COMMUNICATION

Injectable peptide-glycosaminoglycan hydrogels for soft tissue repair: *in vitro* assessment for nucleus augmentation

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James P. Warren[a,b], Ruth H. Coe[a], Matthew P. Culbert[a,b], Andrew R. Dixon[a], Danielle E. Miles[a,b], Marlène Mengoni[a], Paul A. Beales[b], Ruth K. Wilcox[a]*

We report the development of peptide-glycosaminoglycan hydrogels as injectable biomaterials for load-bearing soft tissue repair. The hydrogels are injectable as a liquid for clinical delivery, rapidly form a gel in situ, and mimic the osmotic swelling behaviour of natural tissue. We use a new *in vitro* model to demonstrate their application as a nucleus augmentation material for the treatment of intervertebral disc degeneration.

Introduction

Injectable biomaterials have shown great promise in minimally invasive treatments as carriers for drugs or cells.[1,2]. While they have potential for use directly as devices for tissue repair, there are challenges in meeting the mechanical requirements for load-bearing applications, particularly in musculoskeletal tissues.

In soft tissues such as articular cartilage and the intervertebral disc, the fluid component plays a critical role in governing the mechanical behaviour. These tissues contain high concentrations of proteoglycan macromolecules with negatively charged glycosaminoglycan (GAG) side chains that draw water into the tissue and provide a swelling pressure[3]. Degeneration and disease can reduce the size and quality of the proteoglycan aggregates, resulting in a loss of swelling pressure and a cascade of further biomechanical, chemical and biological changes[4].

Any treatment in which the degenerated tissue is replaced or augmented by a biomaterial must therefore be able to mimic the fluid as well as the solid components of the structure.

In the case of the intervertebral discs, there is an association between these degenerative changes and back pain, especially in the lower spine. [5,6] Back pain is ranked as the leading cause of years lived with disability,[7] and the total costs associated with the condition are estimated to be over US$ 100 billion per year in the US alone.[8] Despite the scale of the problem, there are limited clinical approaches to prevent or treat progressive degeneration of the discs.

The intervertebral discs are the soft tissues between the vertebrae that allow their articulation. They comprise an outer annulus fibrosus, a layered structure of collagen fibres aligned in alternating orientations, and an inner gel-like nucleus pulposus (Figure 1). Degenerative changes cause the nucleus to lose GAGs and result in a loss in the overall disc height. End-stage surgical treatment most commonly involves the fusion of the two adjacent vertebrae, but this may lead to accelerated degeneration at adjacent levels and has relatively poor clinical outcomes.[9] A number of regenerative therapies that aim to restore disc homeostasis have been investigated, but these are challenged by the avascular nature of the tissue, which limits nutrient supply.[10]

We have previously shown that a class of self-assembling peptide hydrogels can be designed to mimic the natural properties of hydrated soft tissues when combined with GAGs.[11,12] Importantly, the presence of GAGs not only mimics the natural tissue's ability to imbibe water, but also enhances the thermodynamic stability and gelation kinetics of the peptide.[11] We have demonstrated that the hydrogels can be formulated to match the mechanical properties of the natural nucleus pulposus and have potential as a treatment for intervertebral disc degeneration (Figure 1).

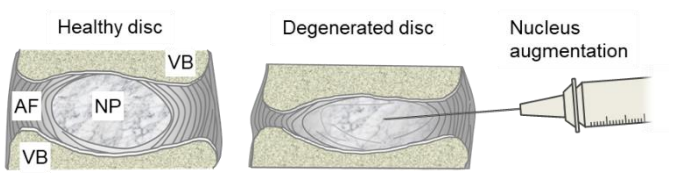

Figure 1: The healthy intervertebral disc comprises a GAG-rich nucleus pulposus (NP) surrounded by the annulus fibrosus (AF). The discs allow articulation between the adjacent vertebral bodies (VBs). In the degenerated disc, there is a reduction in GAGs and the disc loses height. The concept of nucleus augmentation is to increase disc height and restore functionality through the minimally invasive injection of a hydrogel into the NP region of the degenerated disc.

[a] *Institute of Medical and Biological Engineering, School of Mechanical Engineering*
[b] *School of Chemistry, University of Leeds, Leeds, LS2 9JT, UK*

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In a static *in vitro* model, denucleated discs augmented with the peptide-GAG hybrid hydrogels demonstrated properties similar to the native tissue.[11,13] Here, we report the development of peptide-GAG hydrogels that meet the concurrent requirements of being injectable as liquids for clinical delivery, rapidly and reliably forming a gel *in situ*, and mimicking the swelling behaviour of natural tissue. We specifically examine the performance of the gels as a minimally invasive therapy for intervertebral disc degeneration.

The evaluation of the biomechanical performance of biomaterials for nucleus pulposus augmentation or replacement is hampered by the lack of current laboratory testing methodologies.[14] *In vitro* models employing cadaveric or large animal intervertebral disc specimens have been used to mimic the natural physiological environment. [15-20] These models have been tested under cyclic loading, using either biochemical or mechanical approaches to simulate disc degeneration.[14] However, the direct effects of an intervention are often masked by the large variations in mechanical behaviour seen across specimens, due to anatomical variances and changes in specimen hydration.[13] Here, we propose a new accelerated testing approach which enables longitudinal comparisons of the same specimen in different states, while minimising the test durations.

Finally, we report on the use of this methodology to assess the biomechanical performance of the peptide-GAG hydrogels and their ability to restore artificially degenerated tissue to the healthy state.

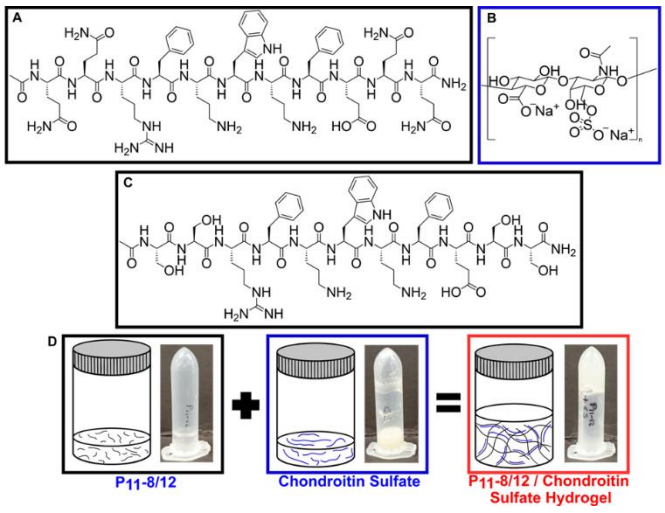

Figure 2 Structural representation of the peptides (A. P_{11}-8 and B. P_{11}-12) and C. chondroitin sulfate. D. Graphical representations and visual photographs of each component and upon mixing.

Results and Discussion

The hydrogels used in this study were based on our previous work, where we found that the specific peptide identity, the peptide:GAG ratio and the mixing process are control parameters that tune the gel mechanical properties.[11,21]We designed two candidate peptide-GAG hydrogels for the nucleus augmentation application to match the rheological properties of the healthy nucleus pulposus, namely P_{11}-12 and P_{11}-8, both with a range of peptide:GAG ratios from 1:2 to 1:20 (where 1:n refers to the molar ratio of one peptide to n GAG dimer subunits).

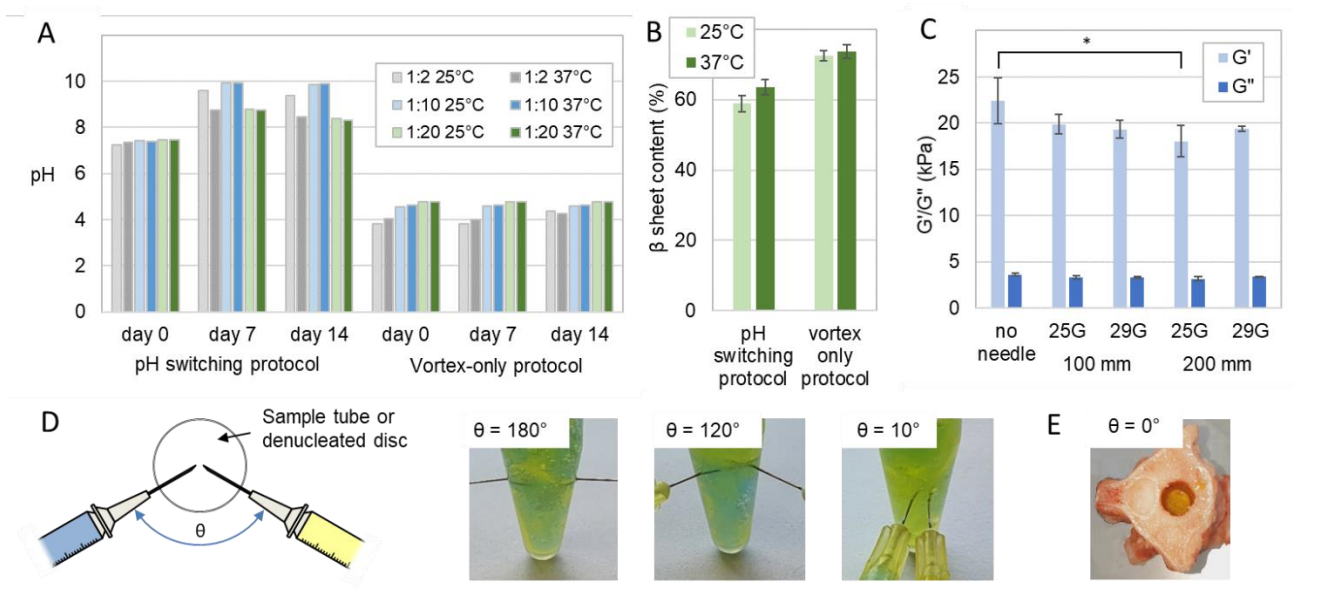

Figure 3 Evaluation of the clinical mixing and delivery of the P_{11}-12 –GAG hydrogel. **A** The pH measured over a period of 14 days of hydrogels prepared at different temperatures using the pH-switching method reported previously[11] and a simplified vortex-only process. **B** The corresponding β sheet content of the 1:20 ratio samples (mean ± S.D., n=3), measured from Fourier transform infra-red spectra. **C** The elastic (G') and viscous (G'') components of shear modulus of 1:20 ratio samples delivered through different needle diameters and lengths (mean ± S.D., n=3), measured with a cone-on-plate geometry rheometer * = significant ($p<=0.05$). **D** The evaluation of different orientations of needle delivery using dye to differentiate the GAG (yellow) and peptide (blue) components. **E** Instantaneous gel formation after injection into a denucleated disc.

For minimally invasive clinical delivery into the intervertebral disc, the gel components must be injected along fine bore The differing peptide:GAG ratios replicated the range of GAG concentrations naturally found in human intervertebral discs. Figure 2 illustrates the hypothesized mechanism of interaction between the peptide and GAG molecules, which results in the self-assembled hydrogel. The peptides were custom synthesised (CS Bio, USA); the GAG used was chondroitin sulfate sodium salt from shark cartilage (Sigma Aldrich, Merck Life Science UK).

We compared the gels prepared using our established laboratory protocol incorporating pH switching and heat monomerisation,[11] and those prepared with a simpler vortex-only benchtop process more suitable for clinical use. We found that there were differences in pH, but no change in the formation of a self-supporting gel, between the two preparation methods. Importantly, with either preparation method, there was little difference in pH or hydrogel structure (measured from Fourier transform infra-red spectra) between samples prepared at 25°C and at 37°C, and the changes were smaller with the vortex-only protocol at higher GAG concentrations (Figure 3A,B). These results indicate that variances in operating room temperatures and handling would not affect the performance of the gel.

needles of sufficient length to reach the centre of the nucleus pulposus from a superio-lateral approach. Previous studies have shown that the mode of agitation during peptide gel transition can dramatically influence the mechanical properties of the resulting gel.[21,22] We hypothesised that the shear forces applied to the monomer and GAG solution during injection could also affect peptide self-assembly the subsequent gelation.

We examined the rheological properties of the hydrogels prepared through a standard benchtop vortexing method and following injection down fine-gauge needles, at a flow rate of 0.22ml/min, with no external agitation. In all cases, self-supporting gels were found to form. The flow rate was kept constant through using an automated syringe driver.

There were differences in the rheological properties between the benchtop prepared hydrogels and those formed following injection through the needles (Figure 3C). The differences were not significant between different needle lengths (100 mm to 200 mm) and diameters (25 to 29G), and the viscosity was comparable to that of the natural nucleus pulposus[23,24] (Figure 3C). We also observed differences in the uniformity of the resultant gel formation when the components were injected sequentially rather than simultaneously, and when the relative orientation of the needles to each other was altered (Figure 3D). Finally, we used a dual needle injection system to deliver the gels simultaneously down 100 mm 25G needles. We showed consistent mixing of the gels in an Eppendorf. Furthermore, we saw instantaneous gel formation when injected into a denucleated intervertebral disc that had been sectioned transversely to allow viewing (Figure 3E).

Using bovine caudal bone-disc-bone units, we developed an *in vitro* degeneration model using injection of a controlled volume of papain followed by injection of an enzymatic inhibitor after 24 hours, as shown in Figure 4.

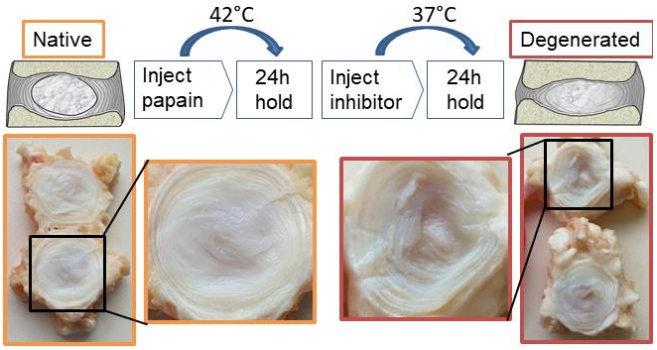

Figure 3 The degeneration model involved two steps: enzyme (papain) injection and inhibitor injection. The degenerated nucleus is highlighted, post-enzymatic digestion.

The degeneration model utilised papain as a broad protease to non-selectively break down proteins, mainly collagen, within the nucleus and inner annulus. The process required an increased temperature to 42°C for the papain to optimally operate. The enzymatic inhibitor deactivated the papain to stop the enzymatic digestion at a known time point. The route allowed accurate control of the digestion process, minimising variation in specimens. The specimens were held at a constant load (39N) during the whole degeneration process.

As shown in Figure 4, this degeneration process caused degeneration of the nucleus as highlighted, which was characteristic of a fluid filled void where the protein structure had previously been.

We minimised variation between specimens by using only the most cranial four levels. Specimens were also imaged and sectioned in a bespoke rig to provide consistent bone lengths at either side of the disc.[13] We developed a mechanical testing protocol that was sufficiently short in duration to enable specimens to be tested longitudinally in their native and degenerated states, as well as subsequently after nucleus augmentation (Figure 5A, B). Specimens were equilibrated under load for 24 hours in phosphate buffered saline (PBS) to ensure consistent hydration and then tested under cyclic loading. The lower and upper limits (356N and 744N) represented the loads experienced during normal activities – unsupported sitting and weighted lifting respectively. We found a significant reduction in specimen height between the native and artificially degenerated specimens ($n = 12$, $p<0.01$) (Supplementary data, Figure S1). Furthermore, we found significant differences in the specimen stiffness after testing for 100 axial loading cycles between the native and artificially-degenerated cases, providing two control values against which to compare the treated cases ($n = 12$, $p<0.01$).

We went on to examine the mechanical performance of the two candidate peptide-GAG hydrogels in the degeneration model. In all cases, specimens were tested longitudinally in the native, artificially degenerated and peptide-augmented states. The hydrogels were injected manually using standard syringes with custom fixtures such that equal volumes of monomer and CS

Conclusion

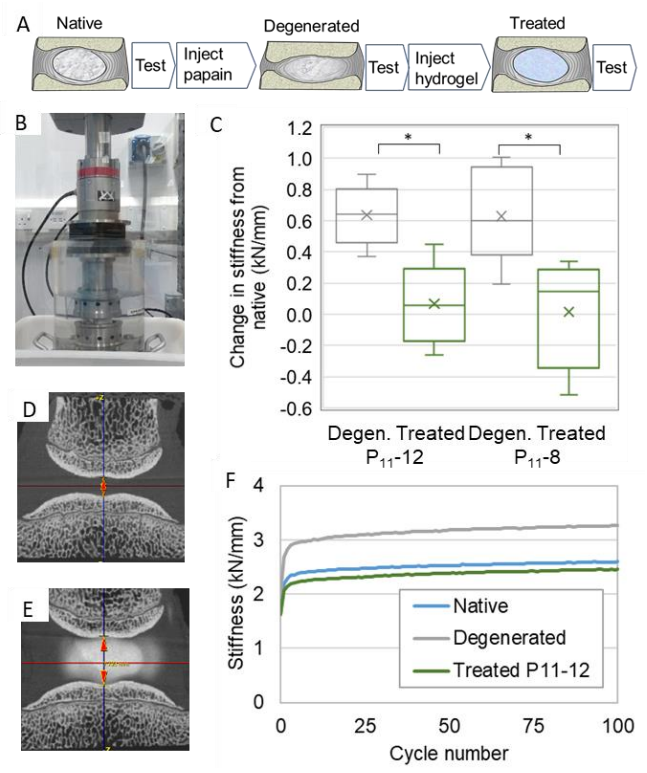

Figure 5. **A** schematic of the *in vitro* longitudinal testing protocol. **B** Specimens were cyclically loaded in a heated fluid bath under axial compression in a materials testing machine. **C:** Change in stiffness following degeneration and treatment. Specimens showed a significant increase in stiffness following artificial degeneration and a reduction to the native levels following treatment. **D, E** MicroCT images of a specimen following artificial degeneration (D) and treatment (E), showing the increase in height following injection of the hydrogel. **F** The stiffness behaviour over the 100 cycles in the three states for a typical specimen.

solution were delivered simultaneously through two 25G needles. A total volume of 0.3 mL was injected in all cases. The stiffness of the peptide-augmented specimens after 100 cycles was found to not be significantly different to the native specimens and significantly less stiff than the artificially degenerated specimens for both P_{11}-12 and P_{11}-8 (Figure 5C, F), indicating that the peptides could restore biomechanical properties to the native levels. Furthermore, testing over longer periods revealed that while the stiffness of the artificially degenerated specimens reached a plateau after less than 8,000 cycles (gradient <0.01 N/mm/cycle), the stiffness of the native and augmented specimens continued to change to beyond 15,000 cycles (Supplementary data, Figure S2). Examination of the disc height using micro computed tomography (microCT) showed that the drop in height caused by the degeneration step was restored following nucleus augmentation (Figures 5D, 5E, S2). No differences in mechanical performance or in height restoration were observed between the two candidate hydrogels. Injectable biomaterials offer potential in the treatment of a number of soft tissue pathologies, but have to be designed to meet demanding requirements relating to both their deliverability and resulting properties. Previously, we have shown that a family of peptide-GAG hybrid hydrogels can be tuned to have appropriate mechanical properties for intervertebral disc nucleus repair. Here we extend the evidence to demonstrate the gels can be successfully delivered through a minimally invasive technique and self-assemble to form a gel in situ. Importantly, the resulting gel properties do not vary through either clinically relevant temperature range or needle size range. Furthermore, the injected hydrogel was shown to restore disc height and stiffness to native levels using a novel *in vitro* sequential testing regime under cyclic loading.

Author Contributions

JPW, RHC, MPC and ARD performed the investigations and the formal analysis with MM. The work was conceptualised by RKW, DEM and PB and supervised by RKW, PB and MM. The manuscript was written, reviewed and edited through contributions from all authors.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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