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Development of Nanofiber-Reinforced Hydrogel Scaffolds for Nucleus Pulposus Regeneration by a Combination of Electrospinning and Spraying Technique

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ABSTRACT: In this work a new method is presented to efficiently produce hydrogel scaffolds reinforced with nanofibers to show enhanced mechanical properties and improved structural integrity. The method is based on a combination of air brush spraying of a hydrogel and electrospinning of nanofibers. With air brush spraying the controllability is enhanced and the potential for scale-up increased. The developed method was used to successfully reinforce gellan gum hydrogels with electrospun polycaprolactone nanofibers. Optical and rheological evaluations were performed and showed that parameters such as the amount of incorporated nanofibers, gellan gum concentration and calcium chloride (crosslinker) concentrations could be used to modulate material properties. Incorporation of a small amount of nanofibers had a reinforcing effect and resulted in a hydrogel with rheological properties similar to the human nucleus pulposus (NP). The method is flexible and carries potential for designing scaffolds for e.g. NP tissue regeneration.
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INTRODUCTION

Many people suffer from back pain, a disability commonly caused by degeneration of the intervertebral disc (IVD). As of today, treatments depend heavily on surgical approaches, such as discectomy, spinal fusion, or complete or partial removal of the damaged disc.[1,2] These are heavily invasive and often result in limited flexibility, as well as an accelerated degeneration of adjacent vertebrae. Finding an alternative treatment would have great impact on socioeconomics as well as on the quality of life for individuals and is therefore the focus of much research.[1–3]

The interest in applying tissue engineering approaches to regeneration of the IVD is mainly due to its limited regenerative capacity.[1–3] The IVD is a complex tissue composed of an inner gel-like material [nucleus pulposus (NP)], an outer fibrous structure [annulus fibrosus (AF)] and an end plate.[4,5] As the IVD is avascular, the end plate has the very important role of providing the NP and AF cells with nutrients.[1,6] The AF has an intricate structure of lamellae composed of aligned collagen fibers functioning to provide mechanical stability and integrity to the whole construct regarding tensile stresses.[6–9] The NP, on the other hand, has a gel-like structure composed of a large part glucosaminoglycans and sparsely distributed collagen fibers in size scale of about 100 nm, both functioning to take up load from forces such as compressive and shear stresses subjected to the back.[10,11] Current repair strategies focus on separate parts of the IVD as well as the complete structure.

When it comes to repair of the NP, a lot of attention has been directed toward the development and use of hydrogels. Hydrogels are commonly used as scaffold materials as they are easy to produce and can be made injectable, thus allowing for minimally invasive surgery.[10] A hydrogel also has the advantage of adequately filling the space of a defect, eliminating the need for

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patient-specific shaping of the scaffold.[10–12] Furthermore, it may be used for encapsulation and delivery of cells and bioactive agents.[10,13] Alginate,[14–17] hyaluronic acid,[18,19] gellan gum,[20–23] and various cellulose derivatives are just a few examples of hydrogel materials investigated for tissue engineering applications. Gellan gum is an interesting polysaccharide which has gained attention during the last couple of years for its gel forming properties.[20–22] It is an extracellular microbial anionic heteropolysaccharide containing repeating units of glucose–glucuronic acid–glucose–rhamnose. It forms a thermoreversible gel in the presence of metallic ions and upon a decrease in temperature.[20–22]

Although hydrogels carry many features that are attractive in tissue engineering applications, there are a few problems that need to be solved. One is that the mechanical stability of hydrogels is generally low, both in tension and compression.[24] When applied *in vivo* the scaffold must withstand the external forces acting upon it, to maintain its integrity and support function without collapsing.[10,12] Especially considering the application of disc regeneration and all the forces the spine is subjected to, mechanical stability is of utmost importance. Another problem with hydrogels is leakage, an issue arising from the fluidic properties of a gel.[24,25] Keeping the gel, and the cells or molecules it may carry, within the wound site is of course crucial for an efficient regeneration. Chemical modification of polymers (e.g., by methacrylation) and crosslinking are possible strategies to increase the strength and stability of hydrogels. Studies of alginates as well as gellan gum have shown that chemical modification and crosslinking increases the mechanical strength and enhances the long-term performance of the engineered structure,[20,22,26] Particles, e.g. hydroxyapatite,[27–29] and carbon nanotubes[30,31] have also been used to reinforce hydrogels. However, considering the structure of a native extracellular matrix (ECM) that is, a combination of nanofibers and gel-like glucosaminoglycans, fiber reinforcement of a hydrogel is a more attractive approach.[24,25] By taking some of the load from the hydrogel structure, the fibers may act to enhance the mechanical properties of the gel and decrease the probability of leakage.[24,25] So far, although deemed attractive, no simple and scalable methods have been presented for the creation of fiber-reinforced hydrogels.

Electrospinning is a commonly used method for creation of nanofibers. It is relatively simple and flexible and produces nanofibrous nonwoven materials closely resembling the natural ECM in structure and size scale.[32–34] Many cell types have been shown to prefer the nanofiber structures provided by electrospun materials.[33,35] Two important benefits of electrospun nanofibers are their large available surface area, suitable for efficient functionalization, and the possibility of making them from a wide variety of polymers, both natural and synthetic.[33,36] Collagen, elastin, poly(-lactic acid), poly(glycolic acid), polycaprolactone (PCL) and cellulose acetate are all among the most widely investigated electrospun polymers in tissue engineering applications.[8,34–37]

One previously studied approach in the creation of nanofiber-reinforced composite materials has been soaking sheets of electrospun fibers in a gel, stacking the sheets on top of each other and crosslinking them.[24] Important problems of such layered structure may, however, present insufficient porosity and inadequate interconnectivity as well as shearing and delamination of the different layers. Another study reported sheets of electrospun fibers that were cut up and physically mixed with a hydrogel.[38] Although this is a simple approach, agglomeration of fibers and potential toxicological effects of the small nanosized particles created by the cutting process may be drawbacks of such method. Recently, an interesting method based on a combination of electrospinning and electrospraying was investigated.[37] Electrospraying is similar to electrospinning but usually is performed with solutions of lower viscosity at higher electric field strengths, both differences resulting in a spraying of drops rather than spinning of fibers. As with electrospinning where a needle comprises the spraying device, electrospraying is limited in its capacity, and furthermore difficult to control with many parameters influencing the result. Therefore, the method described in this study is based on a simpler air brush spraying method instead. With air brush spraying the properties of the solution to be sprayed is of less relevance than in electrospraying as it is the physical force of the pressure applied that is the driving force, rather than the more complex electrostatic force driving electrospraying. In line with that, the quantity of spraying can be readily controlled by simply changing the pressure rather than adjusting electric field strength or solution properties as needed in electrospraying. Additionally, air brush spraying can be readily scaled and is already currently employed at the manufacturing scale for the purpose of painting and coating in the automotive industry.

In this work, a method has been developed for simultaneous electrospinning of PCL nanofibers and air brush spraying of a gellan gum solution, creating a nanofiber-reinforced gellan gum gel with enhanced mechanical properties and with structures mimicking the native NP.

EXPERIMENTAL

All polymers and solvents were purchased from Sigma-Aldrich (USA) and used as received, unless stated otherwise.

2 wt % Gellan gum (Gelzan CM, weight-average molecular weight = 1,000,000), was dissolved in 0.2*M* NaOH in water under stirring at 80°C. PCL, weight-average molecular weight = 80,000, was dissolved in chloroform/methanol (80:20) at a concentration of 13 wt %. A methyl red derivative (MRD; 0.4 wt %) was incorporated into the electrospinning solution to allow for quantification of the amount of fibers in the samples as well as for visualization of the fiber distribution.

Electrospinning/Spraying

The gellan gum solution was loaded into an airbrush (Mini airbrush, Am-Tech, Biltema, Sweden) and sprayed onto a rotating grounded collector at a pressure of 2 Bar. Gellan gum solution (60 mL) was used in each experiment. The collector rotated into a bath of 3–7% $CaCl_2$, allowing for pickup of Ca ions that facilitated gelation over time.

When incorporating PCL nanofibers into the gel, nanofibers were electrospun simultaneously with the gellan gum spraying. The PCL solution was loaded into a syringe capped with a blunt needle (i.d. = 0.6 mm) and charged with 20–24 kV. Pumping rate of the polymer solution was 0.8 mL/h and the distance between the tip of the needle and the grounded metal collector was 12 cm.

Each experiment was carried out for 10 or 15 min, during which time both electrospinning and spraying were carried out,

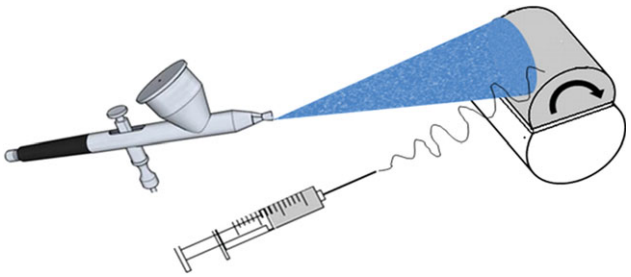

Figure 1. Simultaneous electrospinning of the nanofibers and air-brush spraying of the gellan gum solution. The collector rotated in a $CaCl_2$ solution to allow for gelation of the gellan gum. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to allow for a low (<70 ng PCL/cm^3 gel) and high (<100 ng/cm^3) amount of nanofibers, respectively to be incorporated. The amounts were calculated upon knowing the dimensions of the collected samples and the deposition rates of nanofibers and gel. The same amount of gellan gum was used in all experiments, hence the rate of spraying was adjusted accordingly by manually pausing the spraying in short intervals. Samples for further analysis were punched out from the collected gel using a 17 mm punch.

Rheology

Rheological properties were measured with a rotational rheometer (CS Bohlin Rheometer) in a plate–plate configuration at 25°C. Oscillatory frequency ranged between 0.01 and 10 Hz. Two gels per type were measured and each measurement was carried out three times.

Ultraviolet–visible (UV–vis)

The amount of nanofibers in each sample was determined indirectly by incorporating MRD into the nanofibers and measuring UV absorbance. A known concentration of MRD was mixed into the spin solution and samples were produced as described previously. The samples were thereafter dissolved and diluted in chloroform and absorbance was measured at 433 nm using a PerkinElmer UV–vis spectrophotometer Lamda 14. Loading of MRD into the samples was determined using a calibration curve.

Scanning Electron Microscopy (SEM)

The morphology of the nanofibers distributed in the gels was investigated using low vacuum SEM, FEI Quanta 200F, at a pressure of 80 Pa. No sample preparation was needed, other than cutting the gels in smaller pieces suitable for the sample holder.

RESULTS AND DISCUSSION

Creation of Nanofiber-Reinforced Gels

To create a hydrogel reinforced with electrospun nanofibers, gellan gum solution was sprayed onto a mandrel rotating into a bath of calcium chloride to form a hydrogel, whereas simultaneously PCL nanofibers were electrospun onto the same collector (Figure 1). In contrast to previously studied approaches, the method developed here allows for simultaneous formation of both gel and fibers, thus avoiding layering, and also provides possibilities of scale up.

Successful formation of hydrogels with different amounts of nanofibers was achieved, as seen in low vacuum SEM analysis as well as in incorporation and optical observation of a MRD within the nanofibers. Low vacuum SEM analysis was done to visualize the reinforcing PCL nanofibers (Figure 2). To allow for observation of the fibers the pressure in the low vacuum SEM was set at 80 Pa, resulting in a limited evaporation of sample surface water. This was deemed useful and necessary for revealing and studying the morphology of the fibers, although it may increase the risk of artifacts. It should be noted that the SEM analysis was performed mainly to get a general estimation of fiber sizes and fiber morphology, thus no conclusions can be drawn from the SEM images regarding the overall structure of the constructs. It was found that the fibers in all gels seemed smooth and had diameters in the range of 0.56 ± 0.26 μm and 0.71 ± 0.29 μm for the high [Figure 2(b)] and low [Figure 2(c)] fiber density gels, respectively. Pure PCL nanofibers [Figure 2(d)] had fiber diameters of 0.40 ± 0.17 μm. Although there is usually a distribution in fiber diameters intrinsic to the electrospinning process, it seems as if a higher fiber density resulted in small fiber diameters, with the smallest fibers occurring in a pure nanofiber matrix and the largest fibers occurring in the gel with the lowest fiber density. The reason for this is yet to be understood. During the SEM analysis it was furthermore noted that the occurrence of fibers was indeed higher in the high density nanofiber gel compared to the low density nanofiber gel [Figure 2(b, c)], thus confirming the possibility of incorporating different amounts of nanofibers into the hydrogel.

The incorporation of fibers was further visualized by including MRD in the electrospinning solution, rendering the fibers yellow in color. The incorporation of different amounts of fibers could thereby be confirmed colorimetrically, with stronger yellow color indicating the incorporation of more fibers (Figure 3). Images of the gels indeed shows that a gel with no nanofibers incorporated is transparent [Figure 3(a)], whereas a gel containing a low density of nanofibers is somewhat more yellowish in color [Figure 3(c)] and a gel with a high density of nanofibers is significantly more yellow in color [Figure 3(b)].

The quantification of the amount of MRD was performed using UV–vis. The results confirmed that a longer electrospinning time allows for incorporation of more MRD, that is, more nanofibers are incorporated into the fibers as the electrospinning time increase (Figure 4). This is consistent with the SEM analysis and points to the utility of the presented method for incorporating varying amounts of electrospun fibers into a hydrogel matrix.

Evaluation of the Nanofiber Reinforcement

The IVD is exposed to a lot of external forces, compressive as well as shearing, and an IVD substitute must therefore be evaluated based on this. Rheological measurements were carried out to investigate the mechanical properties of the gels under shear stress and to evaluate the potential effect of incorporation of fibers. The results are shown in Figure 5. From Figure 5, it can be seen that all samples exhibit an elastic behavior under

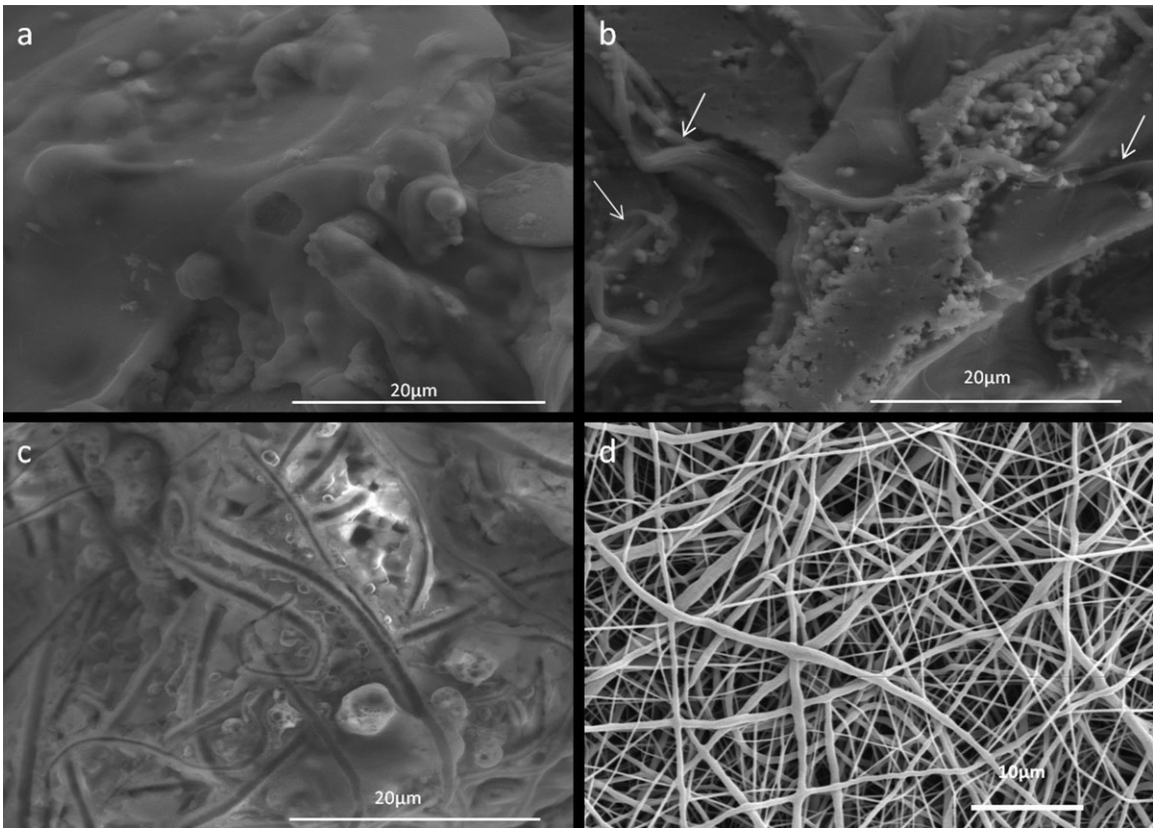

Figure 2. Low-vacuum SEM images of the (a) pure gel, (b) gel with a low density of nanofibers, (c) gel with a high density of nanofibers, and (d) pure nanofibers. The arrows indicate the PCL nanofibers.

dynamic conditions over the range of frequencies tested, as indicated by storage modulus (G') always being larger than the loss modulus (G''). The results for the pure gellan gum gel are in line with what has been previously reported.[21,39] An increase in modulus, both G' and G'', of the gels containing a low density of nanofibers indicates a desired reinforcing effect of the nanofibers. The concentration of gellan gum and $CaCl_2$ are kept constant, as well as the construct dimensions, so the effect achieved is due to the addition of fibers. Considering the extremely small amount of nanofibers needed to achieve this effect, these data shows the potential of the method and the use of nanofibers as reinforcement.

Although a small amount of nanofibers seems to result in reinforcement, the opposite was observed in the constructs with a larger amount of nanofibers. In that case, the modulus decreased compared to both other types of gels tested. This may be a result of a morphological change of the gel structure where the occurrence of nanofibers disrupts the continuity of the gel network, hence resulting in an overall weakening of the construct. Furthermore, kinetics of the gelling process may play part in the observed phenomenon if air pockets are allowed to form. With more fibers giving more possibilities for air to be trapped, an increase in fiber content could result in a weaker material.

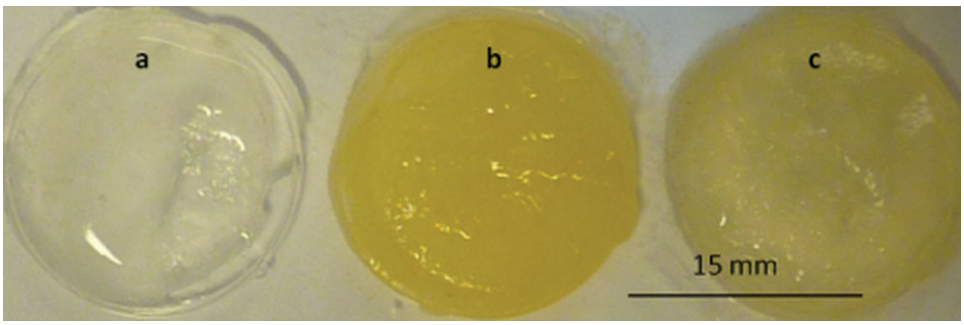

Figure 3. Image of the three different types of gels investigated: (a) pure gellan gum gel, (b) high-density nanofibers in gellan gum gel, and (c) low-density nanofibers in gellan gum gel. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

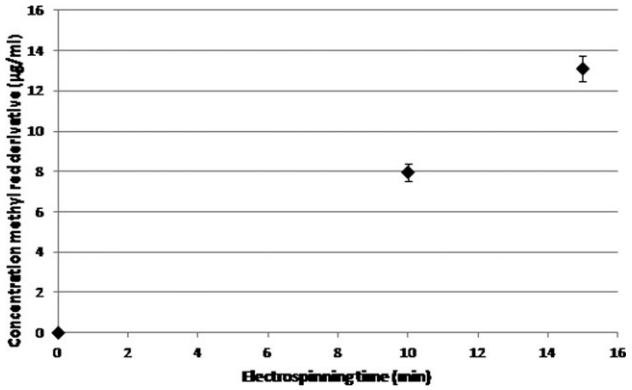

Figure 4. Diagram of the concentration of MRD in the samples at 10 and 15 min of electrospinning as determined by UV–vis.

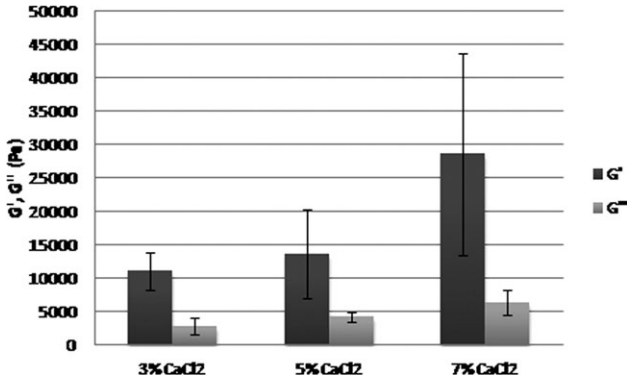

Figure 6. G' and G'' after 10 min of electrospinning and spraying with different $CaCl_2$ concentrations: 3, 5, and 7%. An oscillatory frequency of 1 Hz was used.

In the experiments presented so far, the gellan gum concentration and $CaCl_2$ concentration have both been constant, however, these also affect the properties of the gel–fiber constructs, as reported in previous studies on gellan gum gels.[39] To illustrate, it was shown that when varying the concentration of $CaCl_2$ in the bath in which the collector was rotating the modulus of the gels increased with increasing concentration of $CaCl_2$ (Figure 6; keeping everything else constant, that is, 2 wt % gellan gum and low amount of nanofibers). All the gels retained their elastic behavior over the range of $CaCl_2$ concentrations being investigated (3–7%), with G' constantly being larger than G''. Thus, ion concentration may be a useful parameter in tailoring the properties of the gels. Similar results were seen when varying the gellan gum concentration (results not shown) also in agreement of what has been previously found when studying gellan gum gels.[39] The effect of gellan gum and $CaCl_2$ concentrations thus seems to be the same regardless of the addition of nanofibers. Together with the reinforcing effect of the nanofibers, the $CaCl_2$ and gellan gum concentrations makes up the basis for tailoring material properties.

The achieved results of modulus are in the range of what has previously been reported for native human NP. Iatridis et al.[40] reported a complex modulus of 7.4–11.3 kPa within a frequency range of 1–10 Hz. The comparable values for the reinforced gels of this work are in the range of 5–36 kPa. With mechanical properties being of outmost importance for biological substitutes, limiting the range to better match the native NP may in the end be necessary for construction of a functional tissue. Further evaluations are needed, but it shows the potential and the relevance of the work for creation of hydrogels with adequate mechanical behavior. Furthermore, the use of an air brush to distribute the gel allows for upscaling of the process and using electrospinning opens up for possibilities to tailor features such as fiber morphology, scaffold porosity, material selection, and incorporation and release of biomolecules.

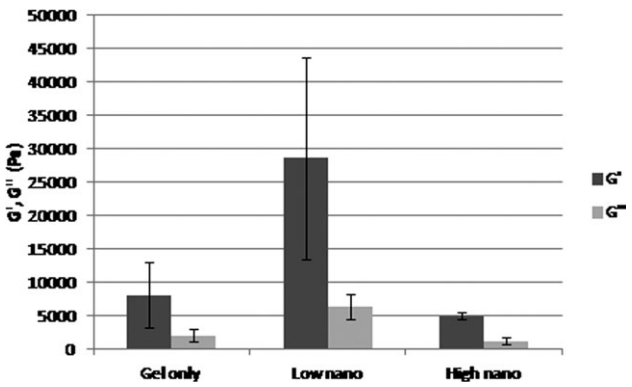

Figure 5. Rheological measurements showing G' and G'' at an oscillatory frequency of 1 Hz for the pure gellan gum gel (gel only) and the gellan gum gel with a low (low nano) or high (high nano) amount of nanofibers.

CONCLUSIONS

In the presented work a method is described by which it is shown possible to create hydrogels reinforced with nanofibers. The nanofiber reinforcement is implemented by spraying a hydrogel simultaneously with electrospinning of nanofibers, allowing for a homogeneous mixture of fibers and gel without layering effects and with potential to be upscaled. It is shown that inclusion of only a small amount of nanofibers have a reinforcing effect and furthermore it is shown that more or less reinforcement can be achieved by changing the amount of fibers alone. A high amount of nanofibers, however, results in a lowering of the shear modulus, potentially as a result of a weakening of the crosslinking of the gel structure. The degree of crosslinking in the gellan gum, affected by gellan gum concentration and $CaCl_2$ concentration can also be used to tailor the mechanical properties of the material to best suit the application. Further design parameters of interest for future applications are e.g. fiber morphology, scaffold porosity, fiber alignment, incorporation of biomolecules etc. The presented method shows potential in creation of nanofiber-reinforced gels with properties suitable for tissue engineering applications.

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