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Enabling Surgical Placement of Hydrogels through Achieving Paste-Like Rheological Behavior in Hydrogel Precursor Solutions

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Abstract

Hydrogels are a promising class of materials for tissue regeneration, but they lack the ability to be molded into a defect site by a surgeon because hydrogel precursors are liquid solutions that are prone to leaking during placement. Therefore, although the main focus of hydrogel technology and developments are on hydrogels in their crosslinked form, our primary focus is on improving the fluid behavior of hydrogel precursor solutions. In this work, we introduce a method to achieve paste-like hydrogel precursor solutions by combining hyaluronic acid nanoparticles with traditional crosslinked hyaluronic acid hydrogels. Prior to crosslinking, the samples underwent rheological testing to assess yield stress and recovery using linear hyaluronic acid as a control. The experimental groups containing nanoparticles were the only solutions that exhibited a yield stress, demonstrating that the nanoparticulate rather than the linear form of hyaluronic acid was necessary to achieve paste-like behavior. The gels were also photocrosslinked and further characterized as solids, where it was demonstrated that the inclusion of nanoparticles did not adversely affect the compressive modulus and that encapsulated bone marrow-derived mesenchymal stem cells remained viable. Overall, this nanoparticle-based approach provides a platform hydrogel system that exhibits a yield stress prior to crosslinking, and can then be crosslinked into a hydrogel that is capable of encapsulating cells that remain viable. This behavior

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may hold significant impact for hydrogel applications where a paste-like behavior is desired in the hydrogel precursor solution.

Keywords

Colloidal gel; yield stress; hyaluronic acid; nanoparticles

Introduction

Hydrogels are a promising class of tissue regenerative materials because of their high water content, 3D structure, tunable mechanical properties, and their ability to be delivered in a minimally invasive manner.[2, 3, 6] However, hydrogels lack the ability to be molded into a defect site by a surgeon because hydrogel precursors are liquid solutions that are prone to leaking after placement,[19, 22] which confounds their ability to be used by surgeons in the clinic. Therefore, although the main focus of hydrogel technology and developments are on hydrogels in their crosslinked form, our primary focus is on the fluid behavior of hydrogel precursor solutions (i.e., the fluid behavior of the hydrogel prior to crosslinking). As an alternative to traditional hydrogels, colloidal gels are mechanically dynamic paste-like materials that can be easily molded into place and will 'set' after placement.[26] Colloidal gels attain their cohesiveness through disruptable particle interactions and our research group has shown that these gels can successfully fill tissue defects, deliver bioactive signals, and promote new tissue formation in non-load bearing cranial defect applications.[4, 23–25] Our recent work has shown that colloidal gels with shear-thinning rheological behavior can be made out of solutions of hyaluronic acid (HA) nanoparticles.[7] These HA-based colloidal gels also have the ability to fully recover after compression to high strains and also after physically destroying and reassembling the gel, which may be attractive for applications such as for cartilage regeneration.[7] However, preliminary work demonstrated that these colloidal gels do not retain their integrity over time in culture. Therefore, we have created a platform system that combines the HA colloidal gels systems with traditional crosslinked HA hydrogels to form a hydrogel suitable for load-bearing applications that is paste-like prior to crosslinking for effective delivery *in situ*. Although other systems, including dermal fillers, employ HA particles with traditional crosslinked HA hydrogels,[9–13, 20, 21] or use alternate means to induce a set strength in injectable materials,[5, 15] our HA nanoparticles (HAnp) are fabricated with a specific molecular weight (MW) designed to achieve paste-like rheological behavior and a yield stress and they have never before been encapsulated within crosslinked HA hydrogels.[7] This yield stress is especially desirable to enable a surgeon to mold the material into the defect site without the concern that the material will flow or leak from the defect, which is the main concern for traditional hydrogel precursor solutions. The HAnp will also allow the surgeon to mold the hydrogel precursor solution to obtain appropriate contouring of the defect site, which in some cases may not be possible with traditional hydrogel precursor solutions. Therefore, combining these HAnp with traditional crosslinked HA hydrogels may allow the material to be implanted *in situ* with appropriate placement and contouring, and the precursor solution can then be crosslinked to form a more rigid structure. Thus, the primary objective of this work was to characterize the rheological behavior of HAnp-incorporated hydrogel precursor solutions. An additional objective was to

ensure that HAnp did not negatively influence the mechanics or cytocompatibility of the hydrogel after crosslinking.

Materials and Methods

Materials

Unless otherwise stated, all materials were purchased from Sigma-Aldrich (St. Louis, MO). EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) was purchased from Thermo Scientific (Rockford, IL). HA (16 kDa and 1 MDa) was purchased from Lifecore Biomedical (Chaska, MN). All cell culture materials were purchased from Invitrogen (Grand Island, NY).

Synthesis and Characterization of Methacrylated HA (MeHA) and HAnp

MeHA was prepared by reacting HA (MW 1 MDa) with 20-fold molar excess glycidyl methacrylate (e.g., 20 mol glycidyl methacrylate per 1 mol HA monomer) in the presence of 20-fold molar excess triethylamine and tetrabutyl ammonium bromide for 12 days stirring in a 50:50 water:acetone solution at 200 rpm. MeHA was then dialyzed against deionized (DI) water for 2 days and was then frozen and lyophilized. The degree of methacrylation was analyzed with ^{1}H NMR (Avance AV-III 500, Bruker) by calculating the ratio of the relative peak area of methacrylate protons to methyl protons.[14] HAnp were prepared using carbodiimide crosslinking chemistry using EDC with adipic acid dihydrazide (AAD) as the crosslinker.[7] Briefly, 300 mg HA (16 kDa) was dissolved in 120 mL DI water in a 500 mL round flask stirring at 300 rpm. Then, 200 mL acetone was added to the flask and stirred for 15 min. AAD (60 mg) was dissolved in 1 mL DI water and added to the flask for 10 min. Similarly, 140 mg EDC was dissolved in 1 mL DI water and added to the flask for 20 min. Another 200mL acetone was then added to the flask and the reaction was allowed to stir for 3 hours. The solution was then dialyzed against DI water for 2 days and the particles were frozen and lyophilized. Repeated batches of HAnp were fabricated in this manner and combined for later testing. Particle size was measured using a ZetaPALS dynamic light scattering instrument (Brookhaven, USA). Particle morphology was examined with Scanning Transmission Electron Microscopy (STEM) images using a FEI Technai G_2 transmission electron microscope at 200 kV.

Preparation of Colloidal Gels

Gels were made by mixing varying weight percents of HA (i.e., MeHA and HAnp) in 0.01M phosphate buffered saline (PBS) containing 0.05% (w/v) Irgacure (I-2959) photoinitiator (e.g., 15% HAnp = 15 mg HAnp in 100 μL PBS). Linear HA (HAlin) at 16 kDa (i.e., the same MW used to make the HAnp) was also mixed with MeHA as a control to discern whether yield stress differences were due to the HA being in the nanoparticulate form or due to the mere addition of extra HA.

Rheological Testing

Prior to crosslinking the hydrogels, the shear stress of the precursor solutions (n=5) were measured over a shear rate sweep of 1–100 s^{-1} using an AR-2000 rheometer (TA Instruments, New Castle, DE) equipped with a 20 mm diameter plate at 37 °C at a gap of

500 μm. Preliminary work suggested that a 15% HAnp solution was sufficient to obtain a yield stress, and 4% MeHA was chosen because it was at the reconstitution limit of MeHA. Formulations tested were 4% MeHA, 15% HAlin, 4% MeHA + 15% HAlin, 30% HAlin, 4% MeHA + 30% HAlin, 15% HAnp, 4% MeHA + 15% HAnp, 30% HAnp, and 4% MeHA + 30% HAnp. The yield stresses of solutions were calculated using a three parameter fitting technique in MATLAB (MathWorks, Natick, MA) to fit the data to the Herschel-Bulkley equation (Eq. 1), where τ is the shear stress, τ_0 is the yield stress, κ is the consistency index, γ is the shear rate, and n is the flow behavior index.

$$\tau=\tau_0+\kappa(\gamma)^{\mathrm{n}} \quad (1)$$

Oscillatory tests were performed first by doing a stress sweep at 1 Hz to determine the linear viscoelastic region of the solutions. Solutions (n=5) were then exposed to three phases of oscillatory shearing at 1 Hz: 5 minutes at a constant shear stress of 10 Pa (i.e., within the linear viscoelastic region of the pseudoplastic solutions), a disruption phase lasting 30 seconds at a constant shear stress of 1000 Pa (i.e., sheared above the yield stress), and another 5 minutes at a constant shear stress of 10 Pa.

Characterization of Crosslinked Hydrogels

Gel solutions of experimental groups containing 4% MeHA were placed in a 2 mm thick mold between glass slides and exposed to 312 nm UV light at 3.0 mW/cm^2 (Spectrolinker XL-100; Spectronics Corp.) for 15 min on each side. Gels were cut using a 3 mm biopsy punch. To calculate the swelling degree, gels were swollen in PBS for 24 hours and then weighed and lyophilized (n=6). The dry weight was recorded after lyophilization and the swelling ratio (Q) was calculated as the ratio of total wet mass to dry mass. To obtain the compressive modulus, gels were swollen in PBS for 24 hours or two weeks (n=6) and were compressed using a RSA-III dynamic mechanical analyzer (TA Instruments) at a rate of 0.005 mm/s until mechanical failure and the elastic modulus was calculated as the slope under the linear portion of the stress-strain curve.

Cell Viability

Rat Bone Marrow-Derived Mesenchymal Stem Cells (rBMSCs) were harvested from the femurs of male Sprague-Dawley rats (200–250g) following an approved University of Kansas IACUC protocol. The rBMSCs were cultured in monolayer until passage 4 for cell seeding. Media consisted of low glucose Dulbecco's Modified Eagle's Medium, 10% Qualified Fetal Bovine Serum, 1% Antibiotic-Antimycotic and was replaced every other day throughout culture. For encapsulation, cells were suspended in the photoinitiator solution at a cell density of 10 million cells mL^{-1} and then mixed with either 4% MeHA or 4% MeHA + 15% HAnp. Hydrogels were then fabricated using the same previously described technique to make acellular gels. After 4 weeks of culture, the gels were stained with live/dead reagent (2 mM calcein AM, 4 mM ethidium homo-dimer-1; Molecular Probes), incubated for 20 min, and then analyzed using fluorescence microscopy on a Zeiss Axio Observer A1 (Carl Zeiss, Oberkochen, Germany).

Statistics

SPSS statistical software was used to compare experimental groups using a single-factor ANOVA followed by a Tukey's *post hoc* test, where $p \leq 0.05$ was considered significant. In addition, SPSS was used to construct standard box plots to eliminate outliers for compression testing. After outlier removal, n=5–6 samples for statistical analysis.

Results

Macroscopic Observation of Hydrogel Formulations

When HAnp (average diameter = 246 nm) were mixed with MeHA (degree of methacrylation = 21%), non-Newtonian paste-like behavior with shape-retention were observed (Fig. 1A–C). In contrast, solutions composed of pure MeHA or MeHA solutions containing HAlin did not exhibit this behavior, and instead exhibited Newtonian or zero yield stress pseudoplastic behavior. STEM images of HAnp confirmed the formation of nanoparticles (Fig. 1D).

Yield Stress Evaluation of Hydrogel Formulations Prior to Crosslinking

The experimental groups containing HAnp were the only solutions that exhibited a yield stress (Fig. 2A–C). Although the yield stress of the 15% HAnp gels was 177 ± 31 Pa (average ± standard deviation), this yield stress was not found to be significantly different from the linear HA groups. However, solutions that contained unreacted HAlin polymer instead of HAnp did not exhibit a yield stress even though they were also fit to Equation (1). The combination of 4% MeHA with 15% HAnp produced a synergistic effect, increasing the yield stress of the HAnp by a factor of 3.4 with the addition of the MeHA ($p < 0.001$).

Rheological Recovery of Hydrogel Formulations Prior to Crosslinking

The storage modulus of solutions lacking HAnp was negligible (i.e., all storage moduli were less than 20 Pa), but the storage modulus increased with increasing HAnp concentration (Fig. 2D). Specifically, compared to the storage modulus of 4% MeHA, the storage moduli of 4% MeHA increased 380- and 770- fold with the addition of either 15% HAnp or 30% HAnp, respectively ($p<0.001$). Recovery was assessed by the restoring of the original storage modulus after the disruption phase. All samples containing HAnp recovered their original storage moduli within 5 min of disruption.

Mechanical Analysis of Gels After Crosslinking

After characterizing the rheological behavior of the gels prior to crosslinking in their precursor solution form, the gels were crosslinked with ultraviolet (UV) light and further characterized as solids. Preliminary tests revealed that crosslinked MeHA was necessary to obtain gels with stable integrity over time in a 37°C saline environment, therefore only gels containing MeHA were characterized after crosslinking. It should first be noted that gels containing 4% MeHA and either 15% HAlin or 30% HAlin were tested to compare with the associated HAnp gels, however, the mixtures containing 30% HAlin remained as solutions after crosslinking, rendering it impossible to cut gels for further testing, so the 30% HAlin mixtures were therefore discarded from further analysis. Although the addition of HAnp

concentration resulted in at least a 5-fold increase in the compressive modulus compared to 4% MeHA gels, the increase was not significant. However, the addition of HAnp did significantly decrease the swelling degree after one day of swelling from 57 for 4% MeHA gels to 25 and 19 with the addition of 15% HAnp and 30% HAnp, respectively ($p<0.001$) (Fig. 3A–B). After 14 days of swelling, the compressive moduli of the MeHA + HAnp gels decreased to a range where they were not significantly different from that of 4% MeHA gels after one day of swelling.

Cell Viability of Cells Encapsulated within Crosslinked Gel Networks

Due to autofluorescence of the HAnp gels, live/dead quantification could not be performed. However, after 4 weeks, rBMSCs encapsulated in the MeHA and HAnp networks were viable as indicated by green fluorescence and minimal cell death (i.e., red fluorescence) was observed (Fig. 3C–D).

Discussion

In this work, we have introduced a method to overcome one of the major drawbacks of using hydrogels in the clinic (i.e., leaking from the defect site) by modifying traditional crosslinked hydrogels with the inclusion of HAnp. The combination of MeHA mixed with HAnp resulted in a hydrogel that exhibited ‘paste-like’ rheological behavior in its precursor solution. Although the underlying mechanism for the resulting paste-like behavior associated with the inclusion of HAnp is currently unknown, it has been hypothesized to be a result of dangling HA chains on the surface of the HAnp [8]. These dangling chains are hypothesized to cause physical entanglements between individual HAnp and entanglements between HAnp and MeHA. The goal of this current experimental work was to first characterize the rheological effect of adding our unique HAnp to MeHA and therefore, further research is necessary to understand the mechanism for the induced paste-like behavior of incorporating HAnp into hydrogel precursor solutions..

This desired paste-like behavior is attributed to the yield stress. The yield stress denotes the threshold where the solution transitions between an elastic solid and a pseudoplastic liquid, and it is desirable because it will prevent the hydrogel from flowing away from the site of interest. In a surgical context, this translates to allowing appropriate shaping and contouring to the defect site of interest. Yield stresses of up to 62 Pa have been previously reported for HA-based solutions,[18] but this yield stress may not be sufficient for topical application. In the current study, we demonstrated the ability to obtain solutions with yield stresses over 700 Pa. For context, the yield stresses for common paste-like materials, such as toothpaste, are approximately 200 Pa. Because the only solutions exhibiting a yield stress were solutions that incorporated HAnp, the yield stress was attributed to the HA being in the nanoparticulate form, as the addition of HA that was the same MW but was linear instead of in nanoparticle form was insufficient for achieving a yield stress. Furthermore, the combination of 4% MeHA with 15% HAnp produced a synergistic effect upon the yield stress. It should be noted that the 4% MeHA with 15% HAnp solution is a 19% overall concentration compared to the 15% HAnp solution, but this small increase in concentration is not assumed to account for the 3.4-fold increase in yield stress when 4% MeHA and 15%

HAnp were combined. Additionally, preliminary work using a lower MW MeHA (16 kDa) did not result in this synergistic effect seen with the 1 MDa MeHA,[1] suggesting the synergistic effect is MW dependent. Results suggest a desirable yield stress can be obtained for various applications by modulating the concentration of HAnp and the concentration and MW of MeHA, and future work will focus on creating a model to predict the yield stress based on these components.

In addition to exhibiting a yield stress, it is desirable for injectable materials to be able to recover rapidly after shearing.[16] All samples containing HAnp recovered their original storage moduli within 5 min of disruption. Additionally, in contrast to the yield stress, which was dependent upon the presence of MeHA and concentration of HAnp, the storage modulus was dependent only on the concentration of HAnp, regardless of the presence of MeHA. Overall, because HAnp gels exhibit a yield stress and recover rapidly, including HAnp in a gel network may allow for precise molding without the risk of material leaking from an implantation site (Fig. 4), making these gels suitable for a variety of topical and minimally invasive applications.

After appropriate shaping and contouring of these hydrogel pastes, it is also important for the pastes to set up to form a rigid hydrogel network, thus emphasizing the importance of incorporating MeHA in the gel precursor solutions. Although the HAnp-incorporated solutions exhibited the desirable yield stress and recovery after shearing, HAnp networks alone disintegrated rapidly in solution without the addition of MeHA. Therefore, we further characterized our MeHA-containing experimental groups as solids after photocrosslinking. The standard deviations of the compressive moduli for gels containing 15% HAlin were much larger than that of the other gels, including gels containing 15% or higher HAnp, which suggests that the mechanical properties of MeHA gels are better controlled with HA when it is in the nanoparticle form rather than in the linear form. Although the incorporation of HAnp did not have a significant effect on the compressive modulus after 1 day of swelling, after 14 days of swelling, the compressive moduli of the HAnp gels decreased to a range where they were not significant from that of 4% MeHA gels after one day of swelling. This decrease in the mean values of moduli for the HAnp groups may alert us to the possibility that the HAnp network may be short-lived, although it should also be noted that the 4% MeHA gels were disintegrated at two weeks, while the presence of HAnp kept the gels intact. In these particular gels, the HAnp are only physically entrapped in the system, so it is possible that chemically crosslinking the HAnp into the system may preserve and increase the mechanical properties if desirable. Furthermore, although the HAnp network may be short lived, the entire purpose of adding these HAnp into traditional hydrogel precursor solutions is to allow for the precursor solution to achieve paste-like rheological behavior, which is only necessary up until the point of crosslinking the solution. After crosslinking, the paste-like rheology is irrelevant to the network, given that we have shown we do not significantly alter the mechanical properties of the HAnp-incorporated hydrogels in their final crosslinked form.

Finally, rBMSCs encapsulated in these HAnp networks were viable at 4 weeks, which suggests that minimal cytotoxicity is feasible for HAnp-incorporated networks. Furthermore, the 4% MeHA gels with cells remained integrated at 4 weeks, suggesting that the inclusion

of cells may be beneficial to the network given the disintegration of acellular 4% MeHA gels within 2 weeks, although it is unknown at this time whether cells were maintaining this network through attachments to the material or through ECM secretion. Although it does appear that there was some cell death in the HAnp networks, due to autofluorescence, the extent of cellular death could not be quantified. However, the goal of cell encapsulation for this study was to show that cells could remain viable in these networks, and future work will in addition consider biochemical content and gene expression of encapsulated cells to further characterize cellular viability and performance. Additionally, because it is likely that these materials will be crosslinked *in situ*, future *in vivo* work with these materials will evaluate the toxicity of UV light to surrounding tissues. However, UV photocrosslinking has already been successfully performed *in situ* without toxicity concerns associated with UV light.[17]

Conclusion

Overall, the present work provides a platform hydrogel system that exhibits a yield stress prior to crosslinking, can recover its network rapidly, and can then be crosslinked into a more rigid hydrogel that is capable of encapsulating cells that remain viable. This behavior holds significant impact for any application of a hydrogel where a paste-like behavior is desired for its precursor solution, including but not limited to healthcare applications. As an example, for applications that cannot tolerate a liquid draining away from an irregularly shaped defect, or spilling from any kind of container at an angle to the direction of gravity, a Herschel-Bulkley or ‘paste-like’ rheology enables placement of the material prior to crosslinking. The yield stress in this platform system can be tailored by modulating the HAnp and MeHA MW and concentration. Furthermore, the MW of MeHA can be adjusted to result in crosslinked hydrogels of desirable mechanical properties, or alternately the HAnp can be crosslinked into the system. Additionally, the current study employed this system comprised of HA, however this platform hydrogel technology may perhaps be fabricated from other various polymers or biopolymers to suit a variety of applications where a paste-like material is desirable over a low-viscosity hydrogel precursor solution.

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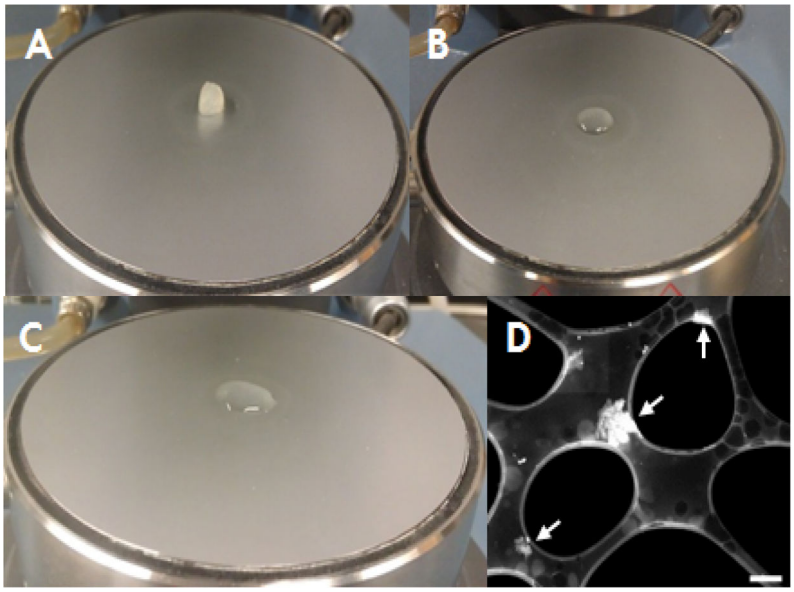

Figure 1.

Hyaluronic Acid Nanoparticle (HAnp)-Incorporated Solutions Impart Paste-Like Gross Rheological Behavior. (A–C) Images of select experimental groups loaded onto the lower rheometer plate prior to rheological testing. (A) 4% Methacrylated hyaluronic acid (MeHA) with 15% HAnp gel solution with shape-retention, (B) 4% MeHA with 15% linear HA (HAlin) and (C) 4% MeHA formulations yielding low viscosity solutions absent of yield stress. Because there were no visible differences between the remaining linear HA groups and Figure 1B, and likewise, no visible differences between the remaining HAnp-containing solutions and Figure 1A, the photographs of these remaining experimental groups were omitted from this figure. (D) Scanning transmission electron microscopy (STEM) observation of HAnp. The scale bar is 200 nm and arrows point to individual HAnps.

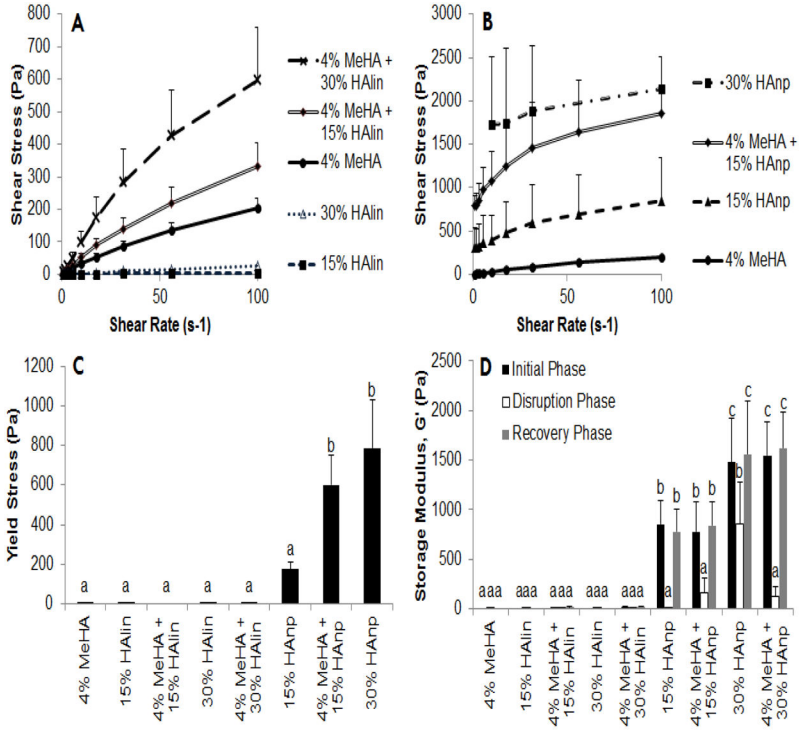

Figure 2.

Rheological Behavior of Solutions Prior to Crosslinking. (A–B) Shear rate sweep of formulations without yield stress (A) and formulations with yield stress compared to 4% Methacrylated Hyaluronic Acid (MeHA) (B). Data points are mean + standard deviation (n=5) and the lines are used to connect the data points to discern between samples. For the 30% hyaluronic acid nanoparticle (HAnp) formulation, shear banding was observed at low shear rates so those data were excluded. (C) Yield stress obtained from fit to Herschel-Bulkley equation. (D) Storage modulus of formulations before, after, and during disruption. Data reported as mean + standard deviation (n=5). Formulations with different letters indicate statistically significant differences ($p<0.05$).

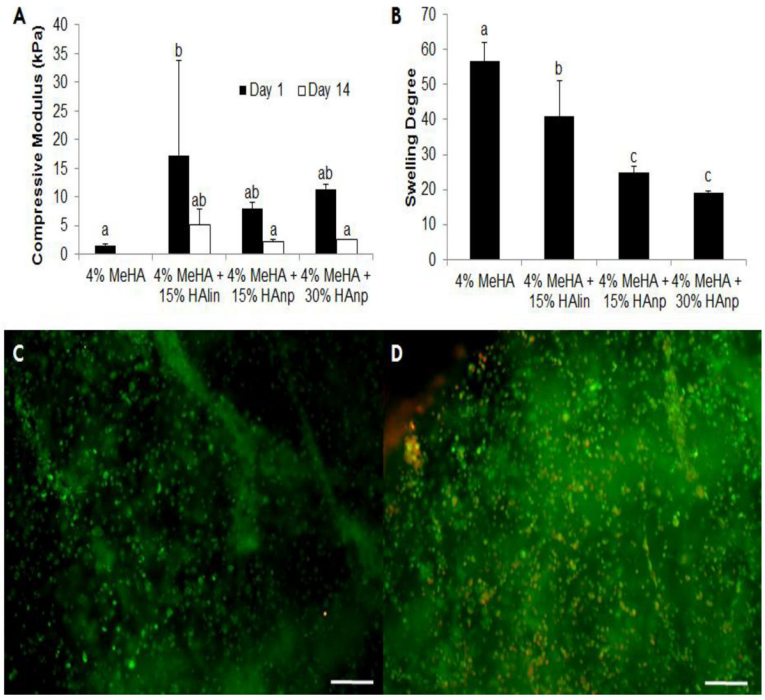

Figure 3.

Characterization of Gels after Crosslinking. Compressive modulus (A) and swelling degree (B) of crosslinked gels. Data are reported as mean + standard deviation (n=6). Formulations with different letters indicate statistically significant differences ($p<0.05$). Live/Dead image analysis of cells encapsulated and cultured for 4 weeks within 4% Methacrylated Hyaluronic Acid (MeHA) (C) and 4% MeHA + 15% hyaluronic acid nanoparticles (HAnp) (D). Scale bars are 100 μm.

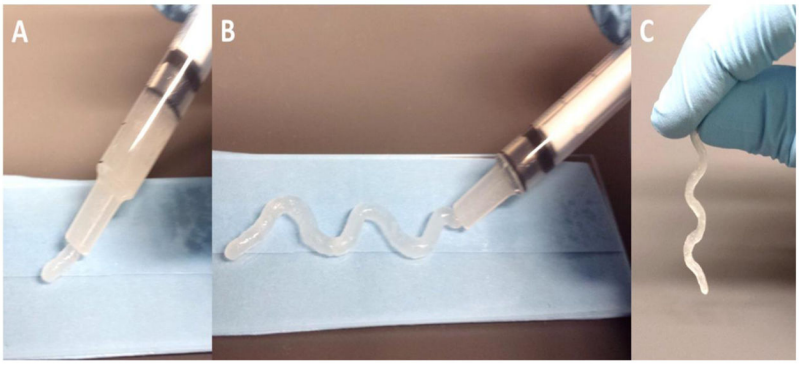

Figure 4.

Hyaluronic Acid Nanoparticle (HAnp)-Incorporated Solutions maintain shaping before and after crosslinking. (A) A solution containing 4% MeHA with 15% HAnp can be readily loaded into a 1 mL syringe and extruded. (B) After extrusion, the HAnp-incorporated solution maintained extruded shaping, which demonstrates that this formulation could be implanted *in vivo* without the risk of leaking from the implantation site. (C) After photocrosslinking the HAnp-incorporated solution, the solution was a crosslinked hydrogel network that retained its original shaping.