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Fiber-reinforced colloidal gels as injectable and moldable biomaterials for regenerative medicine

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ABSTRACT

Hydrogels are the preferred material choice for various strategies in regenerative medicine. Nevertheless, due to their high water content and soft nature, these materials are often mechanically weak, which limits their applicability. This study demonstrates mechanical reinforcement of colloidal gels at microscale using discrete polyester fibers, as confirmed by rheological, compression and nanoindentation tests. This reinforcement strategy results in injectable and moldable colloidal gels with improved mechanical performance. The fully organic gels presented here are cytocompatible and can maintain their mechanical integrity under physiological conditions. Consequently, these gels exhibit a strong potential for applications in tissue engineering and regenerative medicine.

1. Introduction

Hydrogels are the preferred material choice for various strategies in regenerative medicine [1]. These materials can mimic the structure and properties of natural tissues [2], and offer several possibilities to deliver cells [3] as well as various biomolecules such as drugs [4] and growth factors [5]. Recently, injectable hydrogels have gained interest in the biomedical field due to their applicability for minimally invasive treatments [6] and the production of complex hydrogel structures at room or body temperature using techniques such as 3D printing [7] and injection molding [8]. Nevertheless, due to their high water content and soft nature, these hydrogels are often mechanically weak, which limits their applicability for biomedical applications considerably [9]. This mechanical weakness is a major limiting factor for applications which require sufficient strength and stiffness such as regeneration of connective tissues [1, 9]. The most common approach to enhance the stiffness of hydrogels is the increase of their cross-linking degree using non-reversible covalent bonds, which usually renders the hydrogels non-injectable [10–13]. The adjustment of mechanical properties of hydrogels has also been achieved by e.g. controlling the polymer concentration [14], modification of the gelation condition (e.g. temperature) [15], and the variation of the salt concentration in the liquid phase [16]. An emerging strategy for the preparation of mechanically robust hydrogels involves reinforcement of hydrogels with fibers [17–19]. This strategy is based on fiber-based reinforcement mechanisms as observed in nature for tissues such as articular cartilage, the

mechanical properties of which are directly correlated with fiber content and orientation [20, 21]. Incorporation of fibers can increase the stiffness of hydrogels [19], but also enhance their toughness through crack bridging mechanism [22]. However, the majority of synthetic fiber-reinforced hydrogels are made of continuous interconnected fiber meshes or constructs, which hampers their injectability or moldability [18, 23, 24]. Attempts to reinforce hydrogels using discrete fibers are still scarce [19, 25–28]. Furthermore, injectable hydrogel systems which have been reinforced with discrete fibers were only injectable prior to their gelation, requiring a post-injection photo-polymerization [25, 26], chemical cross-linking [19, 27–29], or physical cross-linking [30] step to achieve mechanical stability. For example, in two studies [19, 28], Poveda-Reyes et al. used short polymeric microfibers to reinforce gelatin gels by dispersing up to 1.5 w/v% of the microfibers in a gelatin precursor solution. These injectable dispersions could be then cross-linked enzymatically, resulting in composite hydrogels with storage and Young moduli up to around 500 Pa (at 1 Hz) and 9 kPa, respectively. Nevertheless, the use of higher fiber contents resulted in fiber aggregation which limited the injectability of the dispersions. Moreover, after gelation, these composite materials were not injectable anymore. Consequently, it remains a challenge to produce injectable fiber-reinforced hydrogels.

To this end, colloidal gels are an interesting class of hydrogels in view of their particulate nature [31–35]. These gels assemble into solid-like interconnected networks [36], but they possess injectable, adaptive and self-healing features due to the reversibility of their physical

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interparticle bonds [31, 34]. Nevertheless, similar to other physically cross-linked hydrogels, polymeric colloidal gels suffer from weak mechanical properties [31, 33, 35]. Although mechanical reinforcement of colloidal gels with inorganic nanoparticles was shown to be effective, these nanocomposite gels are still too weak to resist compressive loading [32] or display brittle fracture under compression [34]. For instance, this reinforcement strategy recently delivered a novel silica-gelatin nanocomposite colloidal gel which was highly elastic and could withstand substantial compressive and tensile loads (e.g. compressive modulus ~ 120 kPa) [34]. Nevertheless, various applications such as soft tissue engineering often require biomaterials that are fully organic and biodegradable [37–39]. These requirements necessitate a new strategy for the development of fully organic colloidal gels that are not only injectable, but also are mechanically robust.

Here, we report the development of fully organic fiber-reinforced colloidal gels for applications in regenerative medicine. We used gelatin nanoparticles as colloidal building blocks, which can assemble into colloidal gel networks, and reinforced the colloidal gels with discrete sub-micron poly-L-lactic acid (PLLA) fibers of various lengths in order to achieve mechanically strong hydrogels. Following this strategy, we were able to produce fully organic colloidal gels which were cyto-compatible, injectable/moldable and mechanically strong.

2. Materials and methods

2.1. Materials

Gelatin type B (from bovine skin, gel strength ~ 225 g Bloom) and glycine were purchased from Sigma-Aldrich (Steinheim, Germany). PLLA pellets (PURASORB PL 65) were provided by Corbion Purac (Gorinchem, The Netherlands). Dulbecco Modified Eagle Medium (DMEM) and fetal calf serum (FCS) were obtained from Gibco (Eggenstein, Germany) and Sigma-Aldrich (Steinheim, Germany), respectively. All other chemicals were of reagent grade and were used without any purification.

2.2. Synthesis of gelatin nanoparticles

Gelatin nanoparticles were synthesized using a two-step desolvation method [31]. In the first step, 50 g of gelatin powder was dissolved in 1 L of distilled water at 50 °C. Afterward, 1 L of acetone was rapidly added to the gelatin solution and the mixture was left at room temperature for 1 h to precipitate high molecular weight gelatin. Thereafter, the supernatant was discarded and the gelatin precipitates were dissolved in 800 mL of distilled water and lyophilized for further usage. In the second step, 3.75 g of the lyophilized gelatin was dissolved in 75 mL of distilled water and the pH of solution was set to 2.5 by adding hydrochloric acid. Thereafter, 225 mL of acetone was slowly added to the gelatin solution at 50 °C using a syringe pump with a dripping rate of 12 mL/min during vigorous stirring of the solution at 1000 rpm. To cross-link the gelatin nanoparticles, 555 μ L of glutaraldehyde solution (25 wt%) was added dropwise to the gelatin dispersion at room temperature and left to react overnight while stirring at 600 rpm. In the next day, 300 mL of a 0.1 M aqueous solution of glycine was added to the gelatin dispersion to block unreacted aldehyde groups. After 1 h of stirring, the dispersion was filtered with a nylon mesh (100 μ m mesh size). Subsequently, the dispersion was washed with distilled water through four centrifugation (each at 5000 rpm for 90 min) and redispersion steps. Thereafter, the nanoparticles were dispersed in acetone:water (1:3; V:V) solutions, frozen with liquid nitrogen, and lyophilized for further use.

2.3. Synthesis of PLLA fibers

PLLA pellets were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol at a concentration of 2.5 wt% and subsequently loaded in a syringe

connected to a 21 gauge nozzle. Consequently, sub-micron PLLA fibers were obtained by means of an electrospinning apparatus (Fuecnce Co., Wako, Japan). Electrospinning was performed at a voltage of 20 kV, a flow rate of 5 μ L/h and an air gap of 20 cm. Solidified fiber meshes were collected on an aluminum foil and placed in the chemical fume hood for 24 h in order to remove any residual solvents. Thereafter, the electrospun fiber meshes were aminolyzed by immersion of the meshes in a 5% ethylenediamine solution in isopropanol at 37 °C for 3, 4 or 6 h [40]. After the aminolysis procedure, the suspension of nanofibers was centrifuged and washed two times with distilled water followed by a 1 min sonication step at 100% amplitude and 50% duty cycle using a sonication probe (UP100H, Hielscher Ultrasonics, Teltow, Germany) in order to facilitate the fragmentation of the fiber. Thereafter, the fibers were freeze dried before further use.

2.4. Preparation of hydrogels

Hydrogels with a solid content of 10 w/v% ($\sim 30\%$ particle volume fraction) were prepared by adding water or an aqueous dispersion of fibers (with 0–500 mM of dissolved sodium chloride) to the lyophilized gelatin nanoparticles. Thereafter, the solution/dispersion and nanoparticles were thoroughly mixed using a spatula and vortexing (1 min). Thereafter, the samples were centrifuged at 5000 rpm for 1 min to obtain bulk gels.

2.5. Dynamic Light Scattering (DLS)

DLS measurements were performed using a Zetasizer® Nano-S instrument (Malvern Instruments Ltd., UK) to determine the hydrodynamic size of gelatin nanoparticles and PLLA fibers. The measurements were performed using MilliQ water as the dispersion medium and each test was carried out at 25 °C for 60 s ($n = 3$).

2.6. Zeta potential

Zeta potential measurements were performed using a Zetasizer® Nano-S instrument (Malvern Instruments Ltd.) to determine the net surface charge of gelatin nanoparticles and PLLA fibers. The measurements were performed at 25 °C, and HEPES buffer (5 mM, pH = 7) was used as the dispersion medium. Zeta potential values were calculated by the Zetasizer software based on the measured mobility of nanoparticles according to the Smoluchowski equation ($n = 3$).

2.7. Scanning Electron Microscopy (SEM)

A Field Emission Scanning Electron Microscope (FE-SEM; Sigma 300, Zeiss, Germany) was used to evaluate the morphology of lyophilized nanoparticles, fibers and gels. All electron microscopy samples were prepared by fixing the samples on a sticky carbon tape and sputter-coating with a conductive 10 nm thick chromium layer.

2.8. Mechanical evaluation of hydrogels

2.8.1. Rheology tests

An AR2000ex rheometer (TA instruments, UK) with a flat steel plate geometry (20 mm diameter) was used for rheological evaluation of the hydrogels. The measurements were performed at 25 °C with an operating gap of 500 μ m. Each hydrogel was first characterized by means of frequency sweeps (0.1–10 Hz; at 1% strain), followed by a time sweep (1 min, 1 Hz and 1% strain), a strain sweep (0.1–1000% strain, and 1 Hz) and a time sweep (1 min, 1 Hz and 1% strain) step. The storage modulus (G') of the gels was defined as the average G' during the first time sweep step. The yield strain of the gels was determined as the strain at which G' and G'' were equal. The self-healing capability of hydrogels after shear-induced destruction was evaluated by quantifying the recovery (%) of their storage modulus (G'). The recovery of G' (%)

Fig. 1. Building blocks and formation of colloidal gels. Scanning electron micrographs of (a) gelatin nanoparticles, and PLLA fibers aminolyzed for (b) 6 h, (c) 4 h and (d) 3 h. Schematic illustration of the formation of fiber-reinforced gels is shown in (e).

was determined as the ratio between the storage modulus 1 min after strain sweep-induced gel network destruction (0.1–1000% strain) relative to their original storage modulus. This protocol mimics the gel network destruction by high shear forces during the extrusion through a narrow needle followed by recovery of the gel after the injection procedure. The shear-thinning behavior of the gels was determined through steady flow experiments by measuring their viscosity while varying the shear rate from 0.1 to 100 s^{-1} . Three samples were measured for each experimental condition ($n = 3$).

2.8.2. Compression tests

Compression tests were performed using an LS1 (Lloyd Instruments, UK) universal testing machine equipped with a 10 N load cell. Cylindrical hydrogel specimens were prepared by injecting the hydrogels into cylindrical Teflon molds (diameter: 8 mm, height: 5 mm) and keeping them at 4 °C overnight. The compression tests were carried out at a loading rate of 0.5 mm/min. The elastic modulus of the samples was calculated from the average slope of the initial part (0 to 10% strain) of their stress-strain curves. Three samples were measured for each experimental condition ($n = 3$).

2.8.3. Nanoindentation tests

Mechanical properties of the hydrogels at the microscopic level were measured using a PIUMA nanoindentation instrument (Optics11, The Netherlands). The measurements were carried out using a spherical nanoindentation probe with a stiffness of 0.492 N/m and a radius of 45 μm. The hydrogel samples were filled into cylindrical plastic molds which were fixed on plastic petri dishes using epoxy glue. The petri dishes were filled with MilliQ water and indentations were made in these wet conditions at room temperature with a probe displacement velocity of 9 μm/s at three different spots ($n = 3$) on the surface of each type of hydrogel. Elastic moduli were calculated by the PiUma software using the method provided by Oliver and Pharr for spherical indenters [41, 42].

2.9. In vitro cytocompatibility

The cytocompatibility of the hydrogels was assessed by culturing

mouse fibroblastic NIH 3T3 cells on the gel surface and performing an alamarBlue® assay to assess the metabolic activity of the cultured cells. The nanoparticles and fibers were sterilized by acetone/ethanol rinsing followed by a freeze-drying step. The hydrogels were prepared inside a fume hood using autoclaved MilliQ water and injected into cylindrical plastic molds (diameter: 6 mm, height: 4 mm), which were subsequently placed (including molds) inside a 48 well plate. 1×10^4 fibroblasts were seeded on the top surface of each hydrogel sample located inside the mold. After 10 min, the cell culture medium was added delicately into each well to confine the cells within the mold and the cells were cultured for up to 7 days. As cell culture medium, we used DMEM supplemented with 10% FCS, penicillin (100 U/mL) and streptomycin (10 μg/mL). At each time point, an alamarBlue® stock solution (0.1 mg/mL in PBS) was 10 times diluted with serum-free DMEM. This solution was transferred to each well at a final volume of 1 mL and then incubated at 37 °C for 4 h in the dark. Thereafter, the optical density of the solutions was measured at 570 nm and 600 nm (reference wavelength) using a multi-mode spectrophotometer (Biotek, Winooski, USA). Four samples of each group were analyzed for the experiment. The metabolic activity of cells in each sample was calculated according to the assay protocol (BIO-RAD, Veenendaal, Netherlands) using no-cells blanks as negative control and the cells cultured on pure gelatin gels as positive control. The values were expressed as % relative to the metabolic activity of cells cultured on fiber-free hydrogels at day 1.

2.10. Statistics

All values were presented as average $\pm$ standard deviation. The statistical analyses were carried out using GraphPad Prism 7 software (GraphPad Software Inc., San Diego, CA, USA). Statistical differences between experimental groups were determined by one-way ANOVA with Tukey's multiple comparisons test. $P < 0.05$ was considered as significantly different.

3. Results and discussion

Particles as basic building blocks of colloidal gels play a key role in determining the properties of these materials [36]. Therefore, the

physicochemical properties of the individual particles were characterized in more detail. Fig. 1 shows the scanning electron micrographs of the particulate building blocks that we used in this study as well as a schematic depiction of our methodology to prepare the colloidal hydrogels. The gelatin nanoparticles had an average diameter of 210 ± 64 nm (Fig. 1a), which upon swelling increased to $\sim 332 \pm 3$ nm ($PDI = 0.14 \pm 0.02$) as measured by means of DLS. Previous studies [31, 34] have shown that despite their electrostatic long-range repulsion (Zeta potential of -23 ± 2 mV at pH 7), the gelatin nanoparticles are able to form interparticle bonds when they are in close proximity. Such bonds arise from short-range hydrophobic and ionic interactions between the dangling gelatin chains on the surface of nanoparticles and can lead to the formation of colloidal gel networks. We hypothesized that these dangling chains would also interact with the amine and remaining carboxyl groups of aminolyzed PLLA fibers [40], leading to the formation of physical crosslinks between these fibers and gelatin nanoparticles. As shown in Fig. 1(b–d), the PLLA fibers produced in this study exhibited an average diameter of 795 ± 78 nm and average lengths of 2.2 ± 0.7 , 7.1 ± 3.2 and 19.1 ± 12.1 μ m when aminolyzed for 6, 4 and 3 h, respectively.

To assess the distribution of PLLA fibers within the hydrogels, we lyophilized the hydrogels and evaluated their morphology by electron microscopy. Fig. 2 shows the scanning electron micrographs of various types of lyophilized colloidal gels. These images reveal a homogeneous dispersion of the fibers throughout the hydrogels for all compositions. Nevertheless, in samples prepared with long PLLA fibers (aminolyzed for 3 h), the fibers appeared to protrude from the hydrogel matrix (Fig. 2f). Importantly, imaging of the samples at high magnification showed that the PLLA fibers were covered with spherical gelatin nanoparticles (Fig. 2c), which indeed suggests a high affinity between the fibers and the gelatin nanoparticles.

Subsequently, we investigated the effects of fiber incorporation on the viscoelastic properties of the hydrogels (Fig. 3). Importantly, all hydrogels showed frequency-independent solid-like behavior (Fig. S1). The rheological measurements revealed a significant reinforcement of the hydrogels upon incorporation of the fibers, as evidenced by a 15-fold increase of the storage modulus (G') of the gels upon fiber incorporation. The highest reinforcing efficacy was observed for hydrogels reinforced with fibers aminolyzed for 4 h, which showed

significantly higher storage moduli as compared to the other groups at a fiber/gelatin ratio (w/w) of 1, indicating that the average length of 7.1 ± 3.2 μ m of these fibers was the fiber length which reinforced these colloidal gels most effectively. Furthermore, based on these results, we postulate that longer aminolysis times would result in inferior mechanical properties of the fibers and too low aspect ratios, while shorter aminolysis times might compromise the dispersion. Consequently, there seems to be a balance between dispersion, mechanical properties and dimensions of the fibers for the effective reinforcement of the colloidal gels.

However, the lowest $\tan(\delta)$ and highest yield strain were observed for hydrogels reinforced with the shortest fibers (2.2 ± 0.7 μ m after aminolysis for 6 h). This effect might be caused by a more uniform distribution of these small fibers throughout the gels and a stronger interaction with gelatin nanoparticles as a result of their higher specific surface area. In order to assess the self-healing ability of the hydrogels, we quantified the recovery of the storage modulus after destructive shearing up to 1000% strain. As shown in Fig. 3d, these experiments indicated that the reinforcement of colloidal gels with the PLLA fibers aminolyzed for 4 or 6 h negatively affected their self-healing capacity; upon incorporation of these fibers in the colloidal gels, the recovery of the storage modulus decreased from around 70% to as low as 40%. However, the reinforcement of colloidal gels with longer fibers (19.1 ± 12.1 μ m after aminolysis for 3 h) did not compromise their self-healing capacity since the recovery of the storage modulus remained statistically similar after the incorporation of fibers. These results indicate that the effect of fiber-reinforcement on the self-healing properties of colloidal gels depends on the fiber dimensions. The reduction of self-healing capacity upon incorporation of shorter fibers might result from their stiffer and less flexible behavior which compromised the dynamic nature of colloidal gelatin gels. As a consequence, the restoration of a uniform colloidal network after destructive shearing was hindered.

Since the most effective reinforcement of hydrogels was observed upon incorporation of fibers aminolyzed for 4 h, we selected the hydrogels prepared with these fibers for a more detailed study. To validate the applicability of the fiber-reinforced gels in biological fluids of high ionic strength, we studied the effect of ionic strength on the viscoelastic properties of the hydrogels by dissolving different concentrations of

Fig. 2. Scanning electron micrographs of lyophilized gelatin-PLLA colloidal hydrogels containing different amounts of PLLA fibers with different lengths (controlled by the aminolysis time). R and t correspond to fiber/gelatin (w/w) ratio and the aminolysis time of fibers, respectively.

Fig. 3. (a) Storage modulus (G'), (b) damping factor ($\tan(\delta)$), (c) yield strain, and (d) recovery of G' after destructive-shearing, for hydrogels reinforced with different amounts of fibers aminolyzed for 3, 4 or 6 h.

Fig. 4. (a) Storage modulus (G'), (b) damping factor ($\tan(\delta)$), (c) yield strain, and (d) recovery of storage modulus (G') after destructive shearing of fiber-reinforced colloidal gels of different ionic strength. All hydrogels were prepared from PLLA fibers aminolyzed for 4 h and a fiber/gelatin (w/w) ratio of 1.

sodium chloride in the solutions used for the hydrogel preparation. As shown in Fig. 4, the increase of ionic strength up to 500 mM did not compromise the viscoelastic properties of the hydrogels, as no statistical difference was observed between viscoelastic parameters at different ionic strengths. Since electrostatic interactions are screened at high ionic concentrations [32], these results indicate that other interactions such as hydrophobic and Van der Waals forces can also be responsible

for the formation of interparticle bonds in our gels. In the absence of such hydrophobic and Van der Waals interactions, other polymeric colloidal gels [43] were shown to lose their mechanical integrity at high ionic concentrations.

Importantly, as shown in Fig. 5a and Movie S1, the reinforcement of the gels with PLLA fibers did not compromise the injectability of these materials; the fiber-reinforced fibers showed shear-thinning behavior

Fig. 5. (a) Digital photograph of a fiber-reinforced colloidal gel during extrusion from a syringe. (b) Shear-thinning behavior of fiber-reinforced and fiber-free colloidal gels. (c) Digital photograph of a fiber-reinforced colloidal gel after molding. (d) Stress-strain curves of fiber-reinforced and pure gelatin colloidal gels during compressive loading. The fiber-reinforced gels were prepared at a fiber/gelatin (w/w) ratio of 1, using 4 h aminolyzed fibers.

Fig. 6. (a) Load-displacement curves and (b) elastic modulus (E) of hydrogels with different fiber contents, measured by nanoindentation tests. **indicates statistical difference ($P < 0.005$). The fiber-reinforced gels were prepared using 4 h aminolyzed fibers.

Fig. 7. (a) Metabolic activity of fibroblast cells seeded on the hydrogels prepared from gelatin nanoparticles and PLLA fibers at different time points of cell culture, and (b) viscoelastic properties of the hydrogels after 7 days of cell culture. R indicates fiber/gelatin ratio (w/w). *, **, ***, and **** indicate statistical difference with $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively. The fiber-reinforced gels were prepared using 4 h aminolyzed fibers.

similar to fiber-free gels (Fig. 5b). This unique combination of fiber reinforcement and injectability was obtained owing to a sufficient amount of reversible interactions between the gelatin nanoparticles and PLLA fibers, as previous attempts [19] could not simultaneously achieve reinforcement and injectability upon incorporation of PLLA fibers into polymeric hydrogels. In addition to their injectability, the fiber-reinforced hydrogels also showed excellent moldability (Fig. 5c), which enabled the evaluation of their mechanical properties under compressive loading. As shown in Fig. 5d, these tests demonstrated that the fiber-reinforcement strategy significantly improved the mechanical resistance of the colloidal gels against compressive loading. Upon fiber incorporation, the elastic modulus of the hydrogels was increased almost tenfold from 2.4 ± 0.2 to 23.1 ± 2.1 kPa. The fiber-reinforced gels showed a linear elastic behavior for up to around 20% strain, after which crack formation and propagation occurred and the deformation proceeded plastically (Movie S2). Nevertheless, such visible fracture event was not observed for the fiber-free gels.

Rheological and compression tests describe the bulk mechanical behavior of hydrogels, but direct cell-biomaterial interactions occur at microscale. Therefore, we performed nanoindentation tests on our hydrogels using a micron-size spherical probe to elucidate the effect of fiber incorporation on mechanical properties of hydrogels at microscale. As shown in Fig. 6, upon incorporation of the fibers in the gels, the elastic modulus increased > 150-fold. Importantly, the microscopic mechanical reinforcement effect caused by the fibers occurred uniformly throughout the gel matrix, since standard deviations were low. These results clearly indicate that the fiber reinforcement effect is not limited to the macroscopic level but also occurs at the microscale. This considerable change of elasticity at the microscopic scale can be sensed by cells and direct cellular behavior such as differentiation [44]. For example, Engler et al. [45] have shown that the elasticity of biomaterials can influence the phenotype of mesenchymal stem cells, since matrices with elastic moduli of 0.1–1 kPa, 8–17 kPa, or 25–40 kPa were neurogenic, myogenic, or osteogenic, respectively. These elasticities were in the same range as our colloidal gels, which highlights the potential applicability of our fiber reinforcement strategy for engineering of various tissue types. Nevertheless, more detailed biological studies are required to elucidate the effect of elasticity of fiber-reinforced colloidal gels on cellular behavior.

Although the cytocompatibility of pure gelatin colloidal gels has been shown previously [32, 34, 46], the effect of incorporation of PLLA fibers on the cytocompatibility of these materials has not been studied yet. Therefore, we cultured fibroblastic NIH 3 T3 cells on our colloidal gelatin-PLLA gels. As shown in Fig. 7a, the alamarBlue® assay indicated that the fiber reinforcement of the colloidal gels did not cause cytotoxic effects for the fibroblastic cells. Nevertheless, we measured a decrease in metabolic activity of cells over time for all hydrogels, which can be attributed to e.g. contact inhibition effect upon reaching a high cell confluency [47, 48] and/or the migration of cells towards the inner area of the hydrogels [49]. Importantly, the rheological evaluation of the hydrogels after 7 days of cell culture showed that these materials retained their mechanical integrity during the cell culture period, which proves that the reinforcement of the gels with fibers was still effective after the cell culture period (Fig. 7b). However, after the cell culture, a decrease in storage modulus of fiber-reinforced gels and an increase in $\tan(\delta)$ of the fiber-free gels were observed which might be caused by fibroblasts. It has been shown that the remodeling of collagen gels by fibroblasts through collagen production and the secretion of proteolytic enzymes can stiffen these materials in a frequency-dependent manner [50]. Nonetheless, several studies [46, 51, 52] have demonstrated that proteolytic enzymes can degrade gelatin-based colloidal gels, potentially leading to enhanced swelling and decreased elasticity of such hydrogels. Overall, the cytocompatibility and mechanical stability of fiber-reinforced colloidal gels suggest a great potential for the use of these materials for applications in tissue engineering and regenerative medicine. Nevertheless, more detailed cell culture studies using

different cell types should be performed to characterize the in vitro cell response of these hydrogels in more detail for various application areas in soft tissue regeneration. Particularly, it is important to elucidate the biological effect of the mechanical reinforcement of these hydrogels in a cell-specific manner. Moreover, these in vitro experiments should be followed by in vivo tests in relevant animal models to evaluate the potential for clinical translation.

4. Conclusions

This study demonstrated successful mechanical reinforcement of colloidal gels using PLLA fibers. This reinforcement strategy did not compromise the injectability of these materials, and facilitated the preparation of moldable gels that were mechanically robust. Importantly, the fiber reinforcement of gels was effective at both macroscopic and microscopic levels. Finally, these fully organic gels were cytocompatible and could retain their mechanical integrity under physiological conditions for up to one week. This combination of properties is highly attractive for applications such as soft tissue regeneration. Consequently, these materials exhibit a strong potential for a wide range of applications in tissue engineering and regenerative medicine.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.msec.2018.06.038>.

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References

- [1] N. Annabi, A. Tamayol, J.A. Uquillas, M. Akbari, L.E. Bertassoni, C. Cha, G. Camci-Unal, M.R. Dokmeci, N.A. Peppas, A. Khademhosseini, 25th anniversary article: rational design and applications of hydrogels in regenerative medicine, *Adv. Mater.* 26 (2014) 85–124.
- [2] H. Gecik, F. Xu, X.H. Zhang, S. Moon, U. Demirci, Engineering hydrogels as extracellular matrix mimics, *Nanomedicine* 5 (2010) 469–484.
- [3] S. Utech, R. Prodanovic, A.S. Mao, R. Ostafe, D.J. Mooney, D.A. Weitz, Microfluidic generation of monodisperse, structurally homogeneous alginate microgels for cell encapsulation and 3D cell culture, *Adv. Healthc. Mater.* 4 (2015) 1628–1633.
- [4] T.R. Hoare, D.S. Kohane, Hydrogels in drug delivery: Progress and challenges, *Polymer* 49 (2008) 1993–2007.
- [5] R. Censi, P. Di Martino, T. Vermonden, W.E. Hennink, Hydrogels for protein delivery in tissue engineering, *J. Control. Release* 161 (2012) 680–692.
- [6] L. Yu, J.D. Ding, Injectable hydrogels as unique biomedical materials, *Chem. Soc. Rev.* 37 (2008) 1473–1481.
- [7] R.A. Barry, R.F. Shepherd, J.N. Hanson, R.G. Nuzzo, P. Wiltzius, J.A. Lewis, Direct Write Assembly of 3D Hydrogel Scaffolds for Guided Cell Growth, *Adv. Mater.* 21 (2009) 2407–2410.
- [8] S.C. Chang, J.A. Rowley, G. Tobias, N.G. Genes, A.K. Roy, D.J. Mooney, C.A. Vacanti, L.J. Bonassar, Injection molding of chondrocyte/alginate constructs in the shape of facial implants, *J. Biomed. Mater. Res.* 55 (2001) 503–511.
- [9] S. Utech, A.R. Boccacini, A review of hydrogel-based composites for biomedical applications: enhancement of hydrogel properties by addition of rigid inorganic fillers, *J. Mater. Sci.* 51 (2016) 271–310.
- [10] J.R. Tse, A.J. Engler, Preparation of hydrogel substrates with tunable mechanical properties, *Curr. Protoc. Cell Biol.* 47 (2010) 10.16.11–10.16.16.
- [11] S. Nemir, H.N. Hayenga, J.L. West, PEGDA hydrogels with patterned elasticity: novel tools for the study of cell response to substrate rigidity, *Biotechnol. Bioeng.* 105 (2010) 636–644.
- [12] B. Trappmann, J.E. Gautrot, J.T. Connelly, D.G.T. Strange, Y. Li, M.L. Oyen, M.A. Cohen Stuart, H. Boehm, B. Li, V. Vogel, J.P. Spatz, F.M. Watt, W.T.S. Huck, Extracellular-matrix tethering regulates stem-cell fate, *Nat. Mater.* 11 (2012) 642.
- [13] E. Hachet, H. Van Den Bergh, E. Bayma, M.R. Block, R. Auzely-Velty, Design of biomimetic cell-interactive substrates using hyaluronic acid hydrogels with tunable mechanical properties, *Biomacromolecules* 13 (2012) 1818–1827.
- [14] M. Diba, J. An, S. Schmidt, M. Hembury, D. Ossipov, A.R. Boccacini, S.C.G. Leeuwenburgh, Exploiting bisphosphonate–bioactive-glass interactions for

- the development of self-healing and bioactive composite hydrogels, *Macromol. Rapid Commun.* 37 (2016) 1952–1959.
- [15] N.A. Fletcher, E.L. Von Nieda, M.D. Krebs, Cell-interactive alginate-chitosan bio-polymer systems with tunable mechanics and antibody release rates, *Carbohydr. Polym.* 175 (2017) 765–772.
 - [16] M. Jaspers, A.E. Rowan, P.H.J. Kouwer, Tuning hydrogel mechanics using the Hofmeister effect, *Adv. Funct. Mater.* 25 (2015) 6503–6510.
 - [17] B. Lu, F. Lin, X. Jiang, J. Cheng, Q. Lu, J. Song, C. Chen, B. Huang, One-pot assembly of microfibrillated cellulose reinforced PVA–borax hydrogels with self-healing and pH-responsive properties, *ACS Sustain. Chem. Eng.* 5 (2017) 948–956.
 - [18] Y.W. Huang, D.R. King, T.L. Sun, T. Nonoyama, T. Kurokawa, T. Nakajima, J.P. Gong, Energy-dissipative matrices enable synergistic toughening in fiber reinforced soft composites, *Adv. Funct. Mater.* 27 (2017) 10.
 - [19] S. Poveda-Reyes, L.R. Meller-Ogliadoro, R. Martínez-Haya, T.C. Gamboa-Martínez, J.L. Gómez Ribelles, G. Gallego Ferrer, Reinforcing an injectable gelatin hydrogel with PLLA microfibers: two routes for short fiber production, *Macromol. Mater. Eng.* 300 (2015) 977–988.
 - [20] P. Calvert, Hydrogels for Soft Machines, *Adv. Mater.* 21 (2009) 743–756.
 - [21] G.E. Kempson, M.A.R. Freeman, S.A.V. Swanson, Tensile properties of articular cartilage, *Nature* 220 (1968) 1127.
 - [22] X. Zhao, Multi-scale multi-mechanism design of tough hydrogels: building dissipation into stretchy networks, *Soft Matter* 10 (2014) 672–687.
 - [23] A. Agrawal, N. Rahbar, P.D. Calvert, Strong fiber-reinforced hydrogel, *Acta Biomater.* 9 (2013) 5313–5318.
 - [24] C.-D. Young, J.-R. Wu, T.-L. Tsou, High-strength, ultra-thin and fiber-reinforced pHEMA artificial skin, *Biomaterials* 19 (1998) 1745–1752.
 - [25] A.C. Borges, P.E. Bourban, D.P. Pioletti, J.A.E. Manson, Curing kinetics and mechanical properties of a composite hydrogel for the replacement of the nucleus pulposus, *Combust. Sci. Technol.* 70 (2010) 1847–1853.
 - [26] A. Khoushabi, A. Schmock, D.P. Pioletti, C. Moser, C. Schizas, J.A. Manson, P.E. Bourban, Photo-polymerization, swelling and mechanical properties of cellulose fiber reinforced poly(ethylene glycol) hydrogels, *Combust. Sci. Technol.* 119 (2015) 93–99.
 - [27] O. Regev, C.S. Reddy, N. Nseir, E. Zussman, Hydrogel reinforced by short albumin fibers: mechanical characterization and assessment of biocompatibility, *Macromol. Mater. Eng.* 298 (2013) 283–291.
 - [28] S. Poveda-Reyes, A. Rodrigo-Navarro, T.C. Gamboa-Martínez, J.C. Rodríguez-Cabello, L. Quintanilla-Sierra, U. Edlund, G.G. Ferrer, Injectable composites of loose microfibers and gelatin with improved interfacial interaction for soft tissue engineering, *Polymer* 74 (2015) 224–234.
 - [29] X. Yang, E. Bakaic, T. Hoare, E.D. Cranston, Injectable polysaccharide hydrogels reinforced with cellulose nanocrystals: morphology, rheology, degradation, and Cytotoxicity, *Biomacromolecules* 14 (2013) 4447–4455.
 - [30] K.-K. Alicja, C. Marco, B. Tomasz, S. Krisztina, B. Andrea, B. Jan, Ś. Wojciech, PLA short sub-micron fiber reinforcement of 3D bioprinted alginate constructs for cartilage regeneration, *Biofabrication* 9 (2017) 044105.
 - [31] H. Wang, M.B. Hansen, D.W.P.M. Löwik, J.C.M. van Hest, Y. Li, J.A. Jansen, S.C.G. Leeuwenburgh, Oppositely charged gelatin nanospheres as building blocks for injectable and biodegradable gels, *Adv. Mater.* 23 (2011) H119–H124.
 - [32] H. Wang, M. Bongio, K. Farbod, A.W.G. Nijhuis, J. van den Beucken, O.C. Boerman, J.C.M. van Hest, Y. Li, J.A. Jansen, S.C.G. Leeuwenburgh, Development of injectable organic/inorganic colloidal composite gels made of self-assembling gelatin nanospheres and calcium phosphate nanocrystals, *Acta Biomater.* 10 (2014) 508–519.
 - [33] Q. Wang, L. Wang, M.S. Detamore, C. Berkland, Biodegradable Colloidal Gels as moldable tissue engineering scaffolds, *Adv. Mater.* 20 (2008) 236–239.
 - [34] M. Diba, H. Wang, T.E. Kodger, S. Parsa, S.C.G. Leeuwenburgh, Highly elastic and self-healing composite colloidal gels, *Adv. Mater.* 29 (2017) (1604672-n/a).
 - [35] J. Gu, Y. Zhao, Y. Guan, Y. Zhang, Effect of particle size in a colloidal hydrogel scaffold for 3D cell culture, *Colloids Surf. B Biointerfaces* 136 (2015) 1139–1147.
 - [36] P.J. Lu, D.A. Weitz, Colloidal particles: crystals, glasses, and gels, *Annu. Rev. Condens. Matter Phys.* 4 (2013) 217–233.
 - [37] Q.-Z. Chen, S.E. Harding, N.N. Ali, A.R. Lyon, A.R. Boccaccini, Biomaterials in cardiac tissue engineering: ten years of research survey, *Mater. Sci. Eng. R. Rep.* 59 (2008) 1–37.
 - [38] T.H. Qazi, D.J. Mooney, M. Pumberger, S. Geißler, G.N. Duda, Biomaterials based strategies for skeletal muscle tissue engineering: Existing technologies and future trends, *Biomaterials* 53 (2015) 502–521.
 - [39] S. MacNeil, Biomaterials for tissue engineering of skin, *Mater. Today* 11 (2008) 26–35.
 - [40] A. Polini, D.G. Petre, M. Iafisco, S. de Lacerda Schickert, A. Tampieri, J. van den Beucken, S.C.G. Leeuwenburgh, Polyester fibers can be rendered calcium phosphate-binding by surface functionalization with bisphosphonate groups, *J. Biomed. Mater. Res. A* 105 (2017) 2335–2342.
 - [41] W.C. Oliver, G.M. Pharr, An improved technique for determining hardness and elastic modulus using load and displacement sensing indentation experiments, *J. Mater. Res.* 7 (2011) 1564–1583.
 - [42] W.C. Oliver, G.M. Pharr, Measurement of hardness and elastic modulus by instrumented indentation: advances in understanding and refinements to methodology, *J. Mater. Res.* 19 (2011) 3–20.
 - [43] S.R. Van Tomme, M.J. van Steenberghe, S.C. De Smedt, C.F. van Nostrum, W.E. Hennink, Self-gelling hydrogels based on oppositely charged dextran microspheres, *Biomaterials* 26 (2005) 2129–2135.
 - [44] M. Ahearne, Introduction to cell-hydrogel mechanosensing, *Interface Focus* 4 (2014) 20130038.
 - [45] A.J. Engler, S. Sen, H.L. Sweeney, D.E. Discher, Matrix elasticity directs stem cell lineage specification, *Cell* 126 (2006) 677–689.
 - [46] H. Wang, Q. Zou, O.C. Boerman, A.W.G. Nijhuis, J.A. Jansen, Y. Li, S.C.G. Leeuwenburgh, Combined delivery of BMP-2 and bFGF from nanostructured colloidal gelatin gels and its effect on bone regeneration in vivo, *J. Control. Release* 166 (2013) 172–181.
 - [47] M.G.P. Stoker, H. Rubin, Density dependent inhibition of cell growth in culture, *Nature* 215 (1967) 171.
 - [48] D. Gal, P.C. MacDonald, J.C. Porter, J.W. Smith, E.R. Simpson, Effect of cell density and confluency on cholesterol metabolism in cancer cells in monolayer culture, *Cancer Res.* 41 (1981) 473–477.
 - [49] M. Diba, W.A. Camargo, M. Brindisi, K. Farbod, A. Klymov, S. Schmidt, M.J. Harrington, L. Draghi, A.R. Boccaccini, J.A. Jansen, J.J.J.P.v.d. Beucken, S.C.G. Leeuwenburgh, Composite Colloidal Gels Made of Bisphosphonate-Functionalized Gelatin and Bioactive Glass Particles for Regeneration of Osteoporotic Bone Defects, *Adv. Funct. Mater.* 27 (2017) 1703438.
 - [50] B. Babaei, A. Davarian, S.-L. Lee, K.M. Pryse, W.B. McConnaughey, E.L. Elson, G.M. Genin, Remodeling by fibroblasts alters the rate-dependent mechanical properties of collagen, *Acta Biomater.* 37 (2016) 28–37.
 - [51] H. Wang, O.C. Boerman, K. Sariabrahimoglu, Y. Li, J.A. Jansen, S.C.G. Leeuwenburgh, Comparison of micro- vs. nanostructured colloidal gelatin gels for sustained delivery of osteogenic proteins: Bone morphogenetic protein-2 and alkaline phosphatase, *Biomaterials* 33 (2012) 8695–8703.
 - [52] J. Song, J.C.E. Odekerken, D.W.P.M. Löwik, P.M. López-Pérez, T.J.M. Welting, F. Yang, J.A. Jansen, S.C.G. Leeuwenburgh, Influence of the molecular weight and charge of antibiotics on their release kinetics from gelatin nanospheres, *Macromol. Biosci.* 15 (2015) 901–911.