were transduced to express growth factors by using an adenoviral vector</td>
<td>In Vitro</td>
<td>Culture of IVD cells infected with triple gene combination exhibited the highest increase in proteoglycan synthesis compared to control groups</td>
<td>133</td>
</tr>
<tr>
<td>SOX-9</td>
<td>Intradiscal injection of adenoviral vector bearing the gene for SOX-9</td>
<td>In Vivo</td>
<td>Transduction with AdSOX-9 resulted in an increase in cell anabolic activity both <em>in vitro</em> and <em>in vivo</em></td>
<td>130</td>
</tr>
<tr>
<td>BMP-2 and TGF-β1</td>
<td>Intradural injection of high doses of adenoviral vectors carrying the genes for BMP-2 and TGF-β1</td>
<td>In Vivo</td>
<td>Animals receiving high concentrations of Ad-TGF-β1 developed bilateral lower extremity paralysis.</td>
<td>135</td>
</tr>
<tr>
<td>GFP</td>
<td>Infect NP cells with an adenoviral vector containing a tet-off GFP inducible system</td>
<td>In Vitro</td>
<td>Infected cells expressed GFP but the transgene expression was inhibited in presence of tetracycline</td>
<td>136</td>
</tr>
</tbody>
</table>
Table 1.2 (Continued)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Approach</th>
<th>Environment</th>
<th>Outcomes</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>Infect IVD cells with adeno-associated virus containing a control system (AAV-RheoSwitch GFP)</td>
<td>In Vivo</td>
<td>Infected tissues expressed GFP only in presence of the activator and the expression could be turned off by removal of the ligand</td>
<td>138</td>
</tr>
<tr>
<td>SOX-9 and OP-1</td>
<td>Double gene co-transfection by using an adeno-associated virus</td>
<td>In Vivo</td>
<td>Infected discs displayed an improvement of disc height and a higher expression of proteoglycans and type II collagen</td>
<td>137</td>
</tr>
<tr>
<td>BMPs and SOX-9</td>
<td>Transduce NP cells with adenovirus containing genes for different BMPs and SOX-9</td>
<td>In Vitro</td>
<td>NP cells transduced with Ad-BMP-2 and -7 were the most effective in accumulating proteoglycans while cells transduced with Ad-BMP-4 and -14 displayed higher collagen deposition</td>
<td>131</td>
</tr>
</tbody>
</table>
1.5.4.9 Cell Therapy of the Nucleus Pulposus

Co-culture studies revealed that NP cells can regain their anabolic activity when exposed to other cells such as MSCs and AF.\(^{173,174}\) So the injection of new cells in the disc can increase its anabolism. Unfortunately, this approach has some shortcomings. The first challenge is to identify an ideal cell source. Since implantation of autologous NP or AF cells leads to morbidity of the donor site, the use of xenogenic cells has been considered. Implanted xenogenic cells were shown to survive for as long as six months post-injection.\(^{175}\) Even so, the quantity of cells obtained from a single disc might not be enough to reconstruct the matrix and the microenvironment of degenerating discs,\(^{173,176}\) thereby requiring in vitro expansion. This was shown to alter IVD cells phenotype and compromise their anabolic activity as well as the quality of the matrix they produce.\(^{90,177}\) For this reason, attention has shifted towards the potential use of MSCs in cell therapy of the disc (Table 4). These cells have the ability to differentiate into chondrogenic phenotypes\(^{178-182}\) and their use offers several advantages such as the possibility to expand MSCs in vitro without compromising their differentiation potential and also the ability to access them easily without causing morbidity to the donor site.\(^{183,184}\) Risbud et al designed an in vitro 3D model in which the ability of rat MSCs to differentiate towards a NP-like phenotype was investigated. Following administration of TGF-β1 and under hypoxia conditions (2% O2), rat MSCs acquired a phenotype comparable to that of NP cells.\(^{185}\) Sakai et al studied the potential regenerative effects of MSC transplantation into a rabbit model of degenerative disc. After 24 weeks, the groups that were transplanted with MSCs regained disc height in a much more prominent way than with sham controls. Overall, the MSCs induced higher expression of aggregans and accumulation of proteoglycans, thereby increasing the ability of discs to regain hydration after induced degeneration.\(^{186}\) In another study, Yang et al. revealed that the injected MSCs contributed to lower disc degeneration both by autonomous differentiation and by stimulating endogenous cells to proliferate and produce more matrix.\(^{187}\) However, limitations of cell injections include poor retention of cells at the site of injection and cell death due to loss of anchorage (anoikis).\(^{188}\) Retaining cells in the intended
site is particularly important as it has been shown that cell leakage from the injection site may lead to formation of osteophytes. A possible solution to these issues is to combine the delivery of cells with the use of biomaterials able to shield the injected cells from the harsh environment of the disc while allowing their retention at the site of injection.

1.5.4.10 The Importance of Microenvironments in Cell Therapy

The influence exerted by the microenvironments in which the target cells reside remains under-appreciated. The array of cues intrinsic of proteins or other constituents of the ECM, actively participate in the cross-talk between cells and the ECM. The ECM, in fact, is not only responsible for maintaining the integrity of the tissues but also fulfills a number of other functions. These include allowing diffusion of nutrients to the cells, binding growth factors and therefore acting as a natural reservoir as well as providing instructive cues that influence cell behaviour, dictating cell’s ability to proliferate, differentiate and to produce matrix. Often, disease and trauma are associated with alterations in the structure and properties of the ECM causing aberration of the cell-ECM interactions. But, conventional cell delivery approaches do not restore the microenvironments that surround the targeted cells.

1.5.4.11 ECM-based Scaffolds for Cell Delivery to the Nucleus Pulposus

Delivering cells in ECM-based scaffolds has the advantage of introducing cues typical of their native environments and also offering an enzymatically labile environment that facilitates cell-mediated remodelling of the system. Moreover, the use of biomaterials for cell delivery allows the localization of cells to the implant site. Bertram et al. compared the intradiscal injection of cells resuspended in medium or in an injectable fibrin matrix. After injection, the fibrin matrix was able to polymerize and thus maintain the injected cells in the NP. In contrast, a rapid loss of cells injected in medium was observed. A number of different biomaterials have been studied for cell therapy of the disc. However, as the NP and AF are two regions that are morphologically and functionally very different, tailored approaches are required. Because the NP is a highly hydrated tissue,
hydrogels have generated particular interest. Alginate is one of the most widely used biomacromolecule for designing hydrogels. In an in vitro study, Baer et al. tested the ability of alginate hydrogels loaded with IVD cells to withstand different mechanical stresses. After 21 days of culture, cell-alginate constructs showed significantly lower mechanical integrity than alginate hydrogels with no cells. This indicates that the embedded cells were not able to produce a mechanically functional matrix. An ideal tissue engineering approach should focus not only on delivering cells but should also offer the right conditions for cells to differentiate and form a new tissue. To this end, the use of ECM-based biomaterials appears a promising choice as it mimics the tissue that the cells are trying to restore. Moreover, by using ECM molecules as building blocks for the design of cell delivery platforms, it allows the creation of environments conducive of cell adhesion, proliferation and synthesis of new, functional matrix.

1.5.4.12 Hyaluronic Acid-based Scaffolds

Stern et al developed a fibrinogen/hyaluronic acid (HA) matrix and tested its effects on NP cells in vitro. After 21 days of culture the proteoglycan synthesis in relation to DNA content was significantly higher in fibrin/HA matrix than alginate hydrogels were. But, an issue to overcome in HA-based hydrogels is the loss of HA and the consequent shrinkage of the system in vivo. Park et al. found that the addition of silk to fibrin-HA hydrogels significantly decreased their degradation and prevents shrinkage after four weeks of in vitro culture. Injectability is a desired feature for the cell therapy of the disc because it allows minimal invasive surgeries. Peroglio et al. developed an injectable thermoreversible hydrogel based on modified HA. The modification consisted in the grafting of poly(N-isopropylacrylamide) on a HA backbone. This modification has some advantages over unmodified HA; for example it increases the stability and lowers the viscosity of HA at room temperature. Moreover, below body temperature (32°C) the modified HA undergoes physical cross-linking compatible with cell encapsulation because there are no toxic products or pH shifts in the system. These hydrogels were tested ex vivo by using
bovine NP cells and despite the fact that the hydrogel was tested only for 1 week, a steady increase in GAG/DNA ratio was observed 200.

### 1.5.4.13 Collagen-HA-based Scaffolds

Because of its hydrophilicity and ability to provide cues for cell adhesion and maintenance of phenotype, HA has been used for a wide variety of scaffolds for tissue engineering of the IVD, which is not limited to hydrogels only. For example, Alini et al designed a sponge system composed of type I collagen and HA. *In vitro* characterization revealed that although disc cells were able to express various proteoglycans (aggrecan, decorin, biglycan, fibromodulin, and lumican) and collagens (types I and II), only a small fraction of the secreted biomacromolecules were retained in the scaffold 201. This issue was overcome by designing a hydrogel system using the positively charged chitosan to electrostatically entrap proteoglycans and prevent their diffusion from the hydrogel. The performances of this hydrogel system were tested *in vitro* by using NP or AF cells with different outcomes. In fact, chitosan hydrogels allowed NP survival and retained the majority of proteoglycans produced but failed to maintain AF cells viability 202. This highlights the need for tailored approaches as NP and AF tissues will, most likely, have differing responses to the implant. Collin et al. tailored an injectable hydrogel system for the NP by using type II collagen and HA, two key biomacromolecules of its matrix. To increase the stability of the hydrogel and its resistance to enzymatic degradation, poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG) was used as a cross-linker. Bovine NP cells were embedded in the hydrogel and characterized *in vitro*. NP cells showed high viability for 14 days of culture and the cross-linking did not produce any toxic effects. Furthermore, NP cells maintained a low expression of type I collagen; but their ability to synthesize aggrecan decreased after seven days suggesting that further functionalization of the hydrogel is needed 203. What limits the progress in cell therapy approaches is the dearth of knowledge on NP cells, and the definition of their phenotype which remains unclear. In an attempt to find specific markers of NP cells phenotype, transcriptomic studies on human discs are being done 204. Nevertheless further studies are needed.
Figure 1.3: Schematic representation of strategies to restore disc anabolism. Disc degeneration coincides with the disruption of the equilibrium between anabolism and catabolism of the disc’s matrix. By delivering cells or growth factors, current strategies aim at restoring disc’s matrix anabolism. However, drawbacks of these strategies lead to the adoption of multimodal approaches in which cell and gene therapy are combined in controlled microenvironments (biomaterials) which synergistically contribute to the restoration of disc anabolism.
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Approach</th>
<th>Environment</th>
<th>Outcomes</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVD</td>
<td>Compare intradiscal injections of IVD cells with or without polymerizing fibrin matrix</td>
<td>In Vivo</td>
<td>The fibrin matrix polymerized after the injection and was able to retain IVD cells in the NP and improve their survival</td>
<td>190</td>
</tr>
<tr>
<td>MSCs</td>
<td>Injection of MSCs in a murine model of disc degeneration</td>
<td>In Vivo</td>
<td>MSCs could arrest disc degeneration by autonomous differentiation and stimulatory action on endogenous cells</td>
<td>181</td>
</tr>
<tr>
<td>NP, ASCs</td>
<td>Encapsulation of ASCs and NP cells in an injectable hydrogel system composed of type II collagen/hyaluronan</td>
<td>In Vitro</td>
<td>Crosslinking the hydrogels with 4S-StarPEG did not compromise cell viability. Moreover, NP cells maintained a low expression of collagen I after seven days of culture</td>
<td>197</td>
</tr>
<tr>
<td>Cell Type</td>
<td>Approach</td>
<td>Environment</td>
<td>Outcomes</td>
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<tr>
<td>NP</td>
<td>NP cells seeded in three dimensional type I collagen microspheres</td>
<td>In Vitro</td>
<td>NP cells in three dimensional collagen microspheres maintained a round morphology and, additionally, produced more matrix compared to monolayer controls</td>
<td>204</td>
</tr>
<tr>
<td>MSCs</td>
<td>Injection of human MSCs in hydrogels or in media in a porcine disc model</td>
<td>In Vivo</td>
<td>Xenogenic MSCs could survive six months in the discs and, when injected with a hydrogel, showed higher differentiation</td>
<td>169</td>
</tr>
<tr>
<td>NP or AF</td>
<td>NP or AF cells encapsulated in chitosan-based hydrogels</td>
<td>In Vitro</td>
<td>NP cells showed higher viability in chitosan hydrogels than AF cells; moreover, the hydrogels showed high electrostatic retention of proteoglycans</td>
<td>196</td>
</tr>
<tr>
<td>Cell Type</td>
<td>Approach</td>
<td>Environment</td>
<td>Outcomes</td>
<td>Ref.</td>
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<tr>
<td>MSCs</td>
<td>MSCs transplanted into a rabbit model of disc degeneration</td>
<td>In Vivo</td>
<td>Transplantation of MSCs in degenerated discs lead to an increase in disc height and restored the proteoglycan accumulation</td>
<td>180</td>
</tr>
<tr>
<td>MSCs</td>
<td>MSCs cultured in alginate hydrogels under various conditions to test their ability to differentiate towards NP phenotype</td>
<td>In Vitro</td>
<td>Under hypoxia conditions and in presence of TGF-β1, MSCs acquired a phenotype consistent with that of NP cells</td>
<td>179</td>
</tr>
<tr>
<td>NP</td>
<td>Porcine NP cells were cultured in a fibrin/hyaluronan matrix and compared to culture in alginate hydrogels</td>
<td>In Vitro</td>
<td>The proteoglycan synthesis in relation to the DNA was higher in fibrin/hyaluronan matrix compared to alginate hydrogels</td>
<td>192</td>
</tr>
</tbody>
</table>
### Table 1.3 (Continued)

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Approach</th>
<th>Environment</th>
<th>Outcomes</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>NP</td>
<td>Bovine NP cells were cultured in a thermoreversible hydrogel based on modified hyaluronan</td>
<td>In Vitro</td>
<td>NP cells were viable in the hydrogels. Moreover, they maintained their phenotype and GAGs synthesis was identified</td>
<td>194</td>
</tr>
<tr>
<td>Chondrocytes</td>
<td>Human Chondrocytes were cultured in different formulations of silk-fibrin/hyaluronan gels</td>
<td>In Vitro</td>
<td>Introducing of Silk in fibrin/hyaluronan gels prevented shrinkage and increased their mechanical properties, while allowing GAGs production</td>
<td>193</td>
</tr>
</tbody>
</table>
1.6 Conclusions
The structural complexity of the disc and the coexistence of different microenvironments in a degenerating disc are challenges that regenerative strategies of the IVD are facing. However, it is clear that in a tissue devoid of both cells and paracrine factors, the only way to restore the anabolism is to deliver cells in an environment rich in signals that can direct the formation of the new matrix. The efficacy of growth factors alone in triggering cell anabolism and mitigating catabolism has been proved in a number of studies. Nevertheless, their direct use has significant drawbacks as they lack stability and their effect is short-lived. A biomaterials-based therapy using a combination of cell and biomolecules (nucleic acids/protein) can restore disc anabolism by increasing the disc cells’ population while using their own secretory machinery to produce the desired factors. These approaches will likely result in the design of protein factories thereby stimulating local protein production capable of activating autocrine and paracrine loops that may play important roles in tissue development and physiology. This will be possible by delivering cells in the most appropriate environment and by engineering them with optimal cocktails of genes. However, there are still many problems to overcome. Furthermore, the design of more sophisticated cell therapy platforms is tied in with advances in the understanding of IVD cells phenotype and the signalling pathways that enable them to sustain homeostasis of the disc.

1.7 Hypothesis and Objectives
The ultimate aim of the project was to design a three dimensional cell delivery platform, using biomacromolecules of the NP’extracellular matrix as building blocks, in which stem cells are primed towards desired phenotype and are programmed to secrete specific proteins of interest, thereby acting as a functional cell factory. It is hypothesized that a type II collagen/HA microgel system decorated with a gene delivery reservoir system will allow the delivered cells to manufacture specific proteins, thereby offering a reliable platform for the production of functional cell factories.
This project had three phases. The first was to develop a 3D cell delivery platform by using the biomacromolecules of the NP matrix as building blocks, able to prime the delivered cells towards a NP-like phenotype. The second phase included the development and optimization of a collagen-based reservoir system for the delivery of non-viral gene vectors. The final phase aimed at designing a system for programming cells embedded in 3D microgels to secrete desired proteins.

1.7.1 Phase One (Chapter Two)

**Overall aim:** To design a 3D cell delivery platform able to prime the delivered ASCs towards a phenotype suitable for the NP environment.

**Hypothesis:** 3D microgels fabricated from type II collagen/HA, which mimic the composition of the NP, will exert influence over ASCs phenotype.

**Specific Objectives:**

- To develop a spherical microgel system by using type II collagen/HA as building blocks crosslinked with 4-arm polyethylene glycol succinimidyl glutarate.
- To investigate the effects of macromolecular composition of microgels on their stability in culture conditions.
- To investigate the effects of macromolecular composition of microgels on cell’s viability.
- To investigate the effects of macromolecular composition of microgels on cell’s morphology.
- To investigate the effects of macromolecular composition of microgels on expression of genotypic markers of NP phenotype.
1.7.2 Phase Two (Chapters Three and Four)

**Overall aim:** To fabricate a collagen-based reservoir system for non-viral gene vectors with greater control over its size and shape.

**Hypothesis:** By using the template method collagen microspheres reservoir systems can be fabricated that are uniform in size and are reproducible. The collagen hollow spheres will allow for high loading efficiency and sustained release of non-viral gene vectors.

**Specific Objectives:**

- To coat polystyrene beads template with type I collagen.
- To ascertain that the template is removed and that the microspheres have a hollow core.
- To ascertain that the fabricated microspheres are homogeneous in size and shape.
- To ascertain that the hollow microspheres have no adverse effects on cellular metabolic activity.
- To characterize loading and release of non-viral gene vectors in microspheres reservoirs.

1.7.3 Phase Three (Chapter Four)

**Overall aim:** To design a three dimensional cell delivery system in which cells can be programmed to secrete desired proteins.

**Hypothesis:** a type II collagen/HA microgel system decorated with a gene delivery reservoir system will allow the delivered cells to manufacture specific proteins, thereby offering a reliable platform for the production of functional cell factories.
Specific Objectives:

- To fabricate type II collagen microsphere reservoirs.
- To load the microspheres reservoir with polyplexes (non-viral gene vectors) and ensure they show a sustained release.
- To ascertain that polyplexes-loaded microspheres offer a protective environment, thereby lowering the cytotoxicity associated with polyplexes.
- To optimize the size of polyplexes-loaded microspheres to obtain the highest cell transfection in monolayer culture.
- To optimize the concentration of polyplexes-loaded microspheres to be used in 3D microgels to obtain significant levels of transfection.
- To ascertain that cells transfected in 3D microgels are able to secrete desired proteins, thereby acting as a protein factory.
Schematic Overview of the Project:

Development of a 3D cell delivery platform:
- Development of 3D microgels composed of type II collagen/HA
- Investigate the effects of macromolecular composition on microgels stability
- Investigate the effects of macromolecular composition on ADSCs phenotype

Development of a collagen-based reservoir system for non-viral gene therapy:
- Fabrication and characterization of hollow microsphere reservoirs
- Functionalization of collagen microspheres with non-viral gene vectors
- Characterize loading and release of polyplexes from microsphere reservoirs

Development of a 3D cell factory:
- Optimization of polyplexes-loaded microspheres size to obtain the highest cell transfection in monolayer culture
- 3D microgels functionalization with polyplexes-loaded reservoirs
- To ascertain that cells transfected in 3D microgels are able to secrete desired proteins (luciferase)
1.8 References


Chapter 1


45


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Chapter 2

Development of a 3D Microgel System Tailored for the Delivery of Adipose-Derived Stem Cells to the Nucleus Pulposus

Sections of this chapter have been published:

G. Fontana, D. Thomas, E. Collin, A. Pandit,
Microgel Microenvironment Primes Adipose-Derived Stem Cells Towards a NP Cells-like Phenotype
Advanced Healthcare Materials, 2014
2.1 Introduction

The focus of this chapter is the development of a microgel platform tailored for the tissue engineering of the NP. Tremendous efforts have been made in the hope of regenerating the NP, and cell therapies to date have shown the potential to ameliorate symptoms of its degeneration \(^1\)\(^-\)\(^3\). Among the different sources of stem cells that have been used, mesenchymal stem cells (MSCs) and adipose derived stem cells (ASCs) have shown some promising results \(^4\)\(^-\)\(^6\). ASCs are an easily accessible source and, moreover, these cells are able to differentiate towards a chondrogenic phenotype in the presence of the growth factors from the TGF-beta family, or when co-cultured with NP cells \(^5\), \(^6\). Although the beneficial effects of intradiscal injection of cells have been proven \(^7\), a challenge remains to obtain tissue regeneration at the desired scale. Therefore, a combinatorial approach that encompasses cell therapy and tailored biomaterials is a preferred option. Biomaterials can be tuned for their mechanical properties or their structures modified to obtain a certain cellular response \(^8\). Moreover, biomaterials systems can be functionalized with ligands to control cell adhesion or can encapsulate drugs to modulate the surrounding tissues \(^9\). What is clear now in the evolution of biomaterials is that their function is no longer limited to the delivery of cells but these systems need to recreate the appropriate microenvironment to allow cells to regenerate the diseased tissue. The use of ECM-derived macromolecules for the design of cell delivery systems presents some advantages. In fact, ECM biomacromolecules provide instructive cues that assist and guide cell behavior throughout tissue development, maintenance and regeneration \(^10\), \(^11\). Culturing conditions are also crucial in determining the fate of stem cells. Growing evidence suggests that three dimensional (3D) culture environments are more suitable for chondrogenic or NP differentiation than the traditional monolayer culture \(^12\), \(^13\). For this reason, hydrogels are widely studied in tissue engineering of the disc \(^14\)-\(^16\). In these 3D systems, cells produce more ECM than cells seeded in monolayer \(^13\). 3D hydrogels made by using type I collagen offer a positive 3D environment for MSCs to differentiate towards the chondrogenic phenotype \(^14\); nevertheless, the limitations of these systems is that these undergo drastic changes during in vitro culture such as shrinkage and degradation due to the
Figure 2.1: Schematic representation of the cross-talk between integrins and cytoskeleton. The following schematic depicts how the composition of the ECM can influence cellular cytoskeletal organization. When integrin α10β1 binds type II collagen it triggers a cascade of phosphorylations and activations of a number of intracellular proteins, in particular RhoA and its downstream effector ROCK which are responsible for cytoskeletal organization. It thereby allows cells to respond to the composition of the ECM by changing their shape.
absence of crosslinking. Moreover, as type I collagen is present in the degenerating NP, it does not mimic the composition of the healthy disc 17-19. Although numerous studies have shown the ability of type I collagen to support chondrogenic differentiation 14, 20, type II collagen has proven to be a better environment for NP cells 21, 22. Indeed, type II collagen is one of the most abundant ECM components of NP, and has been shown to strongly influence cells’ phenotype and their ability to differentiate. In fact, type II collagen is known to interact with integrins from the β-1 family, in particular the integrin α10β1 which activates the formation of intracellular complexes that can alter the organization of a cell’s cytoskeleton, thus influencing its morphology. A number of studies have demonstrated that when cells were embedded in type II collagen-based hydrogels, the cells show a rounded morphology with a higher predisposition towards chondrogenic differentiation 23-27. However, another biomacromolecule that plays an important role in both NP homeostasis and functionality is hyaluronic acid (HA). This is a non-sulfated GAG that is usually synthesized on a cell’s surface and has the ability to bind the most abundant proteoglycans in the disc i.e. aggrecans. The properties of HA are varied depending on their physico-chemical properties. High molecular weight of HA for example, is characterized by anti-inflammatory properties 28-30; moreover, via the binding to the cell surface receptor CD44, it is able to promote and maintain chondrogenic phenotype 31-33. Type II collagen hydrogels enriched with HA provide a microenvironment close to that of the NP as they are able to maintain the phenotype NP cells even after seven days in culture 34. The objective of the current study is to optimize a cell delivery platform to prime ASCs by generating a NP-like matrix microenvironment. The delivered ASCs need to acquire a phenotype suitable for the NP microenvironment. This phenotype will be characterized by a rounded morphology and the gene expression characteristic of the NP phenotype. It is thus hypothesized that 3D microgels fabricated from type II collagen/HA, which mimic the composition of the NP, will exert influence over ASCs phenotype.

The specific objectives of this study are to fine tune the macromolecular composition of a microgel system to increase its resistance to enzymatic
degradation, to promote round cell morphology and to favor the expression of genes linked to NP-phenotype such as type II collagen, aggrecan, SOX9, CD44 and integrin α10.

2.2 Materials and methods
2.2.1 Materials and cells
Unless otherwise specified, all materials were purchased from Sigma Aldrich (Ireland). Type II calf articular joint atelocollagen (Affymetrix (UK)), high molecular weight hyaluronic acid (200-750 kDa, Contipro group, (Czech Republic)) were used. 4-arm polyethylene glycol succinimidyl glutarate Mw 10,000 (4S-StarPEG) was purchased from JenKem Technology (USA) and Trinitrobenzenesulfonic acid (TNBS) was purchased from Pierce Protein Research Products, ThermoScientific (Ireland). Quant-iT™ Reagent was purchased from Invitrogen™ (Ireland). ASCs were extracted from rabbit adipose tissue (methodology in Appendix B) and used strictly at passage three for every experiment and were cultured in Dulbecco’s modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS 2%) and penicillin–streptomycin (P/S 1%).

2.2.2 Microgel fabrication
Microgels were fabricated by neutralizing (pH 7.4) type II atelocollagen in sodium hydroxide 1M and phosphate buffer saline (PBS) 10×. The forming gel solution was then enriched with HA (9 to 1 weight ratio collagen to HA) and cells were added to the mixture (10^6 cells/mL final). After addition of 4S-StarPEG 2mM, the forming gel solution was deposited as 2 μL droplets on a hydrophobic surface, (commercial Teflon® tape (Fisher Scientific, Ireland) (this procedure is described in Appendix D). A spherical microgel obtained was then incubated for 1 h at 37°C. To facilitate the dispensing process, a multipipette (Eppendorf, Multipipette Plus, Ireland) was used and tips were modified, as described in Appendix E, to prevent collagen droplets from sticking.
2.2.3 Cell viability
The viability of cells was determined by using the live/dead® assay. Briefly, microgels were washed in PBS and incubated with 10mM calcein AM green (Life Technologies, Ireland) and 1 mM ethidium homodimer-1 (Life Technologies, Ireland) for 30 min. Samples were then imaged with a fluorescence microscope (Olympus BX51, Ireland). Live/dead cells were counted on a subset of 40 captured images at 5 μm distance from one another.

2.2.4 Cell proliferation
Microgel embedded cells were pulsed with 25 μM BrdU (Sigma-Aldrich, Ireland) for two hours, followed by two PBS washes. Microgels were then digested in collagenase (Sigma-Aldrich, C5138, Ireland) for 20 min and sterile filtered to harvest cells. Harvested cells were immediately fixed in ice-cold 100% ethanol. Acid permeabilization was then carried out using HCl, and the cells were blocked in PBS containing 0.1% Triton X-100 solution and 0.5% bovine serum albumin (Sigma-Aldrich, Ireland). The cells were sequentially incubated with anti-BrdU antibody (BD Biosciences, Ireland) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Sigma-Aldrich, Ireland) for 30 min each at room temperature. Before analysis on BD FACS Canto (BD Biosciences, USA), cells were resuspended in propidium iodide/RNase staining buffer (BD Biosciences, Ireland). The percentage of cells in the proliferative phase was ascertained by gating FITC-BrdU positive cells in the S-phase.

2.2.5 Cell morphology
After 2 and 21 days of culture, cell-loaded microgels were incubated in 10mM calcein for 30 min and subsequently washed in PBS. Each microgel was imaged with a fluorescence microscope (Olympus BX51). Stacks were imaged from top to bottom of each microgel at 5μm intervals. Individual stacks were analyzed by an image analysis software (Volocity® 5.0, Perkin Elmer, Ireland). The analysis involved quantification of cell morphological parameters. Cell shape was assessed by quantifying the shape factor index. This index has a scale from 1 to 0 where 1 corresponds to a round
morphology and 0 to a stretched one. To avoid interference from background, the analysis involved only objects in the range of known cell volumes (from 500 to 700,000 μm³). Eventual spatial differences in cell morphology throughout the microgels were tested by comparing morphology of cells located at the center with those at the surface of the microgels. More specifically, each set of stacks was divided into three subsets: top, center and bottom; which included respectively 25%, 50% and 25% of the images. The subsets ‘top’ and ‘bottom’ were considered as ‘surface’ and the remaining images as ‘center’.

2.2.6 Qualitative evaluation of presence of stress fibers in cell’s cytoplasm
The presence of stress fibers was determined by staining the cell’s cytoskeleton with phalloidin. Samples were incubated for 30 min in rhodamine–phalloidin (Invitrogen, Ireland) 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).

2.2.7 Qualitative evaluation of presence of glycogen deposits in cell’s cytoplasm
To ascertain the presence of glycogen in cell’s cytoplasm, the samples were first embedded in epoxy resin and subsequently sectioned and analyzed by using the transmission electronic microscopy (TEM). Samples were fixed in 3% glutaraldehyde in 0.2M sodium cacodylate buffer (SCB) for 1 hour. Samples were then washed in SCB and re-suspended in 1% osmium tetraoxide for 2 h. The excess of osmium tetraoxide was removed by washings in SCB and samples were immersed in a graduated series of ethanol solutions of 30, 50, 80 and 95% for 5 min respectively. Samples were embedded in resin by immersion in a 50:50 resin (Agar resin kit)/propylene oxide mixture for four hours and in a 75:25 mixture overnight. Before proceeding with the thermo-crosslinking of the resin, the samples were washed twice in pure resin for six hours per wash. The resin-
embedded samples were then thermo-crosslinked at 65°C for 48 h. Ultra-thin sections were acquired using a microtome with a diamond knife.

2.2.8 Quantification of expression of genotypic markers of NP phenotype

RNA was extracted by using a modified Trizol isolation protocol (protocol described in Appendix K). Briefly, microgels were collected by centrifugation and re-suspended in TriReagent (Life Technologies™, Ireland). The samples were then mechanically disrupted using a tissue lyser (Qiagen, TissueLyserLT, UK). The phase separation was obtained by addition of chloroform (Sigma Aldrich, Ireland) and subsequently RNA was purified using RNeasy micro kit (Qiagen, UK) following the supplier’s recommended procedure. mRNA was quantified using the Qubit® RNA assay kit (Life Technologies™, Ireland) following the supplier’s recommended procedure (protocol in Appendix K). Reverse transcription was assessed using the ImProm-II RT™ system from Promega (UK) following the supplier’s protocol and using MJ Research PTC 200 Thermal Cycler (Thermo Scientific, Ireland). Quantitative real time polymerase chain reaction (PCR) was carried out by the SYBR® Green method. Gene-specific primers were designed (Table Z.1) and tested to ensure optimal efficiency. The reactions were conducted by using a Step One Plus™ instrument (LifeTechnologies™, Ireland). The gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method and each gene target was normalized in relation to the transcription of 18S as housekeeping gene.

2.2.9 Statistical analyses

All statistical analyses were performed using GraphPad Prism® 5 software and $P$ values < 0.05 were considered significantly different. A one-way ANOVA was performed using Tukey’s post-hoc test to compare all groups. Pearson’s correlation analysis was carried out to highlight positive and negative correlations among the expression of the set of genes analyzed.
2.3 Results

2.3.1 Effects on ASCs viability and proliferation after incorporation in microgels

Spherical microgels were successfully fabricated by depositing a forming gel solution on a hydrophobic surface (Figure 2.2). To increase the stability of the microgels and their resistance to enzymatic degradation without compromising cell viability, 4S-StarPEG was used as a cross-linker. The cytotoxicity effects of high concentrations of cross-linker were tested by live/dead assay. But, the absence of toxicity of this cross-linker was already reported in a number of studies. High concentrations of 4S-StarPEG did not compromise cell viability and the viability was maintained at a level of ± 80% (Figure 2.3).

The spherical shape of the microgels gives the gels a high surface to volume ratio, with an environment supportive of a large number of cells without affecting their viability (Figure 2.3). However, the poor diffusion of nutrients in degenerated discs limits the number of cells that can be used in cell therapy. Previous studies have reported a dose of $10^6$ cells/mL as effective in degenerated discs. For this reason, it was decided to use a concentration of $10^6$ cells/mL. The concentration of type II collagen can influence both microgels’ stability and ASCs’ differentiation towards an NP-like phenotype. Higher concentrations of type II collagen did not affect cell viability (Figure 2.3). Embedded ASCs maintained a relatively high proliferation rate when embedded in microgels even though they were cultured in media containing only 2% of FBS. However, their proliferation rate dropped after four days of culture and maintained a minimal level of proliferation for 14 days of culture (Figure 2.4).

2.3.2 Stability of microgels

The efficiency of cross-linking was assessed (as described in Appendix G) by measuring the amount of free amines using the TNBS assay (Figure 2.5). As concentrations of 4S-StarPEG above 2mM resulted in a degree of cross-linking similar to that of the positive control (0.625% glutaraldehyde), it was decided to proceed with the study by using 2mM as a preferred concentration of cross-linker (Figure 2.5). The stability of the microgels
was tested by enzymatic degradation and measured by Coomassie Brilliant Blue assay (as described in Appendix H). As expected, microgels composed of a higher concentration of collagen showed the slowest degradation rate (Figure 2.5). Similar behaviour was observed when measuring cell-embedded microgels’ diameter over 21 days of culture: 2 mg/mL microgels showed a significant decrease in size, whereas 4 or 5 mg/mL microgels showed only minimal shrinkage (Figure 2.5).

2.3.3 Effects of collagen concentration on cell morphology

As stated above, different concentrations of type II collagen had an effect not only on the stability of the microgels, but also resulted in different densities of ligands and thus different microenvironments. Cells respond to different microenvironments by changing their phenotype, changes that are reflected in different shape, proliferation rate and ECM production. Three-dimensional environments are known to support spherical cell shape, which mimics the round morphology of healthy NP and, more generally, chondrogenic cells. Moreover, it was shown that the onset of chondrogenic gene expression and chondrogenic differentiation is closely connected to cell shape and cytoskeletal organization and it has been suggested that type II collagen induces chondrogenesis by promoting a rounded cell shape. Different morphological features such as cell volume, longest axis and shape factor were quantified as described in the methods section. When embedded in high collagen concentrations, cells maintained a spherical shape for an extended period and took longer to become elongated (Figures 2.6 and 2.7). Overall, ASCs had the tendency to become more spindle-shaped over time in all samples. However, when cultured in higher concentrations of type II collagen for 21 days, cells maintained a more rounded morphology (Figures 2.7). The analysis did not indicate any effect of spatial distribution on cell morphology; the only differences were those attributable to time (results shown in Appendix Z). This disparity with previous findings can be ascribed to the shape of the microgels, which favors homogeneous diffusion of media even at the core of the gels; moreover, the microgels are small enough to ensure that embedded cells are always close to the surface.
2.3.4 Cytoskeletal organization and formation of glycogen deposits

The matrix of the microgels not only enables the cellular physiological functions by permitting diffusion of nutrients as it contains key ECM components such as type II collagen and HA. In addition, the matrix transmits biochemical and mechanical signals to the cells. The mediator of cell–ECM interactions are cell surface receptors known as integrins. These receptors are composed of alpha and beta subunits capable of binding ECM molecules with great specificity. Moreover, integrins are a bridge between the ECM and the cell cytoskeleton allowing extracellular ligands to influence cell behavior and fate. Type II collagen has been shown to be a promoter of round cell shape through β1 integrins-mediated RhoA/Rock signaling pathway. RhoA is part of the Rho GTPases family that, via phosphorylation of a number of downstream effectors such as Rock I, control cytoskeletal organization. However, cell cytoskeleton is known not only to control changes in cell morphology but also to influence the ability of MSCs to differentiate towards chondrogenic phenotypes. In particular, the assembly of actin filaments, also known as stress fibers, was found to severely hamper chondrogenic differentiation and ECM synthesis. Cells seeded on monolayer and in low concentrations of collagen displayed abundance of actin stress fibers in their cytoskeleton (Figure 2.8A-B) (in monolayer more than in 2 mg/mL microgels) whereas in high concentrations of collagen cells were of a more rounded shape and had a cortical disposition of actin filaments (Figure 2.8C-D), a hallmark of chondrogenic phenotype. These results confirm previous findings where 3D culture systems were shown to reduce the formation of actin stress fibers more than those in monolayer culture; additionally high concentrations of type II collagen caused a further decrease (Figure 2.8D). In addition, TEM characterization of ASCs cultured in microgels for 14 days highlighted how these cells had a vacuolated cytoplasm rich in glycogen deposits and with large nuclei (Figure 2.9) typical of NP and chondrogenic cells.
2.3.5 Genotypic characterization of embedded ASCs

In a number of studies, type II collagen was found to prime stem cells for chondrogenic differentiation \(^{23, 24, 26, 54}\). Other studies have highlighted the combined effects of type II collagen and growth factors on cells’ ability to differentiate \(^{23, 24, 45, 54}\). Cellular contact with type II collagen has been shown to enhance cellular response to growth factors such as TGF-β1 that favour chondrogenic phenotype \(^{23}\). Nevertheless, most of the studies undertaken have been qualitative in nature and therefore little is known about the effects of different concentrations of type II collagen on the cellular phenotype. To better understand the effects of different collagen concentrations on cellular phenotype, gene expression was analyzed in two different culture environments: plain media and differentiation media.

2.3.5.1 Expression of morphology-related genes

The mechanism through which cells are more likely to respond to changes in collagen II concentration is the crosstalk between integrins and actin cytoskeleton. Two key proteins that regulate cytoskeletal organization, and thus cell shape, are Rock I and RhoA \(^{40, 43}\). Rock I is a serine/threonine kinase and is a downstream effector of RhoA \(^{46, 47}\). While no major differences in expression of RhoA were found in any condition (results shown in Appendix Z), Rock I expression increased over time, although at the early time point (two days) it was significantly lower in 3D than in monolayer (Figure 2.10).

2.3.5.2 Expression of integrin α10 and CD44

The subunit α10 of the integrin β1 was found to be one of the main integrins to recognize type II collagen \(^{41, 42, 44}\), and although it is expressed at basal levels in ASCs \(^{44}\), high expression of this integrin is a reflection of a chondrogenic phenotype \(^{11, 41}\). Moreover, this integrin has been shown to play a central role in the RhoA/Rock I pathway \(^{11, 24, 43}\). When cells were cultured in 4 and 5 mg/mL microgels, they expressed high levels of integrin α10 after two days of culture in plain media. This expression dropped after 21 days (Figure 2.10), due to the absence of stimuli in the media. Cells displayed a different pattern in differentiation media. In fact, when ASCs
were cultured in 5 mg/mL type II collagen microgels expressed significantly higher levels of integrin α10 (Figure 2.10). To create a more favourable environment, the matrix of microgels was enriched with HA. Previous studies have shown that HA contributes significantly to the maintenance of NP cells’ phenotype 34. Cells are able to sense the presence of HA in the surrounding environment via the receptor CD44 28, 31. Not only is high expression of CD44 a marker of chondrogenic differentiation, 38 but it is also extremely important for the maintenance of the chondrogenic phenotype and to prevent the degenerative state known as chondrolysis 42. Although in plain media no differences in CD44 expression were found, differentiation media boosted its expression in every condition tested (Figure 2.11).

2.3.5.3 Expression of SOX9
Further indication of the supportive environment for chondrogenic phenotype comes from the expression profile of SOX9 (Figure 2.11). In both plain and differentiation media, there was a significant increase in the expression of SOX9 after 21 days of culture. Since the increase in SOX9 expression occurs also in plain media and is more pronounced in 5 mg/mL microgels, it is reasonable to assume that type II collagen can support SOX9 expression. Indeed, SOX9 expression and type II collagen expression were found to be strictly related 23. SOX9 has transcriptional activity and facilitates and enhances the expression of type II collagen 55-58. Furthermore, it is considered to be a “master regulator” of the chondrogenic phenotype 59-62 because SOX9 orchestrates the synthesis of ECM molecules by promoting the transcription of their genes 57, carrying out a bifunctional role as it is also involved in the maintenance of ECM and in the prevention of its degradation 63.

2.3.5.4 Expression of ECM-related genes
An increase in expression of SOX9 in fact resulted in an increase in expression of type II collagen over 21 days, starting at an earlier time point (day two) in 5 mg/mL microgels (Figure 2.12). Further evidence in support of the stronger differentiation that occurred in 5 mg/mL microgels can be
seen in Figure 2.13. In presence of differentiation media, type I collagen expression was significantly downregulated. Maintaining a low level of type I collagen expression is important as its presence in NP coincides with a more fibrotic appearance of the tissue and with a reduced ability to retain water\textsuperscript{64,65}. To maintain its osmotic swelling pressure, the healthy NP is rich in proteoglycans and in a particular aggrecan\textsuperscript{66}. The latter consists of a protein core surrounded by chains of negatively charged chondroitin sulphate and keratin sulphate\textsuperscript{67}. Because of its negative charge, aggrecan attracts Na\textsuperscript{+} ions, thus creating osmotic imbalances with the surrounding tissues. Because aggrecan is too large to redistribute itself, it attracts water in the NP, causing the matrix to swell and to maintain high levels of both hydration and osmotic pressure\textsuperscript{33,68}. Aggrecan expression is mainly controlled by SOX9 expression\textsuperscript{63}. However, in this study, high expression of SOX9 did not translate into high expression of aggrecan after culture in plain media. This can be explained by the fact that to trigger ECM production and fulfil its role, SOX9 needs to act synergistically with other factors\textsuperscript{62}. In presence of differentiation media the expression of aggrecan was significantly increased (Figure 2.12).

**2.3.6 Correlation analysis on mRNA expression**

To illustrate how the expressions of the analyzed genes correlated to each other, analysis of correlation coefficient was done. The results of the analysis are summarized in Figures 2.14 and 2.15. In plain media, a negative correlation was found between integrin α10 and the expression of ROCK I (Figure 2.14), confirming previous findings that highlighted the role of β1 integrins in the RhoA/ROCK pathway. In addition, in plain media, ROCK I was found to positively correlate with changes in cell morphology (Figure 2.14C). An increase in ROCK I expression corresponded to an increase in cell length and thus to a decrease in the shape factor index (Figure 2.14C). The formation of stress fibers and the expression of ROCK I was shown to exert a suppressive effect on chondrogenesis and ECM production mainly via the downregulation of SOX9\textsuperscript{39,47,49,69}. However, despite an increasing expression of ROCK I in 21 days of culture, no suppressive effects were found on SOX9 expression;
on the contrary, its expression increased (Figure 2.14D). As a key regulator of chondrogenic differentiation, SOX9 expression is modulated by a number of different signals and pathways \(^{38, 56}\), thus in this case there probably are other regulatory factors that overcome ROCK I inhibition. Moreover, in the absence of differentiation media, SOX9 activity was limited and its expression was not complemented by external factors for inducing the differentiation of ASCs (Figure 2.14D). To the contrary, expression of both aggrecan and integrin α10 decreased (Figure 2.14D). However, in presence of differentiation media, the expression of chondrogenic markers was observed. Microgels composed of 5 mg/mL collagen showed a higher expression of chondrogenic markers, synonymous with a greater differentiation. Figure 2.15 shows that expression of integrin α10, CD44 and SOX9 synergistically increased the expression of aggrecan and type II collagen and at the same time downregulated the expression of type I collagen. Overall, as also summarized in Figure 2.16, microgels with the higher concentration of collagen II/HA were more stable and also induced the desired cell phenotype.
Figure 2.2: The microgels’ preparation. The forming-hydrogel solution is formed by mixing hyaluronic acid, type II collagen, ASCs and 4S-StarPEG. The gelling solution is then deposited in form of 2 μL droplets on a hydrophobic surface and allowed to gel for 1 h at 37°C.
Figure 2.3: The effects of ASCs’ incorporation in the microgel on their viability. A–C) Effects of collagen concentration, cross-linker concentration and cell density on ASCs viability assessed by live/dead assay, no significant difference was found between the groups ($n = 9$, one way ANOVA, Tukey test $p > 0.05$).
Chapter 2

Figure 2.4: The effects of ASCs’ incorporation in the microgel on their proliferation. A–C) ASCs-loaded microgels after two days of culture and composed respectively of 2, 4 and 5 mg/mL type II collagen enriched with HA; D) Effects of collagen concentration on cells’ proliferation assessed by the BrdU assay, illustrating that cells maintain their ability to proliferate in every condition although the rate of proliferation drops after 14 days of culture.

<table>
<thead>
<tr>
<th>Collagen Concentration</th>
<th>Percentage of Proliferating Cells</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Day 2</td>
</tr>
<tr>
<td>2 mg/mL</td>
<td>8.74%</td>
</tr>
<tr>
<td>4 mg/mL</td>
<td>1.16%</td>
</tr>
<tr>
<td>5 mg/mL</td>
<td>6.13%</td>
</tr>
</tbody>
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Table 2.1 Percentage of proliferating cells over 14 days of culture in microgels

FITC

Count

Non Proliferating Cells

BrdU- FITC -

BrdU + FITC +

Proliferating Cells
Figure 2.5: Enzymatic degradation of microgels and their stability over 21 days of culture. A) Degree of cross-linking of microgels (composed of 5 mg/mL type II collagen enriched with HA) assessed by the TNBS assay; the results are shown as the percentage of free amine groups normalized to non-cross-linked gels, a concentration of 2mM of 4S-starPEG is sufficient to obtain a similar degree of crosslinking of glutaraldehyde ($n = 4$, one way
ANOVA, Tukey test $p > 0.05$); B) The effect of collagen concentration on stability of the microgels was quantified colorimetrically by Coomassie Blue Brilliant assay, results are shown as percentage of dye released over time, microgels composed of higher collagen concentration had a higher resistance to enzymatic degradation ($n = 4$, one way ANOVA, Tukey test $p > 0.05$); C) Variation of diameter of cell seeded microgels was measured over 2, 7, 14 and 21 days of culture, high collagen concentration seems to increase the stability of the microgels as it significantly reduces the shrinkage of microgels.

**Figure 2.6:** Calcein staining of embedded ASCs shows different cell morphologies in different concentrations of collagen. A, B, C) Bright field image of cells embedded in respectively 2, 4 and 5 mg/mL collagen II microgels for two days; D, E, F) Fluorescent image of calcein-stained cells embedded in respectively 2, 4 and 5 mg/mL collagen II microgels for two days.
Figure 2.7: Effect of collagen concentration on cell morphology. A) Cell volume of ASCs embedded in microgels with different collagen concentration; live cells were stained with calcein and z-stacks analyzed by using an image analysis software; cells retain a higher volume when embedded in high collagen concentrations (n = 4, one way ANOVA, Tukey test p > 0.05); B) Measure of the longest axis of cells embedded in microgels, after two days. Cells in lower concentrations of collagen are more stretched but these differences decrease after 21 days of culture (n = 4, one way ANOVA, Tukey test p > 0.05); C) Shape factor index of cells embedded in microgels for 2 and 21 days. This index quantifies the differences in morphology of cells; over time cells showed a tendency to spread but maintained a more rounded morphology after 21 days of culture when embedded in high collagen concentrations (n = 4, one way ANOVA, Tukey test p > 0.05).
Figure 2.8: High collagen concentrations hamper the formation of stress fibers in the cytoskeleton of the cells. A–D) Fluorescent images of cells cultured respectively on monolayer and in 2, 4, and 5 mg/mL microgels for 14 days (scale bars correspond to 50 µm); the nucleus was stained blue with DAPI and the cell cytoskeleton was stained red with Rhodamine/Phalloidin; cells in monolayer and 2 mg/mL microgels showed formation of stress fibers within their cytoskeleton, whereas in higher collagen concentrations the cell cytoskeleton was less organized and moreover showed a cortical actin organization, a hallmark of chondrogenic-like cells.
Figure 2.9: **Embedded ASCs possess a cytoplasm rich in glycogen deposits.** A–C) TEM images of cross-sections of cells embedded in 2, 4 and 5 mg/mL collagen microgels respectively; the cytoplasm of these cells is rich in vesicles and glycogen deposits (indicated by arrows), hallmark of NP and chondrogenic-like cells.
Figure 2.10: Embedding cells in high collagen concentration favours the expression of integrin α10 while delaying expression of ROCK 1. A) mRNA expression of the gene ROCK 1 in plain and differentiation media relative to monolayer control, type II collagen seems to delay the expression of ROCK 1 but this increases after 21 days of culture (n = 3, one way ANOVA, Tukey test p > 0.05); B) Relative mRNA expression of integrin alpha 10 in plain and in differentiation media; in differentiation media the expression of integrin alpha 10 is significantly higher in 5 mg/mL collagen, highlighting a stronger chondrogenic differentiation (n = 3, one way ANOVA, Tukey test p > 0.05).
Figure 2.11: Embedding cells in high collagen concentration favours the expression of SOX9 and CD44. A) Relative mRNA expression of SOX9 in plain and in differentiation media; although there is an increase of SOX9 expression in all the samples, 5 mg/mL collagen microgels showed the highest increase ($n = 3$, one way ANOVA, Tukey test $p > 0.05$); B) Relative mRNA expression of CD44 in plain and in differentiation media; in presence of differentiation media 5mg/mL collagen microgels showed the highest increase of expression of this receptor ($n = 3$, one way ANOVA, Tukey test $p > 0.05$).
Figure 2.12: Embedding cells in high collagen concentration favours the expression of aggregan and type II collagen. A) Relative mRNA expression of aggregan in plain and in differentiation media; in differentiation media the expression of aggregan increases in every sample, showing the highest increase in 5 mg/mL microgels ($n = 3$, one way ANOVA, Tukey test $p > 0.05$); B) Relative mRNA expression of type II collagen in plain and in differentiation media, cells in higher collagen concentration started expressing type II collagen already after two days of culture and after 21 days the expression increased in a similar manner in all the conditions ($n = 3$, one way ANOVA, Tukey test $p > 0.05$).
Chapter 2

Figure 2.13: Embedding cells in high collagen concentration lowers the expression of type I collagen. Relative mRNA expression of type I collagen in plain and in differentiation media, when in plain media the expression of type I collagen increased significantly in cells embedded in high collagen concentrations, but this trend was reversed in presence of differentiation media, highlighting a stronger chondrogenic differentiation (n = 3, one way ANOVA, Tukey test p > 0.05).
Chapter 2

A

Integrin α10 Correlations in Plain Media

Positive correlations

- Integrin α10 2mg/mL
- Integrin α10 4mg/mL
- Integrin α10 5mg/mL

Negative correlations

Rock I

B

CD44 Correlations in Plain Media

Positive correlations

- CD44 2mg/mL
- CD44 4mg/mL
- CD44 5mg/mL

Negative correlations

Aggrecan, Type I Collagen, Type II Collagen

C

Rock I Correlations in Plain Media

Positive correlations

- Rock I 2mg/mL
- Rock I 4mg/mL
- Rock I 5mg/mL

Negative correlations

Cell Volume, Sox 9, Longest axis, Shape Factor, Integrin α10

D

SOX 9 Correlations in Plain Media

Positive correlations

- Sox 9 2mg/mL
- Sox 9 4mg/mL
- Sox 9 5mg/mL

Negative correlations

Aggrecan, Integrin α10
Figure 2.14: Microgels in plain media do not trigger ADSC differentiation. A) Correlation analysis of the mRNA expression of Integrin alpha 10 and other chondrogenic markers in plain media; in high collagen concentrations integrin expression negatively correlated with the expression of ROCK 1; correlation was calculated by using the Pearson coefficient and Prism® software ($n = 6, p > 0.05$); B) Correlation analysis of the mRNA expression of CD44 and other chondrogenic markers in plain media; in low collagen concentration CD44 expression was correlated with the expression of type I collagen while in high collagen concentration CD44 expression was correlated with the expression of type II collagen; C) Correlation analysis of the expression of Rock I and other chondrogenic markers also including cell morphology in plain media; as expected, there is a negative correlation between Rock I expression shape factor and integrin alpha 10 expression; D) Correlation analysis of the expression of SOX9 and other chondrogenic markers in plain media; note that Rock I did not downregulate the expression of SOX9, in addition; the expression of SOX9 did not correlate with the expression of other chondrogenic markers due to the absence of differentiation stimuli.
Chapter 2

A. Integrin α10 Correlations in Differentiation Media

Positive correlations

Integrin α10
2mg/mL
Sox 9
Integrin α10
4mg/mL
Integrin α10
5mg/mL
Type II Collagen
Rock I
Aggrecan
CD44

Negative correlations

Integrin α10

B. CD44 Correlations in Differentiation Media

Positive correlations

CD44
2mg/mL
Aggrecan
Sox 9
Integrin α10
4mg/mL
Integrin α10
5mg/mL
Type II Collagen
Rock I
Aggrecan
CD44

Negative correlations

Rock I

C. ROCK I Correlations in Differentiation Media

Positive correlations

ROCK I
2mg/mL
Type II Collagen
Aggrecan
CD44
Rock I
4mg/mL
Type II Collagen
Aggrecan
CD44
Rock I
5mg/mL
Type II Collagen
Aggrecan
CD44

Negative correlations


D. SOX 9 Correlations in Differentiation Media

Positive correlations

SOX 9
2mg/mL
Aggrecan
CD44
Type II Collagen
SOX 9
4mg/mL
Aggrecan
CD44
Type II Collagen
SOX 9
5mg/mL
Aggrecan
CD44
Type II Collagen

Negative correlations

Integrin α10


92
Figure 2.15: 5 mg/mL collagen microgels offer an optimal microenvironment for ASCs differentiation in presence of differentiation media. A) Correlation analysis of the expression of Integrin alpha 10 and other chondrogenic markers in differentiation media; in 5 mg/mL collagen microgels the expression of integrin alpha 10 is positively correlated with the expression of other chondrogenic markers, showing a stronger differentiation; B) Correlation analysis of the expression of CD44 and other chondrogenic markers in differentiation media; in 5 mg/mL collagen microgels the expression of CD44 had the highest correlation with the expression of other chondrogenic markers, showing a stronger differentiation; C) Correlation analysis of the expression of ROCK 1 and other chondrogenic markers in differentiation media; ROCK 1 expression did not result in downregulation of chondrogenic markers; D) Correlation analysis of the expression of SOX9 and other chondrogenic markers in differentiation media; in 5 mg/mL collagen microgels the expression of SOX9 had the highest correlation with the expression of other chondrogenic markers; the higher SOX9 expression could explain the stronger chondrogenic differentiation in higher concentrations of collagen II.
2.4 Discussion
The main objective of this study was to design a tunable ECM-based cell delivery system that can modulate the differentiation of ASCs towards a NP-like phenotype. In order to maintain the already delicate environment of the NP, it is crucial that none of the components of the delivery system affect cell viability. As shown in previous studies, the use of 4S-StarPEG proved to be an appropriate cross-linking strategy which avoids adverse effect on cell viability. An ideal system should act as a template for the regeneration of the tissue of interest, thus the cells it delivers need to be able to remodel it. ECM-based biomaterials have the advantage of being degraded physiologically without generating toxic products as a result of their degradation. However, their degradation can be rapid, so that cells have insufficient time to synthesize significant amounts of ECM. A cell delivery system that can help remodelling by providing a certain degree of stability is therefore an advantage. Although cross-linking significantly increased microgel stability, the composition of the microgels proved to be another important factor of influence.

2.4.1 5mg/mL is the optimal collagen concentration to engineer microgels
Microgels which had a higher concentration of type II collagen displayed high resistance to shrinking and also to enzymatic degradation (Figure 2.5). When differentiating cells towards a chondrogenic phenotype, an important parameter to consider is their morphology. Moreover, cell morphology can not only facilitate cell differentiation but is also the result of the microenvironment in which cells are present. In fact, differentiated cells in the IVD acquire different morphologies according to the mechanical loads that specific regions will experience. For example, cells in the annulus fibrosus (AF) are more elongated as a result of the tensional forces exerted on the tissue, and cells in the NP are more rounded as a result of compression. Thus it has been hypothesized that different mechanical loads can influence the quality of the ECM that cells produce by influencing their cytoskeletal organization, and thus their morphology. Moreover, cell morphology is also the result of ECM composition as cells can sense and
adapt to their environment via the crosstalk between integrins and cytoskeletal proteins \(^{71-73}\). Presence of type II collagen, for example, has been linked to cell shape \(^{24}\). In fact, microgels with high concentrations of type II collagen helped to maintain a more rounded cell morphology, priming them to differentiate towards an NP-like phenotype and, moreover, to synthesize matrix (results shown in Appendix Z). In fact, in such an environment cells show a higher degree of differentiation than either monolayer or microgels of lower concentrations of collagen. Type II collagen itself was shown to be a conducive environment for chondrogenic phenotype; in the current study, cells embedded in any concentration of type II collagen had the tendency to increase the expression of SOX9 (Figure 2.11). However, while this is an indispensable condition, it is not sufficient to trigger cell differentiation, as shown by the fact that SOX9 is expressed also in other non-skeletal tissues \(^{38, 56}\). Indeed, despite the favourable environment, ASCs embedded in microgels still needed differentiation media to undergo a strong differentiation. Even so, it must be considered that the fact that cells express high levels of SOX9 is positive not only for differentiating them but also because this provides them with the ability to better maintain the already existing ECM. In previous studies, it was reported that expression of SOX9 is key also to the homeostasis of the IVD and its downregulation coincided with a shift towards matrix catabolism via upregulation of MMPs’ expression \(^{63}\). Microgel systems composed of type II collagen and enriched with HA not only mimic the physiological composition of the healthy NP but were shown to influence ASCs’ differentiation. Moreover, by controlling the concentration of type II collagen, it is possible to control both their stability and the phenotype of cells they carry. In particular, type II collagen primed ASCs towards an NP phenotype by influencing their morphology and also by inducing expression of high levels of SOX9. ASCs were able to differentiate in every condition tested but to varying degrees, with stronger differentiation occurring in higher concentrations of type II collagen.
Figure 2.16: 5 mg/mL collagen microgels offer the optimal conditions for ASCs delivery. Schematic representation of the results obtained in the current study. Overall 5 mg/mL microgels have been shown to possess superior stability and ability to prime ASCs’ differentiation towards an NP-like phenotype.
2.5 Conclusions

Cells embedded in microgels of high concentrations of type II collagen had the most prominent differentiation. Here, the expression of key regulators of chondrogenic differentiation such as SOX9, Integrin α10 and the receptor CD44 was upregulated, acting in synergy to increase the expression of ECM molecules such as type II collagen and aggrecan. Additionally, the quality of the matrix produced was higher, as shown by the fact that expression of type I collagen was significantly lower. As summarized in Figure 2.16, 5 mg/mL microgels had the optimal microenvironment in terms of stability and were resistant to enzymatic degradation. These microgels enabled ASCs to achieve a higher degree of differentiation. Hence, microgels of high concentrations of type II collagen show potential for use as a cell delivery system for the regeneration of the nucleus pulposus.
Chapter 2

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Chapter 3

Development of a collagen-based reservoir system

Sections of this chapter have been published in:
S. Browne*, G. Fontana*, B. J. Rodriguez, A. Pandit,
A Protective Extracellular Matrix-Based Gene Delivery Reservoir Fabricated by Electrostatic Charge Manipulation.
3.1 Introduction

Advances in proteomics and genomics and, above all, a better understanding of the intervertebral disc (IVD) physiology have led to the identification of a large number of biomacromolecules with therapeutic relevance. These biologics can influence cell behaviour and trigger a desired response. On the whole, intradiscal delivery of growth factors shows efficacy in attenuating symptoms of disc degeneration \(^1\)-\(^6\). However, these biologics can also have shortcomings such as susceptibility to proteolytic degradation, hydrolysis, chemical modification and denaturation \(^7\). These shortcomings have provided an opportunity in the drug delivery field as limitations of conventional drugs need to be overcome. Advantages of an improved drug delivery system include: (1) continuous maintenance of drug levels in a therapeutically desirable range; (2) a decrease in the amount of drug needed; (3) the facilitation of drug administration, with improved patient compliance and prolonged therapeutic efficacy \(^8\). The use of synthetic materials for the fabrication of drug delivery vehicles has been the preferred choice because it allows precise control over the features of the systems. Indeed, this is a significant advantage especially when designing microspheres and nanoparticles systems because polymeric building blocks enable high degree of uniformity in size and shape and also a high level of reproducibility \(^7\), \(^8\). However, polymeric matrices often undergo hydrolysis in physiological environments which cause bulk erosion of the system and, in turn, dose dumping of the delivered drug \(^8\). Moreover, degradation products of synthetic polymers-based drug delivery systems have undesired cytotoxic effects \(^8\), \(^9\). This issue was in part solved with the introduction of PLGA-based drug delivery systems. In fact, PLGA degradation products can be easily metabolized by the human body, thereby decreasing undesired cytotoxic effects \(^10\). However, cases in which PLGA-based biomaterials evoke an inflammatory response have been reported \(^10\). This is a substantial drawback for IVD applications because the disc is a tissue in which inflammation is difficult to resolve and often becomes a self-perpetuating process that worsens disc degeneration \(^11\)-\(^17\).
3.1.1 The use of ECM-based Drug Delivery Systems

Accessing the vast repertoire of extracellular matrix (ECM) biomacromolecules to engineer drug delivery systems is preferable and has several advantages. In nature, the ECM provides immobilized adhesion cues and acts as a reservoir for growth factors such as for example VEGF\textsuperscript{18}, TGF-\textbeta1\textsuperscript{2}, bFGF\textsuperscript{19} and IGF-1\textsuperscript{20}. This natural reservoir, in response to cell-mediated remodelling, liberates bound growth factors \textsuperscript{21}. Therefore, when using ECM biomacromolecules as building blocks for the design of drug delivery systems, it is possible to obtain release of the drug under spatial and temporal demand of individual cells \textsuperscript{21}. Furthermore, such systems would not trigger cytotoxic responses in the targeted tissues \textsuperscript{8, 9, 22}. Among the various ECM biomacromolecules, type I collagen generated particular interest for applications in drug delivery \textsuperscript{23-25}. The immunogenicity associated with type I collagen is usually eliminated by digesting with pepsin its telopeptides (which contain antigenic epitopes) \textsuperscript{25}. Because of its suitability as a biomaterial as well as a delivery system, various methodologies have been attempted for the development of type I collagen-based reservoir systems such as the emulsion method \textsuperscript{26}, spray-drying \textsuperscript{24}, and microphase separation \textsuperscript{27}. Although promising outcomes are reported from the use of spheres fabricated using the techniques mentioned above, drawbacks to be resolved include low reproducibility and poor control over size. Such problems were encountered also in the current study when collagen spheres were fabricated using methods previously described such as emulsion \textsuperscript{26} and microphase separation \textsuperscript{27}. Recently, it has been demonstrated that it is possible to fabricate hollow spheres of a controlled size and in a reproducible manner with natural polymers using a template method \textsuperscript{28, 29}. However, to date, there have been no reports of hollow reservoir systems that are made from fibrous proteins. It is hypothesized that by using the template method it will be possible to obtain collagen microspheres reservoir systems that are uniform in size and in a reproducible manner. Specific objectives of this study are to coat the polystyrene template with type I collagen, to fabricate collagen hollow spheres with uniform size and shape and to ascertain that they have no adverse effects on cell’s metabolic activity.
3.2 Materials and Methods

3.2.1 Extraction of collagen
Type I atellocollagen was isolated as previously described \(^{30}\). Briefly, bovine tendons were blended, washed in buffer and suspended in 0.5M acetic acid. The resulting solution was then pepsin treated and filtered to remove insoluble collagen telopeptides. The soluble collagen was then purified by repeated salt precipitation and centrifugation, followed by dialysis against 0.01M acetic acid.

3.2.2 Fabrication of collagen microspheres with the emulsion method
Collagen microspheres were fabricated by using the emulsion method \(^{26}\) described in Appendix W. Briefly, a type I collagen solution (5mg/mL in 0.5M acetic acid) was neutralized in 1 M NaOH to obtain a final pH of 7. To stabilize the emulsion Tween 20 was used as a surfactant and was added directly into the neutralized collagen solution. To obtain the adequate density and hydrophobicity, the oil phase was composed of a mix of oils. Paraffin and olive oil were mixed at a ratio of 23:1 respectively and heated to 37°C. The oil phase was then added to the collagen solution at a volume ratio of 4:1. Following this, the emulsion was vortexed until homogenous in appearance. Subsequently, the collagen was allowed to form solid fibrils by 2 hours incubation at 37°C. Collagen microspheres were then washed in ethanol 70% and collected by centrifugation, 5 min at 4500 rpm.

3.2.3 Fabrication of collagen microspheres with the microphase-separation method
The microphase separation is a method \(^{27}\) (described in Appendix X) that allows use of large biomacromolecules for the formation of hollow particles. The method includes three main steps: phase separation, sphere formation, and hollow sphere formation. The first phase involves fast freezing in liquid nitrogen; during this phase a microphase separation occurs where water forms ice crystals and the collagen/acetic acid compartment forms sheet-like structures. When the temperature increases to -20°C, a macromolecular motion occurs because the macromolecular sheets tend to re-organize themselves in more energetically-favorable globular structures.
(less surface interface). Following freeze-drying, these globular structures solidify forming particles. A 2 mg/mL type I collagen solution in 0.25M acetic acid was bathed in liquid nitrogen for 30 seconds. Immediately after this, the solution was incubated at -20°C for 3 hours to allow the annealing phase to start. Following this, the samples were freeze-dried for 18 hours, during which the temperature of the plate was kept constant at -20°C for 12 hours and gradually raised to 0°C over the remaining 6 hours.

### 3.2.4 Fabrication of collagen microspheres with the template method

Hollow collagen microspheres were fabricated using the template method as described in the literature for other natural polymers\textsuperscript{28, 29, 31}. Commercially available polystyrene beads of defined sizes (100nm, 1μm and 10μm in this case), (Gentaur, Chicago, Illinois) were sulfonated to impart a strong negative charge. Following sulfonation, beads were resuspended in 0.5M acetic acid. 5mg/ml of type I collagen solution was added to the beads at a weight ratio of 4:1 (beads: collagen). The resultant mixture was then stirred for four hours at room temperature. Crosslinking of the collagen coating was performed using pentaerythritol poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-PEG), with the active ester groups reacting with the free amino groups of collagen at a ratio of 1:4 (4S-PEG:Free amino groups in the collagen). The mixture was agitated for two hours at room temperature. To produce a hollow sphere, the polystyrene core was dissolved by washing the coated beads with tetrahydrofuran (THF). The suspension of microspheres was diluted at a ratio of 1:1 with THF and agitated for one hour. The washing step was repeated twice to ensure complete removal of polystyrene. Hollow spheres were washed twice with ethanol and twice with water to ensure removal of any remaining THF. Spheres of various sizes were made, ranging from 100 nm to 10 μm.
Figure 3.1: Schematic representation of the emulsion method. Water phase containing the neutralized collagen is mixed with the oil phase. Collagen spheres are obtained following stirring at 37°C for 2 hours.
Figure 3.2: Schematic representation of the microphase separation method. By fast freezing a collagen solution there is formation of collagen compartments surrounding ice crystals. Following a gradual increase in temperature, the collagen will tend to acquire a globular structure because it is energetically more favourable. In this process water is removed by freeze-drying.

**Step 1:**
PHASE SEPARATION
fast freezing (liquid nitrogen)

**Step 2:**
SPHERE FORMATION
annealing (incubation at -20°C)

**Step 3:**
HOLLOW SPHERES FORMATION
lyophilization
Figure 3.3: Graphical representation of the fabrication of hollow collagen microspheres. The process involves the sulfonation of a commercially available polystyrene bead of defined size (100 nm, 1 μm and 10 μm in this case), coating of these beads in a collagen solution, crosslinking of the coating and removal of the polystyrene bead core. (THF: Tetrahydrofuran).
3.2.5 Sample preparation for scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Preparation of samples for SEM analysis consisted of fixation with 3% glutaraldehyde in freshly prepared 0.2 M sodium cacodylate buffer for one hour. After washing the samples in sodium cacodylate buffer, the samples were resuspended in osmium tetroxide for two hours under the fume hood. After a series of washings in their buffer, samples were dehydrated by immersion in a graduated series of ethanol in H$_2$O and hexamethyldisilazane (HDMS) in ethanol solutions of 30%, 50%, 80%, 95% and 33%, 50%, 66%, 100%, respectively. The samples were let dry on the sample holder. TEM samples were embedded in an epoxy-based resin (Agar Low Viscosity Resin kit). The embedding process consisted of incubations of samples in a mixture Resin/Propylene Oxide 50:50 for four hours and then replaced with a mixture 75:25 overnight and lastly in pure resin for six hours. The final step consisted of thermo-crosslinking at 65°C for 48 hours which allows the resins to acquire the hardness needed to cut cross-sections of the embedded samples.

3.2.6 Zeta potential analysis of surface coating during fabrication process

To characterize the surface coating of microspheres, zeta potential was analysed using a zeta sizer (Malvern, Nano-ZS90). Microspheres (500 nm and 5 µm) at various stages of fabrication were analysed to determine the charge of the spheres and confirm the coating process. The four stages at which microspheres were assessed for charge analysis were: polystyrene beads pre-coating, polystyrene beads following collagen coating, polystyrene beads collagen coated and crosslinked, and finally hollow crosslinked collagen microspheres (THF treated to remove polystyrene). The zeta analysis was performed in acetic acid, in which collagen is positively charged.
3.2.7 Size analysis
Size of samples was determined by using ImageJ and measuring the diameter of 60 spheres from SEM images. In order to obtain high accuracy, the analysis was repeated for three different batches of prepared spheres.

3.2.8 Cell’s metabolic activity
The influence of the spheres on the metabolic activity of 3T3 fibroblasts was quantified using the alamarBlue® cell metabolic activity assay. 50μg of microspheres of three sizes (100nm, 1μm and 10μm) were incubated with cells for 48hours and the effect on metabolic activity was measured and compared with the control (cells grown on tissue culture plastic alone).

3.3 Results
3.3.1 Type I collagen spheres obtained with emulsion method and microphase separation
As shown in Figures 3.4 and 3.5, type I collagen spheres were successfully obtained by using the emulsion method and the microphase separation respectively. Each preparation protocol resulted in spheres with different architecture, yield and reproducibility. With the emulsion method illustrated in Figure 3.1, a very heterogeneous population of collagen spheres was obtained. These spheres were filled with a dense network of collagen fibers (Figure 3.4), and characterized by a non-homogeneous appearance and, overall, this protocol had very low yields. Instead, the microphase separation method illustrated in Figure 3.2 allowed obtaining spheres with homogeneous appearance and good yields (Figure 3.5). However, the spheres obtained showed very low stability in water.

3.3.2 Characterization of type I collagen hollow spheres obtained with the template method
By using the template method collagen hollow spheres were obtained. The coating process graphically illustrated in Figure 3.3 is based on an electrostatic interaction between collagen and the negatively charged polystyrene template. Figure 3.6 illustrates the preparation of spheres by the template method using SEM images at each stage of the process. The
coating process is performed in acidic conditions so that the positively charged collagen forms a thin coat around the negatively charged polystyrene beads (Figure 3.7). The spheres obtained have the same shape and size of the polystyrene beads used as the template. Following the formation of a coating around the polystyrene bead, the collagen is cross-linked for stabilization of the coating. Finally, the polystyrene template was removed by washing with THF. This was confirmed by FTIR analysis (data shown in Appendix AA, Figure AA.1). The removal of the polystyrene core leaves a hollow shell of type I collagen; this was confirmed by TEM analysis of cross-sections (Figure 3.8). A more detailed characterization of the spheres’ surface is described in Appendix AA, (Figure AA.1).

3.3.3 Cell viability and interactions with collagen spheres
Considering the ultimate goal is to utilize these hollow collagen spheres as a reservoir system for drug delivery in degenerated intervertebral discs, it is crucial that these spheres were not toxic to cells. To ascertain this crucial aspect, a proof of concept study was done by exposing 3T3 fibroblasts to type I collagen spheres. No spheres of any size exhibited toxicity of any level (Figure 3.9) when compared with the control (cells alone grown on tissue culture plastic). Moreover, because type I collagen is characterized by an abundant presence of RGD sequences, cells were found to adhere to the spheres (Figure 3.9). This will allow the collagen reservoirs to be always in the cell’s proximity, thereby favoring their exposure to the delivered drug. Furthermore, as the reservoirs are expected to be in contact with cells for a considerable period of time in vivo, it is re-assuring that no cytotoxic effects were seen when assessed with the alamarBlue® assay. The cells showed no changes in morphology when compared with a control - further evidence that the collagen spheres do not impair viability, and hence are suitable as reservoirs in contact with cells. Moreover, TEM analysis of fixed samples of 3T3 fibroblasts incubated with collagen spheres showed evidence of uptake of some of the spheres within cells Figure 3.10. When compared with non-treated cells (no spheres), it is clear that some of the spheres have been engulfed and internalized. However, not all of the spheres
were internalized, with large clusters of them remaining adjacent to the cells.

### 3.3.4 Functionalization of collagen reservoirs

**Appendix AA** describes in detail the methodology and the outcomes of type I collagen hollow sphere functionalization with non-viral gene vectors, polyplexes. These are complexes between a cationic polymer and plasmid DNA coding for proteins of interest. Briefly, collagen hollow spheres can be loaded with polyplexes. Independently of reservoir size, the loading efficiency was above 80% (**Appendix AA, Figures AA.3 and AA.4**). A slow and sustained release of polyplexes from the collagen reservoirs was observed, and on average 50% of loaded polyplexes was released over 144 hours (**Appendix AA, Figure AA.4**). An in vitro transfection study confirmed that the released polyplexes maintained efficacy in transfecting cells (**Appendix AA, Figure AA.5**). Interestingly, while allowing levels of transfections similar to those with polyplexes alone, loading polyplexes in collagen reservoirs significantly decreased their toxicity (**Appendix AA, Figure AA.5**).
Figure 3.4: Collagen spheres fabricated by using the emulsion method. (A) TEM image of a collagen sphere filled with a dense network of collagen fibers; (B and C) SEM images of collagen spheres.
**Figure 3.5:** Collagen particles obtained with the macrophase separation method. SEM images of collagen particles fabricated with the microphase separation; although these particles were homogeneous in appearance, they showed very low stability in water environments and were therefore deemed not suitable for use as reservoir systems.
Figure 3.6: Steps of sphere fabrication. SEM images of the coating process, showing (A) polystyrene beads, (B) collagen-coated beads and (C) hollow spheres following treatment with THF to dissolve the template.
Table 3.1 Charge measurements during each step of spheres’ fabrication

<table>
<thead>
<tr>
<th>Size</th>
<th>Polystyrene beads</th>
<th>Collagen-coated polystyrene beads</th>
<th>Crosslinked collagen-coated polystyrene beads</th>
<th>Hollow collagen spheres</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 nm</td>
<td>-29.77 ± 0.67</td>
<td>25.73 ± 2.57</td>
<td>14.07 ± 0.78</td>
<td>31.7 ± 8.1</td>
</tr>
<tr>
<td>4.5 µm</td>
<td>-20.6 ± 0.66</td>
<td>25.37 ± 0.25</td>
<td>8.41 ± 1.03</td>
<td>38.93 ± 3.53</td>
</tr>
</tbody>
</table>

Figure 3.7: Characterization of the template coating. Charge analysis showing the change in zeta potential during the fabrication of hollow collagen spheres of two sizes (500 nm (A) and 4.5 µm (B)) (n=3).
Figure 3.8: Dissolution of the polystyrene template leaves a hollow core. (A) TEM image through polystyrene beads coated with type I collagen. (B) TEM image through type I collagen hollow spheres obtained after the dissolution of the polystyrene template in THF. (C) Size distribution of 500 nm microspheres measured by image analysis on SEM images. While the mean size is around 440 nm, this reduction in size compared to the 500 nm template may be explained by the effect of drying on the sample (n=180).
Figure 3.9: Cell-spheres interactions. (A)* Percentage viability compared with cell alone control as measured by the alamarBlue® assay, data represents mean ± SD (n=3, one way ANOVA, Tukey test p > 0.05), (B) SEM image of type I collagen spheres, (C) SEM image showing the interaction between 3T3 Fibroblasts and collagen spheres. *Data obtained in collaboration with Shane Browne.
Figure 3.10: Spheres’ internalisation. (A) TEM image of a cross-section through a 3T3 cell. (B) TEM image of a cross-section through a 3T3 cell following incubation with 1 µm collagen spheres. A small number of spheres can be seen to be internalized, with many more remaining external to the cell.
### 3.4 Discussion

The generally poor outcome of the first-generation drug therapies, and in particular delivery of growth factors as bolus, could be attributed to their mode of delivery\(^{37}\). Uncontrolled delivery of drugs results in systemic rather than localized responses\(^{38-40}\). Furthermore, uncontrolled delivery might translate into initial excess levels of the drugs followed by a deficit and loss of efficacy caused by their fast degradation/inactivation and clearance\(^{41, 42}\). These drawbacks have generated growing interest in the implementation of strategies in which novel biomaterial systems are used for the controlled delivery of drugs\(^{43-45}\). The use of synthetic polymers allows a high degree of control in the delivery system architecture\(^7, 8\). This, in turn, enables controlled delivery regimes\(^7\). However, from a biological standpoint, synthetic systems are poor performers as they lack adhesion cues and products of their degradation pose risks of overturning the positive effects of the delivered drugs by introducing cytotoxicity or inflammatory signals in the tissue target\(^8, 9, 23, 46\). By contrast, the use of ECM-based drug delivery systems presents multiple advantages. For example, during tissue development, maintenance and remodelling, the ECM plays an active role by providing critical instructive cues to control the behavior of embedded cells that constantly remodel their microenvironment by proteolytic processes\(^{47}\). Moreover, the composition of ECM signals modulates the expression and synthesis of new ECM, establishing a “dynamic reciprocity”\(^{48}\). So far, the limiting factor in using ECM-based drug delivery systems has been the difficulty in achieving a high level of control and reproducibility in some of the system features such as size and shape. Conventional methods for the fabrication of collagen particles were tested in the current study and for each, strong limitations were found. For instance, with the emulsion method there is very little control over the architecture of the produced spheres (Figure 3.4) and, moreover, both yields and reproducibility were extremely low. Slightly better results were obtained by using the microphase-separation method. The population of collagen particles obtained with this method was homogeneous in appearance (Figure 3.5). However, due to the absence of crosslinking, the collagen particles had very low stability in water. Therefore the use of this procedure to fabricate
collagen reservoir systems for long term applications is questionable. In the current study, such issues were overcome by using the template method (described in the methods section) to fabricate collagen hollow spheres.

3.4.1 Fabrication of homogenous collagen microspheres with the template method

As shown in Figure 3.6, fabricating collagen hollow spheres by using the template method allows a high degree of control in terms of size and shape. The collagen coating of the template was controlled and enhanced by harnessing the electrostatic charges of collagen and polystyrene respectively. The coating step was characterized qualitatively by SEM imaging (Figure 3.6) and also by measuring the charge of the spheres in each step of their preparation (Figure 3.7). Following the coating step, the charge of the coated polystyrene beads turns from negative to positive. Overall, collagen hollow spheres were found to possess a positive charge in acidic environments (Figure 3.7) but a negative charge when immersed in PBS (Appendix AA, Figure AA.2). As discussed in Appendix AA, this feature was used to functionalize these collagen reservoirs with polypelexes. The main advantage of using the template method was the possibility to control size and shape of the fabricated spheres simply by selecting the appropriate template. Furthermore, the complete removal of the polystyrene template was ascertained by FTIR analysis (Appendix AA, Figure AA.1) and also by TEM imaging which confirmed that dissolution of the template by THF treatment leaves a hollow core (Figure 3.8). The interaction between cells (3T3 fibroblasts) and type I collagen hollow spheres was characterized in vitro. Here, even when cells were exposed to high amounts of spheres (50µg), no alterations in their metabolic activity were found (Figure 3.9). This is a very important feature as degradation products of synthetic reservoir systems are often found to overturn the beneficial effects of the delivered drugs. Moreover, because of the abundant concentration of RGD sequences in type I collagen macromolecules, cells are able to adhere to the collagen spheres (Figure 3.9 C) thereby, favouring their exposure to the eventual delivered drug. Furthermore, TEM analysis through 3T3 cells incubated with collagen hollow spheres revealed that
some spheres undergo internalization (but the majority of spheres were seen outside, in the cell’s proximity). This suggests that this drug delivery system is particularly suitable as a reservoir of drugs that have intracellular effects, such as polyplexes.

### 3.5 Conclusions

The results of this study demonstrate that hollow collagen microspheres can be fabricated using a consistent and efficient process. In fact, if compared to conventional methods for the fabrication of collagen particles, the template method allows greater control over size and shape with superior yields and reproducibility. Type I collagen hollow spheres did not alter cell metabolic activity even in high concentrations; hence they show potential as an ECM-based drug delivery system. Their functionalization was characterized and optimized by using polyplexes (reservoirs functionalization is discussed in Appendix AA).

### 3.6 References


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Chapter 3


130


Chapter 4

Development of a 3D Cell Factory Platform Tailored for the Nucleus Pulposus

Sections of this chapter have been published:

G. Fontana, A. Srivastava, D. Thomas, P. Lalor, P. Dockery, A. Pandit,

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

Bioconjugate Chemistry, 2014 (in press)
4.1 Introduction

So far, various unimodal strategies have been investigated to promote regeneration in the NP, and these include stem cell therapy \(^1,^2\), implantation of scaffolds \(^3^-^5\) or injection of growth factors \(^6^-^{12}\). The implantation of NP cells for re-population of NP increases the likelihood for regeneration, and this has been reported in a number of studies \(^4,^13\). But, since implantation of autologous NP cells has shortcomings such as morbidity of the donor site and the limited amount of cells that can be retrieved from NP tissues \(^14,^15\), stem cells are considered a most feasible cell source \(^15^-^{18}\) with particular interest shifting towards the use of progenitor cells such as adipose-derived stem cells (ASCs) \(^19^-^{23}\). However, degenerated discs are not only characterized by the depletion of their cell population but also by a phenotypic switch of the resident cells concurrently with lower levels of extracellular signals such as growth factors \(^6,^7\). Intradiscal delivery of growth factors has shown promising results by priming NP cells to synthesize ECM and overall mitigating disc degeneration \(^8,^9,^24\). Even so, because of the short half-life of growth factors \(^25,^26\), these approaches serve only as a short-term solution and require multiple injections of growth factors to retain long-term efficacy \(^27\). In addition, multiple injections can lead to irreparable tissue damage, and thereby weigh negatively from a risk/benefit perspective. These considerations highlight the need of 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. This can be achieved by combining cell therapy and gene therapy approaches.

4.1.1 Non-viral gene therapy approach

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 \(^28\). Hence, desired proteins can be produced by cells for an extended period. In gene therapy, there is growing interest towards the development of non-viral, polymeric-based gene carriers. These are generally cationic polymers with high affinity for pDNA to form complexes also known as polyplexes \(^29\). 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 \(^{30, 31}\). However, a primary concern, that limits their use, is their cytotoxicity \(^{32-34}\). 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 \(^{35}\). 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 \(^{35, 36}\) thereby exerting a protective role on both cells and polyplexes \(^{35, 37}\).

### 4.1.2 A biomaterial system designed to deliver cells to the NP

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 \(^{38}\). This implies that delivering cells and growth factors in biomaterial systems is a preferred choice as it allows higher retention at the desired site \(^{22, 39, 40}\). 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 remodelling, 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 \(^{41-43}\). These promising outcomes have resulted in the adoption of beads and spheroid systems for the culture of NP-like cell lines \(^{44}\). Hydrogel systems that mimic the composition of disc matrix were shown to provide a suitable environment for the synthesis and deposition of new ECM \(^{45-48}\). In particular, hydrogel based on type II collagen and HA seem to be an ideal platform for the cell therapy of the IVD \(^{49-52}\). In Chapter 2 a type II collagen/HA microgel system was optimized to prime ASCs towards a NP-like phenotype. The objective of the current study is to functionalize the optimized microgel platform to program the delivered ASCs 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.

4.2 Materials and methods

4.2.1 Materials and cells
If not specified, all materials were purchased from Sigma Aldrich (Ireland). Type II calf articular joint atelocollagen (Affymetrix® (UK)), high molecular weight (200-750 kDa) hyaluronic acid (Contipro group, (Czech Republic)) were used. 4-arm polyethylene glycol succinimidyl glutarate Mw 10,000Da (4S-StarPEG) was purchased from JenKem Technology (USA). ASCs were extracted from rabbit adipose tissue as previously described22 and used strictly at passage three for every experiment and were cultured in 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 53.

4.2.2 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 35, 36. Briefly, commercial 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 drop wise to the bead suspension at a ratio of 1:4 (collagen:beads), while maintaining the final collagen concentration of 2mg/mL. Although the electrostatic interaction between type II collagen and beads is immediate, the mixture was stirred for four hours 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 crosslinking to occur, the coated beads were then resuspended in PBS 1X and the pH adjusted to 7.4 by using NaOH 1M. The crosslinking was performed using 4S-StarPEG at a ratio of 1:2 w/w
(collagen/4S-StarPEG). The solution was stirred gently for one hour 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 one hour. The washing step was repeated twice to ensure complete removal of polystyrene.

4.2.3 Analysis of charge on microspheres
To characterize the surface coating of microspheres, zeta potential was analysed using a zeta sizer (Malvern®, Nano-ZS90). Microspheres (4.5 µm) at various stages of fabrication were analysed to determine the charge of the spheres and to confirm the coating process. The stages at which microspheres were assessed for charge analysis were: polystyrene beads pre-coating, polystyrene beads following collagen coating (two ratios collagen:beads tested: 1:1 and 1:4), and after crosslinking step.

4.2.4 Size analysis of microspheres
The size of microspheres was determined by using ImageJ 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.

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

4.2.6 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 one hour. 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.
4.2.7 Loading of microsphere reservoirs with polyplexes
The loading of collagen microspheres with polyplexes occurs because of electrostatic interactions. However, polyplexes can not only absorb on the surface of microspheres but are also able to diffuse inside their hollow core. This occurs via diffusion through the collagen layer. 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 four hours 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.

4.2.8 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.

4.2.9 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 one hour. After washing the samples in sodium cacodylate buffer, the samples were resuspended in osmium tetroxyde for two hours under the fumehood. After a series of washings in the buffer, samples were dehydrated by immersion in a graduated series of ethanol in H2O 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.
4.2.10 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 four hours and then replaced with a mixture 75:25 overnight and lastly in pure resin for six hours. The final step consisted of thermo-crosslinking at 65°C for 48 hours which allows the resin to acquire the hardness needed to cut cross-sections of the embedded samples.

4.2.11 ASC encapsulation in 3D microgels

Type II atelocollagen was neutralized (pH 7.4) in 1M NaOH and 10× phosphate buffer saline (PBS) in volumes adjusted to have a final concentration of collagen of 5mg/mL. The forming-gel solution was then enriched with HA (9 to 1 weight ratio collagen to HA). ASCs (10⁶ cells/mL final) and polyplex-loaded microspheres were added to the mixture. After addition of 4S-StarPEG (2mM), 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 one hour at 37°C.

4.2.12 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 the experiment, ASCs were seeded in a 96 well plate (10⁴ 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 two hours at 37°C. The supernatant was collected (100µL) in a clear 96 well plate and the absorbance measured at 550 nm and 595 nm (0.5 seconds per well).

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

4.2.13 Size of microgels
The size of microgels functionalized with different amounts of loaded microspheres was monitored by analyzing images of microgels after two and seven days of culture. 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.

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

4.2.14.1 Quantitative analysis in a monolayer system
To determine the best conditions for transfection, microspheres of different size 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 hours in complete media. Different concentrations of microspheres were then added on them and incubated for 48 hours in complete media. After 48 hours at 37°C, the supernatant (50 µL) was transferred into a new 96 well plate and analyzed for G-luciferase activity.

4.2.14.2 Quantitative analysis in a monolayer system
ASCs were encapsulated as described above; however, in the forming gel solution were added also 10µL of polyplex-loaded microspheres containing 5, 10 and 15 µg of complexed pDNA in 125, 250 and 375 µg of spheres respectively. The supernatant (50 µL) was transferred into a 96 well plate and analyzed for G-luciferase activity.
4.2.14.3 Qualitative analysis in a monolayer system
ASCs were seeded in a 96 well plate (10^4 cells/well) and incubated for 24 hours 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 hours in complete media. ASCs were then fixed in 4% paraformaldehyde for 30 minutes 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).

4.2.14.4 Qualitative analysis in 3D microgels
ASCs were embedded in microgels as described previously. After seven days of culture, the samples were fixed in 4% paraformaldehyde for one hour. 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 immuno-labeling. 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 two hours with FITC-labeled primary anti-GFP antibody (dilution 1:200). Cell nuclei and cytoplasm were stained as described above.

4.3 Results
4.3.1 Fabrication and characterization of type II collagen hollow spheres
Type II collagen hollow spheres were successfully fabricated by using the template based method. As shown in Figure 4.2, the hollow spheres were homogeneous in size and shape. Different batches of 4.5 µm type II collagen hollow spheres were prepared and analyzed in the SEM. Spheres fabricated by using the 4.5 µm template had a size distribution from 4 to 5 µm (Appendix BB Figure BB.1) with an average size of 4.5 µm. As reported previously, the variation in size is attributable to the commercial template used rather than to the fabrication steps. The template coating step was carried out in an acidic environment and it was confirmed by zeta
potential analysis. Different ratios of beads to collagen do not affect the coating and with the 1:4 ratio of beads to type II collagen (Appendix BB, Figure BB.2), a higher amount of hollow spheres can be obtained using the same amount of type II collagen.

4.3.2 Polyplexes loading into type II collagen spheres reservoirs and release kinetics

The intended function for type II collagen spheres is to act as a depot for polyplexes, hence lowering their toxicity while maintaining their efficacy is achieved by releasing them in a sustained manner. Type II collagen, being hydrophilic and a natural reservoir of macromolecules, showed high loading efficiency. In fact, only 50 µg of type II collagen hollow spheres were sufficient to load almost 95% of complexed pDNA (2 µg) (Figure 4.2). Furthermore, a release kinetic study showed that type II collagen hollow spheres allowed a gradual and sustained release of polyplexes, with release kinetics having correlation coefficient ($r^2$) ranged from 0.789 to 0.999. Overall, the 100% of loaded polyplexes was released over 144 hours (Figure 4.2).

4.3.3 Cell viability in monolayer

The aim of introducing a reservoir system for the microgels functionalization is to lower the negative impact that polyplexes have on cell viability. ASCs exposed to type II collagen hollow spheres of different sizes (100 nm, 500 nm and 4.5 µm) for two days did not exhibit any level of toxicity unlike the control (ASCs alone, cultured on tissue culture plastic). When ASCs were exposed to 2 µg of complexed pDNA (or polyplexes) in absence of reservoir, their viability was significantly lower than that of the control; however, when the same amount of polyplexes was loaded into type II collagen hollow spheres, no toxic effects were observed and the cell viability was comparable to that seen in the control groups (Figure 4.3).

4.3.4 Transfection in monolayer

Different sizes of type II collagen hollow spheres were loaded with different amounts of polyplexes and their ability to transfect ASCs in monolayer was
assessed by measuring the luciferase activity. Type II collagen reservoirs allow exposure of cells to high amounts of polyplexes without compromising their viability. Consequently, the highest transfection was obtained by using the highest concentration of complexed pDNA (2 µg). 4.5µm spheres enabled significantly higher transfection in all the concentrations tested compared to smaller size of spheres and also compared to polyplexes alone (Figure 4.4).

4.3.5 Cell internalization of reservoirs
To investigate further the reason behind the higher transfection efficiency of 4.5µm reservoir systems, their interaction with ASCs was observed in TEM and SEM images. Both SEM and TEM analysis revealed that in monolayer, type II collagen reservoirs undergo cell internalization (Figure 4.6). Thus, there are likely to be two mechanisms that allow transfection of ASCs: the release of polyplexes, and the internalization of the reservoirs. Smaller reservoirs (100 nm and 500 nm) are more likely to float in media for longer while larger reservoirs (4.5 µm) have the tendency to precipitate at the bottom of the wells much earlier, and are thus more exposed to interaction with cells. In addition, smaller reservoirs are more likely to be internalized in a single cell whereas larger reservoirs are less likely to be internalized, allowing for the cargo release in cells’ proximity.

4.3.6 Cell behavior in functionalized 3D microgels
4.5 µm type II collagen spheres were used to functionalize 3D microgels systems because of their higher transfection efficiency (Figure 4.7). The highest amount of complexed pDNA used in monolayer (2µg) did not result in higher transfection in 3D (Figure 4.9). Hence, microgels were functionalized with higher amounts of reservoirs loaded with complexed pDNA (5, 10 and 15 µg of complexed pDNA in 125, 250 and 375 µg of spheres respectively). Despite the high concentrations of polyplexes in the system, no toxicity was observed after seven days of culture (Figure 4.8). On the contrary, ASCs exposed to higher amounts of complexed pDNA showed the highest metabolic activity. This was further confirmed by a
greater decrease in size of microgels, due to higher cellular activity (Figure 4.8).

4.3.7 Transfection in 3D microgels
The efficacy of microgels functionalization was assessed by measuring the activity of luciferase released from microgels functionalized with different amounts of complexed pDNA/spheres reservoir. After two days in culture, the luciferase activity in samples loaded with the highest amount of polyplexes was found to be significantly higher than that of the control and also than that of the lower concentrations of polyplexes. Moreover, the luciferase activity in the sample with the highest amount of polyplexes maintained a constant activity even after seven days of culture (Figure 4.9).
Figure 4.1: 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 spheres size and concentration was ascertained in monolayer culture of ASCs. Reservoirs of the largest size allowed the highest level of transfection in monolayer, and were therefore embedded in 3D microgels systems and optimized to obtain functional cell factories.
Figure 4.2: 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 50µg of 4.5µm 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 hours; (n=3).
Figure 4.3: 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).
Figure 4.4: Type II collagen hollow spheres enhances transfection efficiency of polypelexes. Gaussia luciferase assay to assess the ability of spheres reservoir to release active polypelexes capable of transfecting cells in monolayer culture; significantly higher level of transfection was obtained by using 4.5 µm spheres compared to 500 nm and 100 nm or polypelexes alone; (n=3, one way ANOVA, Tukey test p > 0.05).
**Figure 4.5:** ASCs transfected in monolayer expressing GFP. ASCs expressing GFP following treatment with polyplex-loaded spheres (green for GFP, blue for DAPI and red for rhodamine-phalloidin).
Figure 4.6: 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); Figures B and D show the process of internalization that collagen hollow spheres undergo when incubated with ASCs in monolayer.
Figure 4.7: Schematic representation of fabrication of 3D microgels. The forming gel solution is formed by mixing hyaluronic acid, type II collagen, ASCs and 4S-StarPEG. The gelling solution is then mixed with spheres reservoir and deposited in the form of 2 μL droplets on a hydrophobic surface and allowed to gel for 1 h at 37°C.
Figure 4.8: 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); B) Percentage of shrinkage of cell-seeded microgels loaded with different concentration of reservoirs and normalized on day two. The shrinkage is related to cellular metabolic activity and increases with the increasing concentration of reservoir; (n=3, one way ANOVA, Tukey test p > 0.05).
Figure 4.9: Transfection efficiency in 3D is dependent on polyplexes 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 a higher level of transfection over seven 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.
4.4 Discussion

Unimodal strategies for disc regeneration such as intradiscal injection of growth factors or cells have shown encouraging results. However, the limitations of these approaches such as short-term efficacy and leakage of the injected solution need to be overcome. The delivery of cells, in a controlled environment in parallel with system functionalization with non-viral gene vectors, constitutes a multimodal approach that may provide synergistic improvements of such therapies. A microgel system which mimics the NP matrix was developed to provide cells with instructive cues allowing for cellular proliferation, differentiation and synthesis of new matrix. 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, the microgel system was functionalized with a reservoir system for the delivery of non-viral gene vectors as illustrated in Figures 4.1 and 4.7. The gene delivery reservoir consisted of type II collagen hollow spheres fabricated by using the template method. Superfect™ was used as a non-viral gene vector. This is a partially degraded PAMAM 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).

4.4.1 Fabrication of microspheres reservoir with the template method

The main advantage of using the template method for the fabrication of type II collagen reservoirs is that by using monodisperse beads it is possible to obtain spheres of defined size and shape (Appendix BB, Figure BB.1). Moreover, adopting type II collagen as a building block for the fabrication of reservoir systems not only mimics the cues and composition of the ECM of nucleus pulposus but also offers good performance in terms of loading and release of polyplexes (Figure 4.2). This is because type II collagen physiologically fulfills the role of reservoir for a number of macromolecules. 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 4.2, the polyplexes were released gradually and in a sustained manner.

4.4.2 Transfection in 3D microgels
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 4.1). As shown in Figure 4.3, 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). Furthermore, this reservoir system offered a protective environment for the cells from the toxicity associated with polyplexes (Figure 4.3). 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 4.4). When the same amount of complexed plasmids was used with or without reservoir system, the samples with the reservoir system showed higher transfection (Figure 4.4) due to the fact that there were more viable cells to be transfected (Figure 4.3). The size of the spheres was a critical parameter, with higher transfection obtained with bigger spheres (4.5 µm) (Figure 4.4). 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 4.6). Because larger reservoirs tend to precipitate to the bottom of the wells sooner than the smaller ones, the size of 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. Microgels 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.
4.4.4 Lower transfection in 3D microgels requires higher doses of polyplex-loaded microspheres

Three dimensional (3D) microenvironments are also known for influencing the outcome of gene therapies \(^{28, 54-58}\), often affecting their efficacy \(^{54}\). In fact, when functionalizing 3D microgels with spheres containing 2µg 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 4.8, 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 \(^{29, 58, 59}\), and thereby neutralize the micro-acidic environments resulting from cellular metabolic activity within the microgels. 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 seven days. Microgels containing higher amounts of loaded spheres showed higher shrinkage due to increased cell metabolic activity and ability to remodel (Figure 4.8). However, increasing the amount of polyplex-loaded spheres in the microgels successfully increased the transfection efficiency as shown in Figure 4.9, resulting in significantly higher amounts of active luciferase being released in the media. Moreover, in all the samples tested, the transfection rate increased after seven days, suggesting a prolonged effect (Figure 4.9). 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 encoding 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.
4.5 Conclusions

The use of microsphere reservoirs for the delivery of polyplexes shielded the cells from 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 increasing 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 can be transfected for a prolonged period and, as a result, functional transgenic proteins were released from the 3D microgel system. Therefore, the developed platform has the potential to provide cells with instructive cues typical of ECM-derived biomacromolecules and has the capacity to be functionalized with a reservoir system for polyplexes that can 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.
4.6 References


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Chapter 5

Summary and Future Directions
5.1 Introduction
There is strong evidence that low back pain is linked to disc degeneration diseases (DDD)\(^1\)\(^-\)\(^{11}\). This condition has severe consequences on the quality of life of the affected patients\(^{12}\)\(^-\)\(^{15}\). However, current treatments can only provide temporary symptomatic relief without addressing the underlying biological problem\(^ {16}\). Disc degeneration coincides with changes in the composition of the ECM and loss of anabolic potential by the disc cells. This causes a disruption in disc homeostasis with loss of the inherent regenerative potential of the tissue. The unique physiology of the IVD and the disruption of a vast array of intercellular signalling mechanisms (many of which are still not fully understood) in DDD makes regenerative strategies difficult to implement\(^ {17},\)\(^ {18}\). A number of unimodal approaches such as cell delivery, gene therapy or growth factors delivery have had positive outcomes, however with only moderate success\(^ {17},\)\(^ {18}\). Nevertheless, overcoming this multifaceted disease requires the adoption of multimodal strategies. By synergistically combining cell and gene therapy approaches, this project aims at developing a platform that is optimal for generating a microenvironment, tailored for the NP, in which stem cells can be programmed to secrete specific proteins of interest.

5.2 Summary
5.2.1 Phase I – Development of a 3D cell delivery platform
The objective of phase I (Chapter 2) was to design a 3D cell delivery platform that is able to prime the delivered ASCs towards a phenotype suitable for the NP environment. So far, numerous studies have reported on the positive impact that ECM biomacromolecules such as type II collagen and HA have over chondrogenic differentiation\(^ {19}\)\(^-\)\(^{24}\). However, these studies were qualitative in nature, and the effects of different macromolecular concentrations of type II collagen or HA on stem cells phenotypes have been overlooked. In this phase of the study, a 3D microgel system was designed using type II collagen and HA as building blocks. Moreover, the macromolecular composition of the microgel system was tuned to obtain optimal resistance to enzymatic degradation and to promote desired morphological and genotypical changes in the phenotype of the delivered...
ASCs. The use of 4S-StarPEG as crosslinker was shown to be ideal because it increased microgel stability while avoiding any adverse effects on cell viability. Although cross-linking significantly increased microgel stability, the composition of the microgels proved to be an important factor of influence. High concentrations of collagen (5mg/mL) conferred microgels a higher resistance to enzymatic degradation and moreover was shown to prevent significant shrinking in over 21 days of in vitro culture (shrinkage was observed in microgels with lower concentrations of collagen).

Furthermore, the advantages of higher macromolecular concentration of collagen were observed also in the biological response. It was seen that the delivered ASCs acquired a rounded shape when in 5mg/mL collagen microgels while a spindle shape was maintained in microgels with lower concentrations of collagen. Many studies have reported on direct links between cell shape and the stem cells ability to undergo chondrogenic differentiation\textsuperscript{25-28}. In particular, the assembly of actin filaments also known as stress fibers, was found to severely hamper chondrogenic differentiation and ECM synthesis \textsuperscript{25, 29}. This was confirmed also by the genotypic characterization of ASCs embedded in microgels in different conditions. Cell-laden microgels were cultured in plain and differentiation media for 21 days. Although only limited differences were found in plain media, interesting results were observed in differentiation media. Here, cells embedded in microgels of high concentrations of type II collagen had the most prominent differentiation pattern. The expression of key regulators of chondrogenic differentiation such as SOX9, Integrin α10 and the receptor CD44 were upregulated, acting in synergy to increase the expression of ECM molecules such as type II collagen and aggrecan. Additionally, the quality of the matrix produced was higher, as the expression of type I collagen was significantly lower. After these results, it was decided to use microgels composed of 5mg/mL type II collagen and enriched with HA in phase III.
5.2.2 Phase II – Development of a collagen-based reservoir system for non-viral gene therapy

Gene therapy of the IVD relies almost exclusively on the use of viral vectors. As discussed in Chapter 1, the drawbacks relative to the use of viral vectors are generating interest in developing alternative methods. The aim of this phase is to fabricate a collagen-based reservoir system for the delivery of non-viral gene vectors (described in Chapter 3, Chapter 4 and Appendix AA). Commercial transfecting agent (Superfect™) was adopted to form complexes with plasmid DNA (polyplexes), which were used as a non-viral gene vector. To ensure greater control over polyplexes delivery, it is essential that the reservoir system can be fabricated with controlled size and shape and this was possible by using the template method. Commercial polystyrene beads were used as template and by harnessing the electrostatic charges of collagen and polystyrene respectively it was possible to ensure complete coating of the beads. The coated beads were then crosslinked by using 4S-StarPEG as crosslinker and the template was removed by THF washings. TEM characterization revealed that the resulting microspheres have a hollow core surrounded by a shell of collagen. SEM characterization showed that collagen microspheres had similar size and shape to that of the template adopted. Thereby, uniform microspheres of desired size can be obtained simply by selecting the appropriate template. Moreover, the interaction between cells (3T3 fibroblasts) and collagen hollow spheres was characterized in vitro. Here, when cells were exposed to high amounts of spheres (50µg), no cytotoxic effects were found. This is a very important feature considering that as a reservoir system these microspheres are expected to be in prolonged contact with the host’s cells. Loading and release of polyplexes in microsphere reservoirs were performed using Cy5-labeled polyplexes. These studies revealed a polyplex loading efficiency of approximately 90% (about 20µg of complexed pDNA/mg of microspheres) regardless of the microsphere size tested. Furthermore, polyplexes were found to be released from the microsphere reservoir in a sustained manner over a period of 144 hours.
5.2.3 Phase III – Development of a 3D cell factory

The aim of the last phase of this thesis was to design a 3D cell delivery system in which cells can be programmed to secrete desired proteins. This was achieved by decorating the microgel system developed in phase I with the reservoir system for non-viral gene vectors developed in phase II. Type II collagen hollow microspheres of different sizes (4.5 µm, 500 nm and 100 nm) were fabricated and loaded with polyplexes bearing plasmids encoding for luciferase. In vitro characterization on monolayer culture of ASCs revealed that this reservoir system offered an environment for the cells that is protective from the toxicity associated with polyplexes. 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. Surprisingly, the higher transfection efficiency was obtained when using 4.5 µm reservoirs. This determined the choice to use only this size of microspheres for the final phase of the study. 3D microgels were then functionalized with polyplex-loaded 4.5 µm reservoirs. However, because 3D environments are known for affecting the efficacy of gene transfer, higher amounts of polyplex-loaded microspheres were used (5, 10 and 15 µg of complexed pDNA in 125, 250 and 375 µg of microspheres respectively). The highest transgene expression was observed in microgels functionalized with the highest amount of microspheres without affecting cellular metabolic activity. 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 for the production of cell factories that are able to manufacture targeted therapeutic proteins for regenerative therapies of the NP.
Figure 5.1: Summary of the main outcomes from each phase of this thesis

**Phase I**
- Development of a 3D cell delivery platform
  - Embedding ASCs in microgels does not affect their viability
  - The macromolecular concentration of microgels influences ASCs phenotype
  - Elevated concentrations of type II collagen and HA increase microgels stability and prime ASCs towards a NP-like phenotype

**Phase II**
- Development of collagen-based reservoir system for non-viral gene therapy
  - Size and shape-controlled reservoirs were obtained by using the template method
  - Collagen reservoirs did not affect cell viability
  - Collagen reservoirs showed high polyplex-loading efficiency and sustained release

**Phase III**
- Development of a 3D cell factory
  - Microspheres reservoir lowers the cytotoxicity of polyplexes while maintaining their transfection efficiency
  - ASCs were successfully transfected in 3D microgels
  - ADSCs transfected in microgels were able to secrete specific proteins of interest
5.3 Limitations

5.3.1 Phase I

In this phase a 3D microgel system was fabricated by manually depositing a forming-gel solution on hydrophobic surfaces. The obvious limitations to this technique are inconsistency in microgel size, the impossibility of producing smaller microgels and the difficulty in producing high number of samples. An important feature of this microgel platform was its ability to induce expression of SOX-9 in the embedded ASCs even in plain media. However, it was only in presence of differentiation media that SOX-9 seemed to fulfil its function as a mediator of differentiation. SOX-9 in fact, is a permissive factor needed, but not sufficient for ECM-related genes expression. Its activity is dependent on its levels of phosphorylation and also on the concomitant presence of L-SOX-5 and L-SOX-6, proteins needed for the activation of key genes in chondrogenic differentiation. In this study it was not determined if SOX-9 was active in microgels cultured in plain media or if there was co-localization with L-SOX-5 and L-SOX-6. Further limitations of this study were the use of rabbit ASCs instead of human cells and the absence of in vivo characterization. This study was planned concurrently with the development of a rabbit ageing model. However, the degeneration/ageing process of the lumbar discs of the rabbit’s spine was observed to be inconsistent. Therefore, it was decided not to undertake in vivo studies in a model that could prove unreliable.

5.3.2 Phase II

The second phase of this study involved the development and characterization of the microsphere reservoir system for the delivery of non-viral gene vectors. The use of collagen as building block for the fabrication of hollow microspheres showed advantages such as the absence of cytotoxic effects and favoured cells-microspheres interactions (because of the RGD sequences present within collagen macromolecules). As observed in Chapters 3 and 4, in some cases these cells-microspheres interactions resulted in the internalization of the microsphere reservoir. A limitation of this phase is that the mechanisms behind this internalization are not clear, although it is hypothesized that microsphere size could play a role. The role
of reservoir systems is to allow for a sustained release of the cargo. Internalization of a large number of polyplex-loaded microspheres can decrease the amount of polyplexes released and thereby affect the outcome of the therapy. For this reason, this aspect should be investigated further.

5.3.3 Phase III
The parallel use of the 3D microgel system and the collagen microsphere reservoir allowed the programming of delivered cells to secrete specific proteins of interest. One limitation of this study was that only one size of polyplex-loaded microspheres reservoir was used to functionalize 3D microgels. The reason for this is that when tested in monolayer culture, the largest reservoirs allowed for highest transfection. However, it is unknown if different outcomes could have been observed in a 3D environment. Furthermore, cells were transfected with plasmids encoding for luciferase or for green fluorescent protein (GFP), therefore this was a proof of concept study. Although the transfected cells within the microgels were able to secrete transgenic proteins in significant amounts, using plasmids encoding for growth factors or other relevant paracrine factors would have increased the impact of the study. Moreover, as also stated for phase I, the main limitation of the study was the use of rabbit cells instead of human cells and also the fact that the study was conducted only *in vitro*.

5.4 Future Directions
Considering the outcomes of this project and also some of its limitations, it is possible to envisage future studies that not only complement and strengthen the results already shown but also overcome some of the most critical limitations of this thesis.

5.4.1 High-throughput methods for the fabrication of uniform microgels
As discussed in Paragraph 5.3.1, one of the limitations of this study included the method used for the fabrication of type II collagen/HA microgels. This method, in fact, was adapted from a platform developed by Chan *et al.*33-35 and has shown promising results for the tissue engineering
of the IVD. However, only a limited number of microgels can be produced in each experiment with little control over their size as the procedure is entirely manual. Recent efforts are directed towards solving these technological challenges encountered when engineering 3D hydrogels and spheroids systems. A number of simple bench-top microfabrication strategies developed recently are discussed below.

5.4.1.1 Methods for the fabrication of spheroids

Cellular spheroids are an alternative to microgels systems. Numerous studies reported the use of 3D spheroids or cell pellets for the cell therapy of tissues like cartilage or the IVD. Spheroids are basically small aggregates of cells that grow free of foreign materials and one of the latest techniques to fabricate 3D spheroids based on the use of superhydrophobic surfaces. For example, Liu et al. developed a methodology based on ice lithography to produce quasi-spherical microwells to be used for the fabrication of spheroids. In this method, water droplets can be deposited on a superhydrophobic surface by using piezoelectric printing technology. This array of droplets is then frozen at -20°C and a chilled polydimethylsiloxane (PDMS) solution is poured onto this ice mould to prepare the microwells. These microwells are then peeled-off from the superhydrophobic surface and cultured with appropriate concentrations of cells. After few days of culture, cell sedimentation occurs into the microwells with subsequent formation of 3D spheroids. An advantage of this technique is that it enables a high level of control over spheroids size and shape. This can be achieved simply by controlling the volume of the water droplets used for the fabrication of microwells and also by the level of hydrophobicity of the surfaces. However, an alternative option is that of purchasing commercially available microwells such as AggreWell™ used by Markway et al. for the fabrication of homogeneous spheroids. Although a vast range of methodologies such as the liquid overlay technique is also available for the formation of spheroids, these techniques do not allow for incorporating cells into a biomaterial system. Moreover, as shown in Chapter 2, the use of ECM-based biomaterials have shown multiple
advantages including the possibility to control cellular phenotype. For these reasons, different cell delivery strategies need to be considered.

5.4.1.2 Microfluidic devices for cell encapsulation
The development of microfluidic devices is a new emerging trend that enables the handling of fluids in micro and nanoenvironments. Microfluidics, in fact, is being used for the miniaturization of many processes including chemical synthesis, biochemical assays, and provides a promising route to encapsulate cells in microscale hydrogels. Typically, microfluidic devices exploit the formation of non-miscible phases that occur in emulsions to fabricate spherical systems. To produce an optimal and regular stream of droplets, microfluidic chips are designed with different channel geometries such as T-junction (Figure 5.2A) or cross-junction (Figure 5.2B). These chips often share a common mechanism: the phase to be dispersed is injected into a microchannel where it encounters the immiscible or continuous phase streaming from another inlet. But, the junction where the two fluids meet can have different geometries as shown in the examples in Figure 5.2. Microfluidic chips are often used to encapsulate cells within natural polymers such as alginate; however, as shown in Figure 5.2C, these chips can be designed to fabricate type II collagen/HA microgels of homogenous size and shape. In fact, the microgel components can be transported into two different inlets, one for type II collagen, HA and ASCs, and one for the crosslinker (4S-StarPEG). The two inlets will then merge in proximity of the junction to allow crosslinking of the microgel components during the emulsification process. The advantages of using this method for cell encapsulation will be greater control over size and shape of the microgels with elevated reproducibility and high-throughput.

5.4.2 In vivo models and organ culture
One of the challenges for tissue engineering of IVD is the absence of an ideal in vivo model where different regenerative strategies can be tested. This problem is caused primarily by the profound differences between the human spine and those of any other species.
Figure 5.2: Representation of different droplet generation portions generally used in microfluidic devices. Channel geometries can be designed with a T-junction (A) or cross-junction (B); C) Example of a cross-junction that can be used for the design of microfluidic devices for the high-throughput fabrication of microgels.
To overcome the unreliability 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 great control over the disc environment. Because of this advantage, organ culture systems have been used as a tool to elucidate disc biology, to study its response to different mechanical loads or to different nutritional regimes; but, most importantly, these systems are used to assess the effects of potential therapies. Hence, upon optimization of a new high-throughput method for the fabrication of microgels (such as the use of microfluidic devices), this platform can be characterized in an organo culture model. This will allow a better understanding over the reciprocal interactions between the microgels and the surrounding IVD tissue. And, a further advantage is the possibility to study the effects of mechanical loads comparable to those on human discs. However, as discussed in Chapter 1, degenerated discs have a vast array of signals that hamper disc cells’ ability to produce functional matrix and decrease the chances of healing. It is very difficult to reproduce the microenvironment typical of a degenerated disc in an organo culture model. However, degeneration has been induced in relevant in vivo models. Therefore, for reliable characterization, organo culture studies need to be complemented with in vivo studies.

5.4.3 Fabrication of pH-sensitive microspheres reservoirs

Recent advances in the field of drug delivery have fuelled the development of smart reservoir systems that are able to release their cargo under controlled conditions. This is often achieved by crosslinking the delivered macromolecules to the reservoir by using enzymatically labile linkers or short polymers sensitive to changes of pH. The advantages of such approaches are considerable as release regimes can be mediated by cellular activity or progression of disease. As IVD degeneration begins, there is a significant drop in the pH of the tissue. This is a consequence of an altered homeostasis of the IVD and often the degree of pH drop coincides with the severity of disc degeneration. Hence, considering the pathophysiology of disc degeneration, it is advantageous to have a drug
delivery system sensitive to pH. Acetal-type hyperbranched monomers are the candidates for the synthesis of pH-sensitive crosslinkers as the acetal bond is broken under acidic conditions (The synthesis of pH-sensitive crosslinker is described in Appendix Y). When such monomer is functionalized with N-Hydroxysuccinimide (NHS) groups, it can be used for crosslinking of proteins. This crosslinker can be used for the crosslinking of growth factors or peptides to the collagen microspheres reservoirs developed in this study (described in Chapter 3). The covalent link of growth factors to the microsphere system will have the advantage of prolonging the half-life of growth factors \(^{63,64}\) while, at the same time, allowing for a fast release in the acidic environments typical of degenerated discs. Moreover, with this strategy other molecules such as synthetic gene vectors can be delivered. The pH-sensitive crosslinker can be used for the crosslinking of the collagen microspheres. This allows for the fabrication of microspheres able to degrade quickly in acidic conditions. Consequently, their cargo will be released at a similar rate to the rate of degeneration of the IVD. Therefore, pH-sensitive crosslinkers can be used either for the fabrication of microspheres degradable under acidic pH (Figure 5.3A) or for the covalent linking of therapeutic proteins to collagen microspheres (Figure 5.3B).

5.4.4 Gene therapy approach to program cell’s paracrine activity

In the healthy IVD, the paracrine activity of notochordal cells stimulates NP cells to produce ECM \(^{17,65}\). Co-culture studies also revealed that NP cells rely on the presence of paracrine factors, in particular growth factors, to produce new ECM \(^{66}\). In Chapter 4 it was shown that by decorating a microgel system with polyplex-loaded microsphere reservoir it is possible to program the delivered cells to secrete target proteins. Hence, this platform allows for the engineering of cell factories with controlled paracrine activity. This strategy can be used to program delivered cells to secrete growth factors. However, because the disruption of intercellular communication in disc degeneration encompasses a wide range of growth factors \(^{67-69}\), the transfection of delivered cells with only one gene would have limited regenerative effects.
Figure 5.3: Schematic representation of the possible applications of pH-sensitive crosslinkers. A) Collagen microspheres can be crosslinked by using pH-sensitive crosslinkers, the resulting reservoir will have a high degradation rate in acidic environments, thereby the release rate of the cargo (for example polyplexes) can be tuned to be proportional to the pH of the environment; B) pH-sensitive crosslinkers can be used to link proteins or peptides directly to microspheres reservoirs. This link can be tuned to be degradable in an acidic environment thereby causing release of the cargo in a pH-dependent manner.
A number of studies showed that the use of multiple genes coding for different growth factors for the gene therapy of the IVD leads to improved outcomes. For example, Moon et al. investigated the biologic response of human IVD cells to gene therapy cocktails. In this study IVD cells were transfected with TGF-β1, IGF-1 and BMP-2 genes. Although in each case the proteoglycan synthesis improved significantly compared to the control group, the highest increase was observed when there was combined nucleic acid transfer with all three genes at the same time. Hence, the functionalisation of microgels with microsphere reservoirs loaded with a cocktail of gene vectors bearing genes encoding for different growth factors is a good strategy. The major challenge lies in using the appropriate combination of growth factors as this will weigh significantly on the outcome of the therapy. In fact, Zhang et al. demonstrated how transfection with different combinations of BMPs can lead to different outcomes. So while co-transfection with BMP-2 and BMP-7 was most effective in stimulating proteoglycan accumulation, the combination of BMP-4 and BMP-14 instead allowed greater type II collagen accumulation. Thereby the choice of the appropriate combination of genes will be determined in in vitro pilot studies and then characterized in in vivo or organ-culture models. However, some studies reported that there are some growth factors which have a particularly high influence on ECM deposition rate. These are TGF-β1 and Osteopontin-1 (OP-1) and have been shown to stimulate the production of proteoglycans in both in vivo and in vitro models. In particular, TGF-β1 was found to increase the rate of cellular proliferation and the rate of matrix synthesis by as much as five folds. On the other hand, OP-1 was found to promote ECM repair where even a single injection of OP-1 into degenerated discs was sufficient to increase their proteoglycan content significantly. Therefore, TGF-β1 and OP-1 will be among the growth factors to be initially tested.
5.4.5.1 Down-regulation of pro-inflammatory cytokines in the nucleus pulposus

The appearance of symptoms of back pain coincides with the presence of pro-inflammatory cytokines in degenerated discs. In particular, abundance of IL-1β and TNF-α was shown to have detrimental effects for IVD as these cytokines promote aggrecan degradation 77, stimulate angiogenesis (which in turn allows macrophage infiltration and perpetual inflammation) 78, and sensitize nociceptive pathways 79. For these reasons, strategies aiming at lowering the catabolic activity in the IVD focus on counteracting the effects of IL-1β and TNF-α with the introduction of soluble receptors for TNF-α 80-82 or by programming cells to overexpress IL-1β receptor antagonist 83, 84. However, there are still very few strategies that aim at the direct down-regulation of IL-1β and TNF-α. The silencing of specific proteins can be done with the cytoplasm delivery of specific microRNA (miRNA) or small interfering RNA (siRNA) 85. These are short, double stranded RNA sequences used by the cells to regulate protein expression. SiRNA, in fact, can target specific mRNA molecules and trigger their degradation, thereby lowering the expression of specific proteins 85. Synthetic non-viral gene vectors are often used for the delivery of siRNA molecules 86. Because adult NP cells have a very low proliferation rate, the nuclear delivery of genetic material is very difficult and often non-viral gene vectors have low transfection rates 87. However, the advantage of siRNA delivery is that cytoplasm delivery is sufficient to allow for the silencing of protein expression 85 and hence this approach is suitable for gene therapy of the IVD.

5.4.5.2 Collagen microspheres as a reservoir for dual delivery of SiRNA and plasmid DNA in degenerated IVD

The advantages of using collagen microsphere reservoir systems for the delivery of non-viral gene vectors were discussed in Chapter 4 and in Appendix AA. But, while those strategies are oriented towards transfection of the delivered stem cells, the programming of the host’s NP cells can be achieved in parallel with the delivery of siRNA. The number of commercially available complexing agents for siRNA is increasing and
polymeric systems such as Superfect™ could be easily loaded in collagen microspheres reservoirs\textsuperscript{88, 89}. Considering the different behaviour of stem cells and NP cells, and in particular the difference in their proliferation rate, it is obvious that such cell populations require different gene therapy strategies. Hence, type II collagen/HA microgels can be easily functionalised with reservoirs containing different cargos such as polyplexes bearing the genes coding for growth factors (aimed at transfecting stem cells) and also polymeric vectors for the delivery of siRNA (aimed at downregulating proteins in NP cells). Specific siRNA can be designed to silence the expression of cytokines in the NP such as IL-1β or TNF-α, or can as well target their downstream effectors such as IKK kinases responsible for NF-κB activation\textsuperscript{90-92}. However, with such an approach, some difficulties can be encountered. For example, while the transfection of delivered stem cells within the microgels was shown to be feasible, the delivery of siRNA to the surrounding NP tissue might be difficult. But, the use of pH-degradable microspheres reservoirs (discussed in Section 5.4.3) may allow higher siRNA release regimes. Furthermore, with such approach the release of siRNA will be proportional to the degree of NP degeneration, hence allowing for a high degree of control over the release.
Figure 5.4: Schematic depicting future directions of the project.
5.5 References


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Chapter 5

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Appendix
### Appendix

**A. List of reagents and instruments**

<table>
<thead>
<tr>
<th>Reagent/Instrument</th>
<th>Supplier/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type II Collagen</td>
<td>Affymetrix (UK)</td>
</tr>
<tr>
<td>Poly(ethylene glycol) Ether Tetrasuccinimidyl Glutarate (4S- StarPEG)</td>
<td>JenKem Technology, Allen, TX, USA</td>
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<tr>
<td>Hyaluronic Acid (200-750 kDa)</td>
<td>Contipro group, CPN (Czech Republic)</td>
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<tr>
<td>FITC-conjugated Anti-Mouse IgG Antibody</td>
<td>Sigma Aldrich®, Dublin, Ireland</td>
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<td>BrdU (B5002)</td>
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<tr>
<td>NaCl</td>
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<tr>
<td>KCl</td>
<td></td>
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<tr>
<td>Na₂HPO₄</td>
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<tr>
<td>Anti-Mouse FITC – conjugated IgG Antibody (F0257)</td>
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<tr>
<td>KH₂PO₄</td>
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<tr>
<td>Glutaraldehyde</td>
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<td>Chloroform</td>
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<td>HBSS</td>
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<td>DMEM</td>
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<tr>
<td>Penicillin/Streptomycin</td>
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<tr>
<td>Collagenase Type II C-5138</td>
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<tr>
<td>ITS</td>
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<tr>
<td>Dexamethasone</td>
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<tr>
<td>β-Glycerophosphate</td>
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<td>Ascorbic Acid</td>
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<td>Manufacturer</td>
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<td>Plasmid Labeling Kit</td>
<td>Mirus</td>
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196
B. Isolation of adipose derived stem cells (ASCs) from rabbit adipose tissue

1. Sacrifice a three to four month-old rabbit of (four to five kg) using pentobarbitone injection (140mg/kg).
2. Apply a sterile field on a bench.
3. Use 70% ethanol to disinfect the lower midline of the rabbit abdomen.
4. Use scissors to cut the skin. Locate the adipose tissue which is just below the skin, at the level of the groins.
5. Harvest the tissue and collect it in transport media.
6. Wash the tissue several times with Hank’s Balanced Salt Solution (HBSS) until all traces of blood are being eliminated.
7. Incubate the tissue with collagenase type I at 0.025% for 1h at 37°C under agitation.
8. Add a volume of complete media to inactivate the collagenase.
9. Allow the digested tissue to settle under the hood, after several minutes formation of 2 phases will occur:
   - Top phase with fat and adipocytes
   - Bottom phase with the stromal fraction
10. Centrifuge for 5 minutes at 1,200 rpm (300g).
11. Discard the top phase.
12. Resuspend the last phase and filter on a cell strainer 70µm.
13. Centrifuge for 8 minutes at 1,200 rpm.
14. Wash the cells with complete media.
15. Dilute the cells 32 times and count them.
16. Seed cells at 10^6 cells/mL (the excess of cells is due to the fact that the majority of them will not adhere).
17. After 24h, change the media to eliminate vascular cells and adipocytes (they require at least 72h hours to adhere).
18. Change the media every 2 days and maintain the cells sub-confluent to avoid spontaneous differentiation.
Types of media

Transport media
- HBSS/DMEM (1:1)
- 10% FBS
- 1% Penicillin/Streptomycin (P/S)

Complete media
- DMEM
- 10% FBS
- 1% P/S

C. Assessment of adipogenic, osteogenic and chondrogenic potential of the extracted ASCs

C.1 Adipogenic differentiation
1. Seed rabbit ASCs at a density of $3 \times 10^4$ cells/cm$^2$ in a six-well plate. As the surface of a single well is 9.6 cm$^2$ 288,000 cells per well should be seeded.
2. Incubate in complete medium (DMEM with 10% FBS and 1% P/S) for 24h.
3. After 24h, wash in HBSS and add adipogenic media (composition described below).
4. Change the media twice a week for 14 days.

Adipogenic differentiation media
- DMEM
- 10% FBS
- 1% P/S
- Insulin (ITS) 10µg/mL
- 0.5M isobutylmethylxanthine (IBMX)
- 1 µM dexamethasone
- 200 µM Indomethacine

C.2 Osteogenic differentiation
1. Seed ASCs at a density of $10^4$ cells/cm2 in a six-well plate.
2. Incubate in complete medium (DMEM with 10% FBS and 1% P/S) for 24h.
3. After 24h, wash in HBSS and add osteogenic media (composition described below).
4. Change the media twice a week for 28 days.

**Osteogenic differentiation media**
- DMEM
- 10%FBS
- 1%P/S
- 10 mM β-Glycerophosphate
- 0.05 mM Ascorbic acid
- 1 µM dexamethasone

**C.3 Chondrogenic differentiation**
1. Culture ASCs in complete medium (DMEM with 10%FBS and 1%P/S).
2. At passage 3, trypsinise and wash cells in DMEM.
3. Centrifuge for 5 minutes at 1,200 rpm and resuspend the cells in differentiation media (composition described below).
4. Count cells, add 10⁶ cells in a 15 mL tube and create a pellet by centrifuging for 5 minutes at 1,200 rpm.
5. Loosen the lid of the tube to allow air flow and incubate the pellets at 37°C for 21 days.
6. Change the media every 3 days.

**Chondrogenic media**
- DMEM
- 10 ng/mL TGF-β1
- 6.25 μg/mL ITS
- 50 nM Ascorbic acid

**D. Preparation of hydrophobic surfaces**
1. Wash and disinfect glass slides.
2. Under the hood wrap each slide with Teflon tape and place each in a petri dish.
3. Cover each slide with ethanol 95% and let it dry under the hood.
4. Close the petri dishes containing the slides, seal them by using parafilm and store them.
5. Before using the slides, incubate them under UV for at least 30 minutes.

E. Coating tips with Polydimethylsiloxane (PDMS)
When dispensing forming gel solution in form of droplets the collagen has the tendency to stick on the side of the tips thus requiring 1 tip per droplet. By coating the tips with hydrophobic PDMS, it is possible to use on average 1 tip per 200 droplets (enough to cover one slide with microgels).

1. Dissolve the PDMS in hexane and stir for 1 hour (hexane is very volatile, thus possibly use a glass vial and close the cap).
2. By using a brush, apply the PDMS solution twice on each tip.
3. To crosslink the coating and also to sterilize the tips at the same time, autoclave the tips (to crosslink the PDMS, a temperature higher than 65°C is required).
4. Dry the autoclaved tips for at least one day and store.

F. Type II collagen/Hyaluronan microgels fabrication
The preparation of collagen microgels consists mainly in 2 phases: the combination of all the reagents to obtain a forming-gel solution, and the deposition of this solution on hydrophobic surfaces in form of droplets.

F.1 Preparation of reagents
1. Reconstitute type II collagen in 0.05M acetic acid at a final concentration of 7mg/mL.
2. Store overnight at 4°C with gentle rocking (vigorous stirring may cause the collagen to denaturate).
3. The next day, measure the concentration of the collagen solution (this step is necessary as the solution swells overnight, altering the final concentration of collagen).
Appendix

4. Dissolve hyaluronan (HA) in NaCl 0.4M at a final concentration of 10mg/mL (HA will take several hours to dissolve completely).
5. Prepare and filter PBS10X.
6. Prepare and filter glucose 4g/L in PBS1X.

Table F.1 Preparation of 200 µL of type II collagen hydrogel

<table>
<thead>
<tr>
<th>HA in NaCl 0.4M</th>
<th>PBS 10X</th>
<th>NaOH 1M</th>
<th>Collagen II 7mg/mL</th>
<th>Cells 20 million/mL</th>
<th>StarPEG in PBS/Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>2mg/mL</td>
<td>4.44</td>
<td>19</td>
<td>1.1</td>
<td>60</td>
<td>100 + 1mg</td>
</tr>
<tr>
<td>4mg/mL</td>
<td>8.89</td>
<td>19</td>
<td>2</td>
<td>114.3</td>
<td>37.81 + 1mg</td>
</tr>
<tr>
<td>5mg/mL</td>
<td>11.11</td>
<td>19</td>
<td>2.5</td>
<td>142.88</td>
<td>20 + 1mg</td>
</tr>
</tbody>
</table>

1. Dispose of all the solutions in the hood and make sure to have sufficient hydrophobic surfaces and modified tips (one of each per set of microgels).
2. Mix HA with PBS 10X and NaOH 1M and vortex the mix for 3 seconds.
3. Add type II collagen to the mix and vortex for 3 seconds.
4. Add cells to the mix and vortex for 3 seconds.
5. Resuspend starPEG in PBS/glucose, add it to the mix and vortex for 3 seconds.
6. By using a multipipette, aspire all the forming-gel solution and dispense 2 µL droplets on the hydrophobic surface (if the droplets are less than 2 µL they may dry before gelation occurs).
7. Close the petri dish and incubate for 1 hour at 37°C.
**Appendix**

**Figure F.1:** Type II collagen/HA microgels deposited on a hydrophobic surface.

**G. Amine quantification by TNBS assay**

1. Prepare a standard curve with glycine in sodium bicarbonate pH 8.5 (0.1M) (H2N-CH2-COOH) (100nmol, 50nmol, 25nmol, 10nmol, 5nmol and 0nmol).
2. Add 250μL of 0.01% TNBSA in 0.5mL of each sample. Mix well. The dilution of the stock solution concentrated at 5% in the sodium bicarbonate buffer.
3. Incubate at 37°C for two hours to allow the reaction to occur.
4. Add 250μl of 10% SDS plus 125μl of 1M HCl.
5. Measure the absorbance at 335nm.

To break the microgels and release the TNBS reagent, the samples should be incubated at 120°C for 15 minutes. The absorbance of each sample can be read at 335 nm by using a Varioskan Flask plate reader (Thermo Scientific).

**H. Microgel stability assay**

The stability of microgels was quantified colorimetrically by Coomassie Brilliant Blue (CBB) assay. CBB is an anionic dye with high affinity for proteins. Break-down of collagen by collagenase will result in release of the dye, thus more stable microgels will release the dye in a sustained manner.

1. Incubate microgels in 0.1% CBB (w/v) for 6 hours with gentle agitation.
2. De-stain for 2 hours in de-staining buffer.
3. Transfer the microgels to multi-well plates and incubate with 0.5-1mg/mL collagenase in 50mM CaCl\textsubscript{2} prepared in 0.1mM Tris HCL.

4. At chosen intervals, collect the supernatants and measure them spectrophotometrically at 595nm.

**I. Bromodeoxyuridine (BrdU) proliferation analysis**

Time and concentrations (primary and secondary antibodies) for the BrdU treatment must be optimised before performing a complete experiment. A final concentration of 10 – 25 µM BrdU can be added to the cells (25 µM BrdU is adequate for stromal cell lines).

**Solutions**

1. PBT – Blocking buffer made fresh on the day – PBS + 0.5% BSA + 0.1% Tween 20. (in 50ml – 50ul of Tween and 250mg of BSA).

**I.1 Pulsing, harvesting and fixing cells from microgels**

1. Remove media from the wells containing microgels.
2. Add 2µL of BrdU solution into 2 mL fresh media (final concentration of 25 µM) and add to the well containing microgels.
3. Pulse the cells with BrdU for the optimised amount of time (usually 2 hours).
4. 15 minutes before the end of the 2 hours, add collagenase to the wells (0.5mg/mL) and monitor the digestion of the microgels.
5. Once the microgels are completely digested, transfer the cells from all the wells into a falcon tube.
6. Incubate the tube at 37°C for 5 minutes.
7. Centrifuge the tube at 1500rpm for 10 minutes.
8. Remove the supernatant and resuspend the pellet in a suitable volume of PBS (according to the size of the pellet).
9. Filter the cell suspension through a 70 µm filter.
10. Collect the cell filtrate and centrifuge at 1200 rpm for 15 minutes.
11. Discard the supernatant and resuspend the cell pellet in 300 µL PBS.
12. Slowly, transfer the cell suspension to a sterile 15 mL falcon tube containing 700 µL of ice-cold 95% ethanol while being vortexed.
13. Store the cells for up to 1 week at 4°C and for longer periods at -20°C.

I.2 BrdU-PI staining
NB: Prepare PBT-blocking buffer fresh on the day (PBS + 0.5% BSA + 0.1% Tween 20).
1. Remove the fixed cells from storage, add 3 mL of PBS to the cells and vortex to mix.
2. Centrifuge the cell suspension at 1,200 – 2,000 rpm for 10 minutes at RT/4°C.
3. Aspirate and discard the supernatant.
4. Resuspend the cells in 1 mL of PBS.
5. Add 1 mL of 4N HCl and mix by brief vortex.
6. Incubate the cells for 15 minutes at RT.
7. Immediately, centrifuge the cell suspension at 1,200 – 2,000 rpm for 10 minutes at RT/4°C.
8. Aspirate and discard the supernatant.
9. Resuspend the cells in 1 mL of PBS.
10. Centrifuge the cell suspension at 1,200 – 2,000 rpm for 10 minutes at RT/4°C.
11. Aspirate and discard the supernatant.
12. Resuspend cells in 1 mL of PBT.
13. Centrifuge the cell suspension at 1,200 – 2,000 rpm for 10 minutes at RT/4°C.
14. Aspirate and discard the supernatant.
15. Resuspend the cells in 200 µL of PBT containing the optimised dilution of the anti-BrdU antibody (1:20).
16. Incubate the cells at RT for 30 minutes in the dark.
17. Add 1 mL of PBS to resuspend the cells.
18. Centrifuge the cell suspension at 1,200 – 2,000 rpm for 10 minutes at RT/ 4°C.
19. Aspirate and discard the supernatant.
20. Resuspend the cells in 200 µL of PBT containing the optimised dilution of the anti-mouse FITC conjugated antibody (1:20).
21. Incubate the cells at RT for 30 minutes in the dark.
22. Centrifuge the cell suspension at 1,200 – 2,000 rpm for 10 minutes at RT/ 4°C.
23. Aspirate and discard the supernatant.
24. Resuspend the cells in 500 µL of PI/RNase staining buffer.
25. Incubate the cells at RT for 30 minutes in the dark.
26. Analyse the sample by flow cytometry.

**J. Samples preparation for electronic microscopy (TEM and SEM)**

**J.1 Reagents**

1. Sodium cacodylate buffer 0.4M (stock solution) – 21.4g sodium cacodylate dissolved in 250 mL H₂O.

2. Sodium cacodylate buffer 0.2M (working solution) – 50 mL of 0.4M sodium cacodylate stock + 45 mL H₂O, adjust pH to 7 and bring up to 100 mL of final volume by using H₂O.

3. Glutaraldehyde 3% in 0.2M sodium cacodylate buffer.

4. 2% Osmium Tetroxide (OsO₄, diluted in sodium cacodylate buffer).

5. Various concentrations of Ethanol.

6. HDMS.

**J.2 Samples fixation**

1. Add samples in 3% glutaraldehyde in 0.2M sodium cacodylate buffer for 1-2 hours.

2. Gently, spin down the samples (1000 rpm for 5 minutes) to obtain a pellet.

3. Wash in 0.1M cacodylate buffer (5 minutes x 2 washes).

4. Resuspend cells in 1% OsO₄ for 1-2 hours in a fumehood.
5. After fixing in OsO₄, spin down the sample gently to obtain a pellet.
6. Remove the supernatant and store safely for disposal (NB: OsO₄ is hazardous, read the safety assessment before using it).
7. Wash the sample in 0.1M sodium cacodylate buffer (5 minutes x 2 washes).

**J.3 Samples dehydration for SEM**
Dehydrate fixed samples in the following sequence for 15 min each:
- 30% Ethanol (EtOH)
- 50% EtOH
- 80% EtOH
- 99% EtOH
- 33% HDMS in EtOH
- 50% HDMS in EtOH
- 66% HDMS in EtOH
- 100% HMDS (let dry overnight)

**J.4 Embedding samples in epoxy resin for TEM**
Dehydrate fixed samples in the following sequence for 15 min each:
- 30% Ethanol (EtOH)
- 50% EtOH
- 80% EtOH
- 99% EtOH

**J.5 Resin preparation**
Mix in the following order the components of the resin:
- LV resin 48g
- VH1 Hardener 10g
- VH2 Hardener 42g
- Accelerator 2.5mL

1. Aspirate all the ethanol and replace it with propylene oxide (2 washes x 20 minutes, work in a fumehood).
2. Aspirate the propylene oxide and place the samples in a mixture of 50% propylene oxide and 50% resin.
3. Incubate for 4 hours at room temperature.
4. Replace the resin with a mix of 75:25 resin and propylene oxide.
5. Incubate overnight.
6. Replace the resin with pure resin and incubate for 4 hours.
7. Place the samples in a mold and replace the resin with freshly prepared resin and incubate for 30 minutes.
8. Crosslink the resin for at least 2 days at 60°C.

K. Isolation of Total RNA

Despite the small size of collagen microgels, the extraction of RNA can be difficult and result in very low yields. Therefore, to increase the yield, it is necessary to combine mechanical disruption with the Trizol protocol and the Qiagen RNeasy® micro kit.

1. Clean the hood with RNAseaway.
2. Heat a water bath to 55°C.
3. Add 1mL of Trizol to the samples and transfer in tissue raptor tubes.
4. Add 2 steel beads per tube and run the tissue raptor for 10 minutes at maximum speed.
5. Aspire the samples by using a syringe (according to the size of the microgels different needles may be needed to avoid clumping the syringe, usually 23G).
6. Add 10 µL of pre-heated Proteinase K (37°C) and heat the sample at 55°C for 10 minutes.
7. Add 0.2 mL of Choloroform per mL of Trizol.
8. Shake vigorously for 15s by inversion.
9. Incubate for 15 min at RT.
10. Centrifuge at 12000g for 15 min at 4°C.
11. There will be three phases, in the upper phase (translucent) there is the RNA, the DNA and proteins are in the other phases.
12. Recover the upper phase and resuspend with an equal volume of 70% ethanol (generally 650µL) and mix.
13. Apply the sample in a column (RNeasy® micro spin column (Quiagen), ideal volume is 700 µL; note- exceeding this volume may affect the yield) and centrifuge for 15s at 8000g. Discard the flow-through. Repeat for remaining sample.
14. Add 350µL of RW1 buffer and centrifuge for 15s at 8000g. Discard the flow-through.
15. Add 10µL of DNase stock solution to 70µL of Buffer RDD and add the DNase (fragile, mix gently) incubation mix gently directly onto the Rneasy column. Incubate at RT for 15 min.
16. Add 350µL of RW1 buffer and centrifuge for 15s at 8000g. Discard the flow-through.
17. Add 500µL of RPE Buffer, centrifuge for 15s at 8000g. Discard the flow-through.
18. Add 500µL of RPE Buffer, wait 1 minute and centrifuge for 2 min at 8000g. Discard the flow-through.
19. Transfer the column in a normal 1,5mL tube and centrifuge at full speed for 5 min to dry the membrane. Discard the flow-through.
20. Place the column in a new tube and add 20µL of RNAse-free water directly to the center of the spin column membrane and incubate for 1 minute. Centrifuge for 1 min at full speed to elute the RNA (should obtain at least 12 µL of elute).

L. Quantification of mRNA
A reliable method to quantify the concentration of messenger RNA (mRNA) is by using the Qubit™ RNA assay kit.

1. Prepare the working solution by diluting the Qubit™ reagent 1:200 in Qubit™ RNA buffer (each sample will have a final volume of 200µL of which at least 190 µL will be of working solution and minimum 1µL of sample).
2. Prepare 2 standards (positive and negative control) by adding 10µL of each in 190µL working solution.
3. Mix (for all the solutions) by vortexing 2-3 seconds.
4. Incubate the tubes at room temperature for 2 minutes.
5. Calibrate the Qubit™ fluorometer by using the standards.
6. Run the samples.

M. Synthesis of cDNA
This step consists in a reverse transcription of RNA to cDNA. The cDNA will be used for real time PCR.

M.1 Preparation before reverse transcription
1. Clean the work surface and spray RNAse away.
2. Wide dry all the pipettes and gloves with RNAse away.
3. Use sterile nuclease free tubes which are pre-chilled on ice.
4. Use 1µg of RNA template and 0.5µg of random primers.
5. Denature the mRNA and primers by incubation at 70°C for five minutes.
6. Quick-chill on ice for five minutes.

M.2 Preparation of reaction mix for reverse transcription
1. Prepare the mix and keep in ice until incubation.
2. Follow Table M.1 for volumes.
3. Add the reverse transcriptase enzyme at the end.
4. After combining all components, vortex gently to mix.
5. After mixing, add the samples and mix again.
6. Spin down at max speed for 5 seconds.
7. Run the program described in Table M.2.
8. When the reaction is completed, store the cDNA at -20°C.
Table M.1  Reagents and their volumes required for a single reverse transcriptase reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction (µL)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease Free Water</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>ImProm-II™ 5X Reaction Buffer</td>
<td>4</td>
<td>1X</td>
</tr>
<tr>
<td>MgCl₂ 25 mM</td>
<td>2.4</td>
<td>3 mM</td>
</tr>
<tr>
<td>dNTP mix 10mM</td>
<td>1</td>
<td>0.5mM</td>
</tr>
<tr>
<td>RNAsin Ribonuclease Inhibitor</td>
<td>1</td>
<td>1U/µL</td>
</tr>
<tr>
<td>ImProm-II™ Reverse Transcriptase</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Final Reaction Volume</strong></td>
<td><strong>15</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table M.2  Reverse transcription program

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annealing</td>
<td>25</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Extension</td>
<td>42</td>
<td>60 minutes</td>
</tr>
<tr>
<td>Heat Inactivation Reverse Transcriptase</td>
<td>70</td>
<td>15 minutes</td>
</tr>
<tr>
<td>Cool Down</td>
<td>4</td>
<td>Forever</td>
</tr>
</tbody>
</table>
N. Polymerase Chain Reaction (PCR)

Dilute cDNA samples so to obtain a final concentration of 20ng per well

1. The cDNA volume should not exceed 10% of the final volume.
2. Prepare the master mix by mixing the components listed in Table N.1.
3. For each sample consider preparing a negative control by replacing cDNA with nuclease free water.
4. Mix well the master mix by pipetting and add to each well to obtain final volume of 25µL.
5. Add a plastic cover on the PCR plate.
6. Centrifuge for 1 minute at 1400 rpm.
7. Place the plate in the PCR machine and run.
8. A general program is listed in Table N.2.

Table N.1 Polymerase chain reaction components

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Reaction (µL)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Quantifast® SYBR Green PCR Master Mix</td>
<td>12.5</td>
<td>1.5mM</td>
</tr>
<tr>
<td>Forward Primer, pmol</td>
<td>0.25</td>
<td>1µM</td>
</tr>
<tr>
<td>Reverse Primer, pmol</td>
<td>0.25</td>
<td>1µM</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1.49</td>
<td>≤ 100 ng/reaction</td>
</tr>
<tr>
<td>Nuclease Water (bring volume to 25 µL)</td>
<td>10.51</td>
<td>1U/µL</td>
</tr>
<tr>
<td>Final Volume</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>
### Table N.2 General PCR program

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Ramp Rate</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Step</td>
<td>50</td>
<td>2 minutes</td>
<td>Maximal/Fast Mode</td>
<td>1</td>
</tr>
<tr>
<td>PCR Initial Activation Step</td>
<td>95</td>
<td>5 minutes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two Steps</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>10 seconds</td>
<td>Maximal/Fast Mode</td>
<td>35</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60</td>
<td>30 seconds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Step</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>15 seconds</td>
<td>Maximal/Fast Mode</td>
<td>1</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60</td>
<td>20 seconds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Denaturation</td>
<td>95</td>
<td>15 seconds</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**O. Electrophoresis in 1% agarose gel**

1. Mix 1g of agarose in 100mL of Tris-Acetate-EDTA buffer (TAE) 1X.
2. Place the beaker in a microwave and boil the mixture until complete dissolution of the agarose.
3. Add SybrSafe (Dilution 1/10,000).
4. Pour gel in the mold and wait until full polymerization.
5. Place a comb to create wells within the gel.
6. Remove the comb and load the samples.
7. Run at 90V for 1h and read under UV using the transilluminator.
P. Sulfonation of polystyrene beads

Sulfonation allows an increase in the negative charge of polystyrene beads, thus favoring their interaction with positively charged macromolecules.

1. Incubate 1g of polystyrene spheres in 35 mL of sulfuric acid for 16 hours at 16°C (use an oil bath).
2. Centrifuge at max speed for 10 minutes and wash with 95% ethanol at least twice.
3. Resuspend and store in water or PBS (note water and PBS have different densities, thus when using small size of beads, the yield is lower after centrifugation when using PBS).

Q. Fabrication of collagen hollow spheres

Although type I and type II collagens have slightly different physical properties (e.g., Hydrophilicity), they possess a similar charge and thus the same protocol can be used to fabricate hollow spheres.

1. Centrifuge the sulfonated beads (sulfonation protocol in appendices P) and resuspend in 0.5M acetic acid.
2. Sonicate sulfonated beads to break aggregates and to obtain a uniform solution.
3. Stir the beads solution gently and dropwise add collagen solution at a weight ratio of 4:1 (beads to collagen). Note: to obtain the highest yield, it is best to use a final concentration of collagen of 2mg/mL.
4. Stir the mixture for 4 h at room temperature.
5. Centrifuge at 4500 rpm for 10 minutes and discard the supernatant (to eliminate the excess of collagen).
6. Resuspend the pellet in 0.5M acetic acid and stir for 5 minutes.
7. Centrifuge at 4500 rpm for 10 minutes and discard the supernatant.
8. Resuspend in PBS 1X and by using NaOH adjust the pH to 7.4.
9. Add 4S-StarPEG to the solution at a 1:2 w/w (collagen/4S-StarPEG).
10. Stir gently for 1 h at 37°C.
11. Centrifuge at 4500 rpm for 10 minutes and discard the supernatant.
12. To dissolve the polystyrene template, resuspend in H₂O and THF at 1:1 v/v.
13. Stir for 1 h at room temperature.
15. Resuspend in 70% ethanol and stir at high speed to break eventual aggregates.
16. Store at 4°C.

**R. Polyplex formation**

1. Mix Superfect™ and the plasmid of interest at a 9:1 w/w (superfect/plasmid) ratio and use serum free DMEM media to obtain a final concentration of plasmid of 15µg/mL.
2. Incubate at room temperature at least 10 minutes.

**S. Loading of collagen hollow spheres with polyplexes**

1. Resuspend collagen hollow spheres in ethanol 95%.
2. Stir gently for 1 h at room temperature.
3. Centrifuge at 4500 rpm for 10 minutes.
4. Use the polyplex solution to resuspend the spheres (note that the final ratio spheres to plasmid has to be 50:1 w/w for type I collagen spheres and 25:1 w/w for type II collagen spheres).
5. Vortex vigorously and incubate at 37°C for 4 h.
6. Centrifuge at 4500 rpm for 5 minutes and resuspend in complete DMEM media.

**T. Plasmid labeling**

1. Warm the plasmid labeling kit to room temperature.
2. Prepare the labeling reaction (total volume 50 µL):
   - 35 µL Sterile ddH₂O
   - 5 µL 10X labeling buffer A
   - 5 µL of 1 µg/µL DNA sample
   - 5 µL Label IT reagent
3. Incubate at 37°C for 1 h.
4. Purify by spinning through microspin column.
   - Use 50 µL per column
   - Spin down at 3000 rpm
III. Vortex to resuspend the resin
IV. Loosen slightly the cap and pull out the bottom plug
V. Place the column into a 1.5 mL centrifuge tube
VI. Spin for 1 minute at 3000 rpm
VII. Discard the buffer and place the column in a new tube
VIII. Add the sample on the top of the resin
IX. Spin at 3000 rpm for 2 minutes

5. The sample concentration is approximately the same as the starting solution.
6. Store protected from light at -20°C.

U. Protocols for transfection in-vitro

U.1 Transfection in monolayer
1. 24 h before transfection, seed cells in a 96 well plate at a density of approximately 10000 cells/well.
2. The following day prepare polyplexes and load the hollow spheres as described in appendices R and S.
3. Add the loaded hollow spheres (in complete media) in order to have between 0.5 and 2 µg of plasmid per well.
4. Incubate for 4 h (in the incubator at 37°C).
5. Remove the solution.
6. Wash the cells with HBSS.
7. Add fresh complete media.

U.2 Transfection in 3D collagen microgels
1. Prepare polyplexes and load the hollow spheres as described in appendices R and S.
2. Because the polyplexes are loaded in the hollow spheres, it is possible to change the concentration of polyplexes in solution simply by spinning down and resuspending in the appropriate volume.
3. Ideally 10µL of the loaded hollow spheres solution should contain 15 µg of plasmid, therefore the final concentration of this solution should be 1.5 µg/µL.
4. Prepare microgels as described in appendices F but, when mixing the components, add one extra step as described in Table U.1.

Table U.1 Preparation of 200 µL of type II collagen hydrogel functionalized with polyplex-loaded microspheres

<table>
<thead>
<tr>
<th></th>
<th>5mg/mL microgels</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA in NaCl</td>
<td>11.11 µL</td>
</tr>
<tr>
<td>PBS 10X</td>
<td>19 µL</td>
</tr>
<tr>
<td>NaOH 1M</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Collagen II 7mg/mL</td>
<td>142.88 µL</td>
</tr>
<tr>
<td>Polyplex-loaded microspheres</td>
<td>10 µL</td>
</tr>
<tr>
<td>Cells 20 million/mL</td>
<td>10 µL</td>
</tr>
<tr>
<td>4S-StarPEG in PBS/Glucose</td>
<td>20 µL + 1 mg</td>
</tr>
</tbody>
</table>

U.3 Quantification of transfection via G-luciferase assay

1. Prepare 1X G-luc assay solution by using dilution buffer 1 to 100.
2. Add 100 µL dH2O to black opaque 96 well plate.
3. Add 50 µL of sample into each well.
4. Add 50 µL of the G-luc solution to each well.
5. Read within 5-10 seconds.

V. AlamarBlue™ assay

1. Wash the cells to be assessed 3 times with HBSS.
2. Make up the solution of alamarBlue™ in HBSS (ratio 1:9 v/v respectively).
3. Add 100 µL of the alamarBlue™ solution per well.
4. Add also controls which are 4 wells with HBSS and 4 wells with the alamarBlue™ solution.
5. Incubate for 2 hours at 37°C.
6. Transfer 100 µL from each sample into a clear 96 well plate.
7. Measure the absorbance at 550 nm and 595 nm (0.5 seconds per well).
8. Calculate a viability value according to “simplified method of calculating percent reduction” available in the alamarBlue™ handbook.
9. Subtract the absorbance values of HBSS only from the absorbance values of the alamarBlue™ in HBSS (ratio 1:9). AOLW = absorbance of oxidized form at lower wavelength, and AOHW = absorbance of oxidized form at higher wavelength.
   a. Calculate the correlation factor: RO.
   b. RO = AOLW/AOHW.
   c. To calculate the percentage of reduced alamarBlue™:
      \[ ARLW = ALW - (AHW \times RO) \times 100 \]

W. Emulsion method for the fabrication of collagen microspheres
1. Dilute a type I collagen solution in acetic acid 0.5M in to have a final concentration of 5mg/mL.
2. Prior to use, neutralize 2 mL of type I collagen by using NaOH 1M (final pH 7).
3. Add 1 mL of PBS 1X to the neutralized collagen solution.
4. Add 0.2 mL of tween 20 to the collagen solution (the surfactant is necessary to stabilize the emulsion).
5. Prepare the oil phase by mixing paraffin oil and olive oil at a ratio of 23:1 respectively and heat at 37°C.
6. Add the pre-warmed oil solution to the neutralized collagen solution at a ratio of 4:1 v/v oil:collagen.
7. Vortex vigorously for 1 minute; the emulsion should appear homogeneous and have a milky color.
8. Stir the emulsion for at least 2 hours at 37°C.
9. Collect the collagen microspheres by centrifugation, 5 minutes at 4500 rpm.
10. Wash the collagen microspheres twice in ethanol 70% and collect by centrifugation as described above.

11. Store at 4°C.

X. Microphase separation method for the fabrication of collagen microspheres

1. A solution of 2mg/mL of type I collagen in 0.25 M acetic acid was bathed in liquid nitrogen for 30 seconds.

2. Immediately after this, the solution was incubated at -20°C for 3 hours to allow the annealing phase to start.

3. The annealing phase was completed during the lyophilization step; briefly the freeze-dry program was set to have the temperature of the plate constant to -20°C for 12 hours; following, a gradual increase in temperature to 0°C over a total period of 18 hours.

Y. Synthesis of pH-sensitive crosslinker

Acetal-type hyperbranched monomers with N-Hydroxysuccinimide (NHS) functional groups were synthesized by deactivation-enhanced atom transfer radical polymerisation (DE-ATRP) reaction. Because the acetal bonds of the monomer are broken under acidic conditions, this monomer is suitable for the crosslinking of proteins molecules to protein-based drug delivery systems such as collagen microspheres (described in Chapter 3). Therefore, such system would be able to release protein molecules at slightly acidic conditions. However, the characterization of this pH-sensitive crosslinker is limited to the synthesis steps.

Y.1 Crystallization of toluene sulfonylic acid (catalyst)

It is crucial for the synthesis of the pH-sensitive crosslinker to work in the absence of water. The catalyst contains a significant amount of water and therefore a crystallization step is needed to purify it. Because toluene sulfonylic acid is a salt, it forms crystals of different colors according to the presence of water:

White – no water
Pink – water
The whole process consists in boiling the catalyst and distilling it (collect part of the vapours).

1. Pour the catalyst in a flask and immerse it in a hot oil bath (120°C) and stir.
2. Add toluene as a solvent (2/3 of the total volume).
3. Recondense the vapours by using a column.
4. Collect the vapours by using a beaker in ice.
5. Incubate for 10 minutes.
6. There will be formation of 2 phases: white (crystallized catalyst) and pink (melted catalyst).
7. Collect the white phase by filtration.

Y.2 Reaction setup

1. 24 hours prior the reaction, incubate each reagent (anisoaldehyde, hydroxyethylacrylate and toluene solufonic acid) in molecular sieve.
2. Mix 3.3g of anisoaldehyde, 19.7 g of hydroxyethylacrylate and 0.8 g of purified catalyst in a beaker filled with molecular sieve (1/3 of the volume).
3. Add the beaker in ice and incubate for 5 minutes under argon.
4. Seal the beaker and stir gently for 15 hours in ice (it is important to maintain the reaction cold).
5. Before stopping the reaction, the presence of a product needs to be confirmed by TLC (aluminium).
6. Place a drop of the reaction mix on a TLC and use 1:1 ethyl acetate:hexane as a solvent.
7. If there is a product, there will be formation of 3 bands.
8. Add 3.6g of TEA to stop the reaction.

Y.3 Extraction of the organic phase

1. Add NaCO₃ 1M and mix by shaking.
2. When mixing organic and water phases, there will be a formation of gas, so after shaking for a number of times, open the valve to release the gas.
3. Let the solution rest for 5 minutes, until 2 phases will form.
4. Collect the organic phase (at the bottom) and discard the water phase.
5. Repeat the above procedure 3 times.
6. Filter and collect the filtrate; if the filter is obstructed, use dichloromethane to wash it.

![Figure Y.1: Reaction setup. Anisoaldehyde, hydroxyethylacrylate and toluene sulfonylic acid are mixed after being dehydrated for at least 24 hours. The reaction is carried out for a minimum of 15 hours in ice.](image)

**Figure Y.1: Reaction setup.** Anisoaldehyde, hydroxyethylacrylate and toluene sulfonylic acid are mixed after being dehydrated for at least 24 hours. The reaction is carried out for a minimum of 15 hours in ice.

**Y.4 Purification**

1. This step removes all the residues of dichloromethane from the mix.
2. Transfer the solution in a spheric beaker and, by using a rotavap, remove the dichloromethane at room temperature.
3. When the volume of the solution remains constant, it will be possible to proceed with separation of the monomers by using a column.
4. Set up the column as shown in **Figure Y.2**.
5. Wash the column first with hexane : ethyl acetate 3:1 respectively.
6. Add the solution with the product on the top of the column.
7. By using TLC, monitor the presence of the product.
8. Confirm the presence of product by doing NMR.

**Figure Y.2:** Schematic representation of the column setup used for the purification.

**Figure Y.3:** NMR spectra of the reaction reveal the presence of product.
Z. Supplementary information for Chapter 2

Figure Z.1: A) 5 mg/mL microgels after deposition on a commercial Teflon® tape; B) SEM image of 5 mg/mL microgel seeded with ASCs and cultured for 7 days.
Figure Z.2: Cell volume of ASCs embedded in microgels with different collagen concentrations; live cells were stained with calcein and z-stacks analyzed by using image analysis software; cell volume was influenced by collagen concentration and time but no significant differences were observed in cell volume of cells at the surface or the center of the microgels ($n = 4$, one way ANOVA, Tukey test $p > 0.05$).
Figure Z.3: Length of ASCs embedded in microgels with different collagen concentration; cell longest axis was influenced by collagen concentration and time but no significant differences were observed at the surface or the center of the microgels ($n = 4$, one way ANOVA, Tukey test $p > 0.05$).
Figure Z.4: Shape factor was influenced by collagen concentration and time but no significant differences were observed in cell morphology at the surface or the center of the microgels ($n = 4$, one way ANOVA, Tukey test $p > 0.05$).
Figure Z.5: A, B, C) Brightfield image of cells embedded in, respectively, 2, 4 and 5 mg/mL collagen II microgels for 7 days; D, E, F) Fluorescent image of calcein-stained cells embedded in, respectively, 2, 4 and 5 mg/mL collagen II microgels for 7 days.
Figure Z.6: Comparison of shape factor index after 21 days of culture in different types of microgels. The shape factor index was significantly higher in ASCs embedded in microgels composed of high concentrations of type II collagen (average number of cells analyzed = 750 from 4 independent experiments, one way ANOVA, Tukey test $p > 0.05$).
Figure Z.7: mRNA expression of the gene RhoA in plain media relative to monolayer control. The culture of cells in different concentrations of collagen does not seem to significantly influence the RhoA expression.
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**Table Z.1.** Nucleotide primers used for reverse transcription-polymerase chain reaction.
Figure Z.8: Cells in microgels produce ECM. A) H&E staining of ASCs cultured in a pellet for 21 days in presence of differentiation media; B) H&E staining of ASCs embedded in 5 mg/mL collagen microgels for 21 days in plain media; C) H&E staining of ASCs embedded in 5 mg/mL collagen microgels for 21 days in differentiation media; D–F Alcian Blue staining of ASCs cultured in a pellet for 21 days in presence of differentiation media (D), in 5 mg/mL collagen microgels for 21 days in plain media (E) and in 5 mg/mL collagen microgels for 21 days in differentiation media (F). The staining revealed a more substantial deposition of GAGs for cells cultured in differentiation media while a light deposition of GAGs is observed when the cells are cultured in plain medium.
### Table Z.2: Correlation analysis of the mRNA expression of key genes such as Integrin α10, CD44, Rock I and SOX9 and other chondrogenic markers in plain media; in the table are reported only significant correlations and their strength represented by gradients of colors; correlation was calculated by using the Pearson coefficient and Prism® software ($n = 6, p > 0.05$).
Table Z.3: Correlation analysis of the mRNA expression of key genes such as Integrin α10, CD44, Rock I and SOX9 and other chondrogenic markers in differentiation media; in the table are reported only significant correlations and their strength represented by gradients of colors; correlation

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was calculated by using the Pearson coefficient and Prism® software \((n = 6, p > 0.05)\).

**Figure Z.9:** Flow cytometric analysis of microgel embedded ASCs pulsed with BrdU. Cells stained with (blue) isotype control (FITC) or (red) monoclonal antibody against BrdU (FITC).
AA. Supplementary information of chapter 3*

*The majority of this section has been published:
S. Browne†, G. Fontana†, B. J. Rodriguez, A. Pandit,
A Protective Extracellular Matrix-Based Gene Delivery Reservoir Fabricated by Electrostatic Charge Manipulation.
Molecular Pharmaceutics, 9 (2012) 3099–3106. (†Equal Contribution)

AA.1 Introduction

Gene therapy, through the up- or downregulation of disregulated genes, has been suggested as therapy for various disease states and disorders. However, research in this field is still far from achieving clinical acceptance due to adverse effects and concerns of delivery systems used, both viral (safety) and non-viral (efficacy and toxicity). Recently, there has been a shift towards the development of non-viral, polymeric-based gene carriers. These are generally cationic polymers that form complexes (polyplexes) with the negatively charged DNA. These complexes enable protection of the DNA but also facilitate its cellular uptake and intracellular trafficking towards the nucleus. Moreover, the use of polyplexes can help overcome some of the limitations typical of viral-vectors such as the size of DNA that can be packaged, as well as limitations of immunogenicity, mutagenesis, reproducibility and scale-up. Nevertheless, the use of polyplexes for clinical applications is hampered by their toxicity and lack of stability in vivo, which can lead to a low efficiency of transfection. Cytotoxicity of polyplexes has been studied extensively, and it is generally known that these systems can potentially destabilize cell membranes, induce autophagy, apoptosis and necrosis. Polymer molecular weight, charge of polyplexes, polyplex size and its ability to be cleared by the organism have all been associated with cytotoxicity. To address these issues, many modifications to the formulations of these polymeric systems have been attempted. These include the addition of poly (ethylene glycol) (PEG) groups, change in the molecular weight of the polymers, and addition of biodegradable backbones to the polymer. However, despite these advances, toxicity still remains a major obstacle yet to be fully overcome. In the current work, another approach is explored to address cytotoxicity and stability of polyplexes by optimizing the method of delivery rather than by modifying the polyplexes themselves. It is hypothesized, that the toxicity of
polyplexes can be masked by controlling the delivery rate by creating a reservoir system that is of uniform and controlled size composed of an extracellular matrix based protein: type I collagen. It is hoped that a reservoir of this kind will extend the duration of polyplex release (lengthening the duration of transgene expression), and decrease the associated toxicity. From current literature, it is known that the use of natural polymers for gene delivery, in both particle and matrix form, is advantageous due to their inherent non-toxic and biodegradable nature\textsuperscript{13}. In fact, matrices composed of natural polymers have been previously used and shown to prolong the transgene expression both \textit{in vitro}\textsuperscript{14} and \textit{in vivo}\textsuperscript{15}. It was also hypothesised that the modulation of the polyplexes release may decrease their toxicity and also prolong their ability to transfect cells by increasing their stability by protection from the extracellular environment. To ensure a uniform release, it is essential to have control over both the size and the architecture of the reservoir system. The composition is yet another important aspect to consider. A reservoir system composed of type I atellocollagen (collagen type I digested with pepsin to eliminate telopeptides containing antigenic epitopes)\textsuperscript{16, 17} will have several advantages over those of a system composed of other polymers. This is because it would be recognised \textit{in vivo} as an extracellular matrix constituent rather than as a foreign matter and would, moreover, impart site specificity to the delivered gene carrier since collagen can be recognized by cells\textsuperscript{18, 19}. The development and fabrication of such a system was discussed in Chapter 3 where, with the use of the template method, type I collagen hollow spheres with controllable size and shape were obtained. Control over size and homogeneity are two important criteria for clinical translation. It is hypothesized that a uniform type I collagen reservoir will allow efficient transgene expression, while limiting any toxic effects of the loaded polyplex cargo.

AA.2 Methods

AA.2.1 Surface analysis of spheres
Amplitude modulation atomic force microscopy (AFM) was utilized to analyse the surface of the 10 μm collagen spheres using a commercial
system (NanoWizard-II, JPK Instruments, Germany). A 100 μL drop of aqueous solution containing hollow collagen-coated spheres was pipetted onto a glass microscope slide and air-dried. After the slide was placed on the AFM sample stage, an inverted optical microscope (Eclipse Ti-E, Nikon, Japan) was used to locate isolated or clusters of spheres. The region of interest was positioned directly under the AFM tip prior to imaging in air. Silicon cantilevers (PPP-NCH, Nanosensors, Germany) with nominal spring constants and resonance frequencies of 42 N m\(^{-1}\) and 314 kHz, respectively, and typical tip radii of ~ 7 nm were used. The images were recorded with a resolution of 512 x 512 pixels and a 1 Hz scan rate. Images were processed using WSxM software (Nanotec).

**AA.2.2 Analysis of polystyrene removal**

To verify complete removal of the polystyrene template following THF treatment, an infrared spectrum analysis of the spheres was performed. Particluar attention was paid to the characteristic peaks of polystyrene. Three samples were examined: uncoated polystyrene beads, collagen coated polystyrene beads and hollow collagen spheres (washed with THF). Samples were examined following drying using a Fourier transform infrared spectrometer (FTIR – Varian 660-IR).

**AA.2.3 Labelling of plasmid**

*Gaussia princeps luciferase* plasmids (pCMV-GLuc; New England Biosciences, Ipswich, USA) were propagated and isolated using standard techniques, as described elsewhere\(^1^4\). These plasmids were then fluorescently labelled with the Cy5 dye using a Cy5 labeling kit (Mirus, Madison, USA). Briefly, the dye was incubated with the plasmid in the provided buffers for one hour. Following the incubation, the plasmid was eluted through a microspin column to remove any unbound dye. The recovered labelled plasmid was then stored in a light-protected environment at -20°C until use.
AA.2.4 Polyplex formation
Polyplexes were prepared by incubating the labelled plasmid with a partially degraded PAMAM dendrimer (Superfect™, Qiagen) (SF). The weight ratio of the partially degraded dendrimer to the pCMV-GLuc used was 9:1. As a control, polyethyleimine (PEI, Branched, MW=25,000) complexes were formed at a weight ratio of 1:1 (PEI:pDNA).

AA.2.5 Loading of microsphere reservoirs with polyplexes
Loading was quantified using a method for detecting complexed pDNA previously validated and standardized 21. 100 μg of collagen spheres were resuspended in 500 μl of phosphate buffered saline (PBS). To this, 2 μg of Cy5 labelled complexed pDNA was added. This mixture of spheres and labelled complexes was agitated on a mechanical shaker for four hours at room temperature. The collagen spheres were spun down and the supernatant removed. This supernatant was then measured spectroscopically in a black well plate (excitation=649 nm, emission=670 nm) in a Varioskan Flask plate reader (Thermo Scientific, Ireland). The supernatant was compared with a standard curve to determine the amount of pDNA remaining which enabled quantification of loading efficiency.

AA.2.6 Release study
The release profile of Cy5 labelled polyplexes was characterized in PBS at 37°C. Loaded spheres reservoirs were spun down; the supernatant was collected and replaced at various timepoints. The collected supernatant was frozen until it was assayed for polyplex content using a plate reader as before for loading efficiency.

AA.2.7 Transfection
Polyplex-loaded collagen spheres of three sizes (100 nm, 1 μm and 10 μm) were incubated with 3T3 fibroblasts in complete media (10% serum) to assess the ability of the microspheres to release polyplexes that transfect cells in vitro. Briefly, 10,000 cells were seeded into a 96 well plate and incubated with 50μg spheres reservoir loaded with 2μg of complexed plasmid for 48 hours. In addition, polyplexes alone formed using 1 μg
pDNA and SF and PEI, (at weight-to-weight ratios previously specified), were used as positive controls. At 48 hours, 50μl of the supernatant was removed and assayed using a gaussia luciferase assay kit to quantify the expression of the excreted luciferase protein. Furthermore, to microscopically determine the ability of spheres to release bioactive polyplexes and transfet 3T3 fibroblasts in vitro, protein expression was also assessed by delivering an SF complexed green fluorescent protein plasmid (pCMV-GFP) loaded within collagen spheres and visualizing the expression using an inverted fluorescence microscope after 48 hours.

AA.2.8 Cell proliferation
The effect of collagen microspheres size on the proliferation of 3T3 fibroblasts over 48, 120 and 168 hours was assessed. As per alamarBlue® assay as reported previously, following incubation with collagen spheres for the three time periods specified, media was removed and cells washed with HBSS. The cells were then subjected to 3 cycles of freeze-thaw at -80°C in DNAse free water, and the supernatant analysed for DNA content as per the manufacturers’ instructions. Each size was compared with the control, cells grown on tissue culture plastic alone.

AA.2.9 Toxicity of polyplexes
To assess the potential of the collagen spheres to buffer the cytotoxicity associated with large doses of transfecting agents, the effect of polyplexes formed using two commonly used transfecting agents, SF and PEI, at the same weight-to-weight ratios previously specified, was assessed both loaded within microspheres (1μm) and directly in the cell culture media of 3T3 fibroblasts. A 5μg dose of pDNA was used in both cases, and compared with the control (cells alone) using an alamarBlue® assay at 48 hours.

AA.3 Results
AA.3.1 Collagen spheres characterization
Dissolution of the template was achieved by washing in THF and confirmed by FTIR analysis (Figure AA.1). AFM analysis of the structure of the surface of the sphere showed evidence of a nanofibrous coating (Figure
AA.1. While the surface structure is not readily apparent, the curvature of the sphere is visible. The surface roughness of the sphere has been determined to be 1.5 nm rms by selecting a 1.4 x 1.4 μm² area and performing a second order flattening to subtract the effect of the curvature of the sphere. The amplitude and phase AFM images (Figure AA.1 (B) and (C)) have also been flattened to reveal the surface structure. The surface of the spheres exhibited topographical features associated with the matrix assembly process used in the fabrication of the microspheres. Furthermore, as shown in Figure AA.2, when immersed in PBS, type I collagen hollow spheres acquire a negative charge. This will facilitate their functionalization by electrostatic interaction with the cationic polyplexes.

AA.3.2 Characteristics of loading and release
Loading and release properties of the spheres reservoir are critical as they determine the efficiency of the delivery system. The intended application for these spheres is as a reservoir system that is capable of releasing polyplexes over time thus extending the lifetime of transgene expression. The first step was to assess the loading efficiency. Each of the three sizes of spheres displayed an ability to efficiently load polyplexes at an efficiency of about 85-90% (Figures AA.3 and AA.4) regardless of the sphere size tested (for 2 μg per 100 μg hollow collagen spheres). Hence, the spheres have a high loading capacity, with an ability to efficiently load about 20 μg of complexed pDNA per 1mg of reservoirs.

Each of the three sizes of spheres showed an ability to delay the release of polyplexes, and therefore this system was suitable to extend the release of polyplexes and transgene expression over time. Each size showed a similar release pattern of polyplexes over 144 hours (Figure AA.3). Overall, the spheres showed an ability to delay the release of polyplex cargo over the first 96 hours, varying from 40-60%.

AA.3.3 Protein expression
Measurement of luciferase activity was used to assess the ability of the spheres to release bioactive polyplexes and transfect 3T3 fibroblasts in vitro. There was a correlation between size of spheres and transfection ability. Of
the three sizes, the 1μm and 10μm spheres showed the highest ability to transfect in vitro and a similar level of transfection to the polyplex alone and PEI controls. However, cells incubated with 100nm microspheres showed significantly less transfection than the polyplex alone control, and even less than the plasmid alone control (Figure AA.4 (A)). Fluorescent images showed GFP expression in 3T3 fibroblasts (Figure AA.4 (B)) following incubation with the collagen spheres which further illustrates the ability of this collagen reservoir to release bioactive polyplexes.

AA.3.4 Reduction in polyplex toxicity
High doses of the commercially available transfecting agents, SF and PEI, had a negative effect on the viability of 3T3 fibroblasts as measured using the alamarBlue® assay, with metabolic activity decreasing to 40% and 50% of control, respectively. However, when the same dose was loaded within 1μm spheres, there was no negative effect on the metabolic activity when compared with the control (Figure AA.4 (C)). These results illustrate that the collagen hollow spheres can deliver a large dose of transfecting agent to cells without compromising cellular viability.

AA.3.5 Maintenance of cell proliferation
As Figure AA.6 shows, there was no significant effect on the proliferation of 3T3 fibroblasts over any of the timepoints following incubation with any size of collagen microsphere tested when compared with the control. These results confirmed the conclusions that collagen hollow spheres do not alter cells’ ability to proliferate.
**Figure AA.1**: Characterization of spheres’s surface and template removal. AFM image of the (A) surface topography of a hollow collagen sphere. Amplitude modulation AFM (B) amplitude and (C) phase images of the same sphere, displaying the fibrous structure present on the surface of the spheres. (B, C) Images have been 2nd-order flattened to subtract the curvature of the sphere and reveal the surface structure; (D) FTIR spectrum showing the removal of the polystyrene core following treatment with THF. The characteristic peaks of polystyrene (dotted lines) are removed from the sample following THF treatment. (THF: Tetrahydrofuran). *Data obtained in collaboration with Shane Browne.
Collagen spheres have a negative charge when immersed in PBS. Zeta analysis of collagen microspheres in PBS; all three sizes were seen to be negative, with the largest size, 10 µm, being more negative than the others. (n=6). *Data obtained in collaboration with Shane Browne.
**Figure AA.3*: Reservoir characterization.** (A) Loading efficiency of polyplexes within three sizes of hollow collagen spheres shows no significant difference, while release profile in PBS over 6 days, (B), shows an ability to delay the release of the polyplexes over time. Data represents mean ± SD (n=3). *Data obtained in collaboration with Shane Browne.
Figure AA.4: Characterization of spheres’ loading with polyplexes. (A) TEM image of a cross-section through collagen hollow spheres before incubation with polyplexes; (B) TEM image of a cross-section through collagen hollow spheres after incubation with polyplexes.
Figure AA.5*: Transfection and polyplex toxicity. (A) Gaussia Luciferase assay to assess the ability of the microspheres to release bioactive polyplexes capable of transfecting cells in vitro. In this case, the 1 µm and 10 µm spheres have displayed an ability to release polyplexes capable of transfecting 3T3 Fibroblasts with a similar ability to polyplexes alone. (B) 3T3 Fibroblasts expressing GFP following treatment with polyplex-loaded spheres (Green – GFP, Blue - DAPI). (C) Cell metabolic activity is dramatically reduced following incubation with cationic polymers. However, this effect is removed following loading of the polymers with collagen microspheres (1 µm). Data represents mean ± SD (n=3). (PEI: Polyethyleimine, SF: dPAMAM, Superfect™, Qiagen). *Data obtained in collaboration with Shane Browne.
**Figure AA.6*: Collagen hollow spheres do not affect cell proliferation.**

Cell Proliferation as measured by DNA content using the PicoGreen™ assay. It can be seen that there is no significant effect on the proliferation of 3T3 fibroblasts over 48, 120 and 168 hours when compared to the control (n=3). *Data obtained in collaboration with Shane Browne.
AA.4 Discussion

The use of polyplexes is one of the most promising alternatives to viral vectors for gene therapy. Despite the introduction of new features such as biodegradability, addition of PEG groups, or change in the molecular weight, there are still many issues to address including toxicity and duration of transgene expression. The current study addresses the issues of toxicity and dose from a different prospective: instead of introducing modifications to the polyplexes, its aim is to modulate their delivery both in terms of rate and retention within the microenvironment. In many cases, polyplexes are used in an environment in which cells fail to secrete a certain protein or in an environment in which a population of cells lacks the ability to renew themselves following injury. With the above considerations in mind, it is desirable to have a system with minimum toxicity, as otherwise even high transfection efficiency would be masked by cell death. For this reason, this system was designed by using one of the most abundant ECM molecules: type I collagen. This system not only allows for reduction in toxicity, but also can be recognized by the cells, thus facilitating their interaction with the loaded polyplexes. The AFM analysis shows a nanofibrous pattern of the coated surfaces and a smooth surface on the polystyrene beads (Figure AA.1), thus indicating the presence of fibrous protein on the surface of the spheres. The dissolution of the polystyrene template by washing the spheres in THF was confirmed by FTIR analysis that showed the absence of polystyrene from the samples. Considering that small particles and molecules may diffuse inside the lumen of the spheres (Figure AA.4), their hollowness is an advantage in terms of loading because it almost doubles the surface area available to interact with the cargo relative to solid particles.\(^2\)

AA.4.1 Collagen microspheres showed high loading of polyplexes and sustained release

*In vitro* analysis revealed that a high amount of loading of pDNA with an efficiency of the 85% (about 20 µg of DNA per mg of collagen) is possible in the fabricated systems. Characterization of the loading of three different sizes of spheres showed a uniform loading efficiency over the sizes tested,
thus confirming independence between the loading ability and the size of the spheres. Zeta analysis of collagen microspheres showed that these spheres are negatively charged in PBS (Figure AA.2). Hence, the high loading efficiency is due to the fact that spheres and polyplexes are incubated in PBS. Positively charged polyplexes interact electrostatically with the negatively charged spheres. This electrostatic interaction explains the high loading efficiency of the collagen reservoirs. In addition, the phosphates of PBS create a temporary cloud of negative charges around the collagen molecules. Together, these properties allow for the random diffusion of polyplexes inside the hollow spheres. Moreover, type I collagen is a molecule rich in aspartic acid, a negatively charged amino acid, which further enhances the loading capacity. Indeed, this interaction is of importance to the loading ability of the system and the amount of nucleic acid loaded is vital for the success of any gene delivery reservoir. Considering that many in vivo studies frequently deliver as little as 2 µg of complexed pDNA and with the fabricated collagen spheres it is now possible to deliver 20 µg of complexed DNA per mg of collagen, this system now enables a high dosing regimen. The polyplex release curves demonstrate a prolonged release of the loaded cargo over 144 hours in vitro (Figure AA.3). While it is likely that the release profile will be different in the extracellular space in vivo, due to interactions with various proteins and degrading enzymes, this collagen reservoir system holds great promise in prolonging the release of polyplexes.

**AA.4.2 Microsphere reservoirs lowered the cytotoxicity associated with polyplexes while maintaining high transfection efficiency**

An outcome that is closely linked to loading and release of polyplexes is transfection. It is of vital importance that the released polyplexes are capable of transfecting cells in vitro, and that the dose of polyplexes released is enough to see meaningful transfection. When examining the ability of the spheres to transflect 3T3 fibroblasts in vitro, it was seen that both the 1 µm and 10 µm loaded spheres are capable of transfecting the cells. However, the 100 nm spheres showed a much lower transfection in
Appendix

comparison. With no difference seen in the release profiles, this could be linked to the uptake of the smaller size spheres, which would result in a lower transfection as polyplexes would be released within individual cells, resulting in toxicity, rather than staying outside cells and releasing the loaded polyplexes to many cells. However, as Figure AA.5 showed, following release over five days, polyplexes from 100 nm spheres showed no transfection. Therefore it seems that 100 nm collagen spheres may either interact with polyplexes in such a way that they reduce their bioactivity and are incapable of transfecting cells, or due to the smaller size they do not protect the polyplexes from degradation in the way that the larger spheres seem able to. The ability of the 1 µm and 10 µm spheres to transfect was visually confirmed by using the GFP reporter plasmid, while for the 100 nm spheres no protein expression was evident. One of the hypotheses behind this study was that by modulating the rate of delivery of polyplexes, the cytotoxic effect of the transfecting agents can be reduced. This is especially crucial when delivering polyplexes to cells which have a limited ability to regenerate following injury as seen in nucleus pulposus cells in the intervertebral disc \(^{26}\). This was examined by using two known cytotoxic transfecting agents in high doses and comparing these with the same transfecting agents but loaded within microspheres. As seen in Figure AA.5, there is a significant effect on cell metabolic activity using the transfecting agents on their own. However, this effect is buffered by the utilization of polyplex-loaded spheres and the metabolic activity is maintained as similar to the control. This shows that by altering the release pattern using an ECM-based reservoir system, the cytotoxic effects of known transfecting agents \textit{in vitro} can be shielded. It is likely that this ability will be displayed \textit{in vivo}, with the microspheres capable of delivering a high dose of polyplexes to a target tissue without causing toxicity in the local microenvironment.

**AA.5 Conclusions**

The results of this study demonstrate that hollow collagen microspheres displayed the ability to efficiently load a large amount of polyplexes and release them over a prolonged period. Polyplex-loaded collagen spheres
efficiently transfected 3T3 fibroblasts similar to as seen with polyplex alone control; and in addition, reduced the cytotoxic effect of large doses of polyplex when compared with a bolus dose. Hence, collagen hollow spheres show potential for use as a reservoir for polyplexes for extended transfection and gene expression.

**BB. Supplementary information for Chapter 4**

**Figure BB.1:** Collagen hollow spheres can be produced with uniform size. Graph showing the diameter of collagen hollow spheres measured by analyzing SEM images of 25 spheres fabricated using 4.5 µm templates. This analysis was repeated for four different batches of spheres, one way ANOVA, Tukey test p > 0.05).
Figure BB.2: Differing ratios of beads/collagen do not affect the charge on the spheres. Charge analysis of 4.5 µm spheres was conducted during each step of their fabrication. Following collagen coating, the charge of the polystyrene beads used as template turn positive, independently of the ratio of beads/collagen used. The surface charge of the spheres decreases after crosslinking. (n=3)
Figure BB.3: No internalization of the reservoirs was observed in three-dimensional (3D) microgels. TEM images of cross sections through ADSCs embedded in 3D microgels functionalized with a polyplex-loaded collagen reservoir system.
CC. References


Appendix

DD. Research outputs

DD.1 Manuscripts


DD.2 Book chapters


DD.3 Research poster and oral presentations


Thomas D., Fontana G., O’Brien T., Pandit A. “An Optimised Microgel for Maintaining a Microniche Environment for Human Mesenchymal Stem Cells”, Oral presentation at the 9th World Biomaterials Congress (WBC); 1rst-5th June, 2012; Chengdu, China.


Browne S., Fontana G., and Pandit A. “Sequential-Release of Nucleic Acid from a Collagen Scaffold with Hollow Microspheres that Avoid Phagocytosis by Activated Macrophages” Poster presentation at the 3rd Tissue Engineering and Regenerative
Appendix

Medicine International Society (TERMIS) World Congress, Vienna, Austria, 2012


Thomas D., **Fontana G.**, Chen X., Sanz C., Madden K., Quondamatteo F., O’Brien T., Pandit A. “A Tuneable Microgel Platform for In Vivo Delivery of Mesenchymal Stem Cells”, Oral presentation at The 25th European Society for Biomaterials (ESB), 8th-12th September, 2013; Madrid.

DD.4 Research awards and prizes

Winner of the Wiley Functional Material Award for best poster presentation at The 24\textsuperscript{th} European Society for Biomaterials (ESB), 4\textsuperscript{th}-8\textsuperscript{th} September, 2011; Dublin.