Mechanically Stiff Nanocomposite Hydrogels at Ultralow Nanoparticle Content

Manish K. Jaiswal,† Janet R. Xavier,† James K. Carrow,† Prachi Desai,† Daniel Alge,†‡ and Akhilesh K. Gaharwar*†§

†Department of Biomedical Engineering, Texas A&M University, College Station, Texas 77843, United States
‡Department of Materials Science and Engineering, Texas A&M University, College Station, Texas 77843, United States
§Center for Remote Health Technologies and Systems, Texas A&M University, College Station, Texas 77843, United States

ABSTRACT: Although hydrogels are able to mimic native tissue microenvironments, their utility for biomedical applications is severely hampered due to limited mechanical stiffness and low toughness. Despite recent progress in designing stiff and tough hydrogels, it is still challenging to achieve a cell-friendly, high modulus construct. Here, we report a highly efficient method to reinforce collagen-based hydrogels using extremely low concentrations of a nanoparticulate-reinforcing agent that acts as a cross-link epicenter. Extraordinarily, the addition of these nanoparticles at a 10 000-fold lower concentration relative to polymer resulted in a more than 10-fold increase in mechanical stiffness and a 20-fold increase in toughness. We attribute the high stiffness of the nanocomposite network to the chemical functionality of the nanoparticles, which enabled the cross-linking of multiple polymeric chains to the nanoparticle surface. The mechanical stiffness of the nanoengineered hydrogel can be tailored between 0.2 and 200 kPa simply by manipulating the size of the nanoparticles (4, 8, and 12 nm), as well as the concentrations of the nanoparticles and polymer. Moreover, cells can be easily encapsulated within the nanoparticulate-reinforced hydrogel network, showing high viability. In addition, encapsulated cells were able to sense and respond to matrix stiffness. Overall, these results demonstrate a facile approach to modulate the mechanical stiffness of collagen-based hydrogels and may have broad utility for various biomedical applications, including use as tissue-engineered scaffolds and cell/protein delivery vehicles.

KEYWORDS: nanocomposites, hydrogels, mechanical stiffness, nanoparticles, tissue engineering

Hydrogels are highly hydrated three-dimensional polymeric networks that can mimic the native tissue microenvironment; however, their utility for biomedical applications is severely hampered due to limited mechanical stiffness and low toughness.2–6 Despite recent progress in designing stiff and tough hydrogels, it is still challenging to achieve a cell-friendly, high modulus construct.7–10 A range of chemical and physical mechanisms have been proposed to enhance the mechanical stiffness of hydrogel networks including double-network hydrogels,11 supramolecular or topological hydrogels,12 microsphere-cross-linked hydrogels,13 nanoclay-cross-linked hydrogels,14–16 and hybrid physically/chemically cross-linked hydrogels.17 Nevertheless, most of these modifications either require the hydrogels to be formed under conditions that are not suitable for cell encapsulation or need a high concentration of reinforcing agent. Thus, there has been unprecedented interest in developing advanced strategies to design and develop mechanically stiff hydrogels for engineered tissue structures.

Recently, collagen-based hydrogels have been extensively investigated for cell and tissue engineering applications owing to their ability to encapsulate cells, nonimmunogenic properties, ability to mimic the native extracellular matrix (ECM) and biodegradable characteristics.18 There have been several efforts to fabricate mechanically robust collagen-based hydrogels by covalent cross-linking, interpenetrating networks, and nanocomposite structures.19–22 However, these techniques cause significant decreases in porosity and hydration degree, which are important drawbacks. Recently, one-dimensional nanomaterials such as carbon nanotubes (CNTs)20,21 and two-dimensional nanomaterials such as graphene, graphene oxide (GO),19 and synthetic silicate nanoparticles22 have been incorporated into collagen-based (gelatin) hydrogels to enhance the mechanical stiffness of these materials.20,21 These nanoparticles physically and/or chemically interact with polymer and enhance the

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机械强度在更高纳米材料浓度（3-20 mg/mL）时可提高2-3倍。这些进步虽然为在纳米材料浓度下工程力学性质的组织提供了条件，但尽管如此，基于胶原的水凝胶仍然没有被广泛用于制造能够发生塑性变形并保持水分以支持细胞渗透的机械性质的组织。在这里，我们报告了一种高效的方法，可以使用极低浓度（5 μg/mL）的纳米颗粒增强胶原基水凝胶。我们假设，多个聚meric链通过与纳米颗粒表面的交联点连接，将提供对水凝胶网络属性的控制。预计机械性质可以通过改变纳米颗粒的大小和浓度来完全控制。预计由于这种易于调整机械性能的方法而无需改变聚合物网络的化学性，这些纳米工程化的水凝胶可以用于异步化生物材料的化学和结构与细胞行为的关系。这些纳米工程化的水凝胶可能在各种生物医学应用中具有广泛的应用，包括用于组织工程的支架和细胞/蛋白质输送载体。

**RESULTS AND DISCUSSION**

**Synthesis and Characterization of Nanoparticle Cross-Linker.** To develop the chemically reinforced nanocomposite networks, oleic acid coated iron oxide (Fe₃O₄) MNPs with three different sizes (4, 8, and 12 nm) were synthesized using methods reported in the literature.23−25 The surface of the MNPs was modified with nitro-dopamine. Subsequently, poly(ethylene glycol)−dopamine (COOH-PEG-COOH) was used to create EDC-NHS chemistry. TEM images of MNPs with different diameter and size distribution. SQUID data of MNPs with different sizes indicating superparamagnetic characteristics. Zeta potential and electrophoretic mobility of MNPs indicate highly stable nanoparticles in aqueous solution.
glycol) (PEG) diacid was conjugated via carbodiimide-N-hydroxysuccinimide (EDC-NHS) chemistry, as shown in Figures 1a, S1 and S2. Transmission electron microscope (TEM) images indicate the formation of monodisperse MNPs with sizes of 4.4 ± 0.5, 8.6 ± 0.8, and 12.6 ± 0.7 nm (Figure 1b). According to the magnetization profiles (M-H), the MNPs were superparamagnetic, as no hysteresis was observed (Figure 1c). The 12 nm MNPs had the maximum magnetization of 65 emu/g at 2 kOe. The 4 nm MNPs exhibited higher magnetization than 8 nm, likely due to the smaller-sized particles being able to easily aggregate in aqueous media. The magnetization profiles also indicated that adding the nonmagnetic coating (i.e., the nitro-dopamine-PEG ligand) onto the MNP surfaces reduced the absolute magnetization values of Fe3O4. The coating is also responsible for diamagnetic characteristics at higher fields, which were more pronounced in the 4 nm MNPs. The zeta potential and electrophoretic measurements of the MNPs (Figure 1d) indicated high stability of the nanoparticles in aqueous solvent, which can be attributed to the presence of the hydrophilic PEG chains on the nanoparticles.

Reinforcing Hydrogels with Nanoparticle as a Cross-Link Epicenter. To fabricate nanocomposite hydrogels, denatured collagen (gelatin) was selected as the base polymer. Notably, gelatin contains cell-binding domains and can be easily modified to obtain covalently cross-linked hydrogels that mimic the native tissue microenvironment. Brieﬂy, the primary amine groups present on the gelatin backbone were partially modified with methacrylate groups to obtain photo-cross-linkable gelatin methacryloyl (GelMA), according to previously published reports. The degree of methacrylation was 80%, indicating that 80% of amine groups present on gelatin backbone were transformed into a methacrylamide group. Covalently cross-linked gelatin hydrogels (Gel) were produced by exposing a prepolymer solution of 5 wt % GelMA to ultraviolet (UV)
radiation (320–500 nm; 30 mW/cm²) in the presence of a photoinitiator (0.5 wt % Irgacure 2959). Nanocomposite hydrogels were fabricated (Figure 2a) by combining 5 wt % GelMA prepolymer solution with different sizes (4, 8, and 12 nm) and concentrations of the functionalized MNPs (0.1–5 μg/mL of iron (Fe)) and then performing UV cross-linking. Cross-linking of the MNP-reinforced nanocomposite hydrogels (5 μg/mL of Fe in 50 mg/mL GelMA) was evaluated by using a rheometer to monitor shear modulus evolution over time during photo-cross-linking (Figure 2b). As expected, the uncross-linked prepolymer solution containing GelMA and MNPs exhibited a low storage modulus (‘G’ before UV exposure (<1 min). After UV exposure (>1 min), which initiated crosslinking of polymer network, a sudden increase in ‘G’ was observed, indicating the formation of a covalently cross-linked network. In general, G’ reached a plateau after 1 min of UV exposure, indicating that the network was fully cross-linked. However, the final storage modulus (‘G’’) obtained was strongly influenced by the size of the functionalized nanoparticles, suggesting interactions between the ligands present on the MNPs and the collagen. In addition, we observed that the incorporation of MNPs resulted in enhanced network stability as determined from frequency sweep and stress sweep data (Figures S3 and S4).

The presence of MNPs within the cross-linked Gel network was confirmed by X-ray photoelectron spectroscopy (XPS) (Figure 2c) and electron diffraction pattern (TEM) (Figure S5). The presence of peaks at ~712 and 724 eV corresponded to Fe 2p3/2 and Fe 2p1/2, respectively, in the nanocomposites; these peaks were absent in Gel hydrogels that did not contain MNPs. XRD data also indicate presence of MNPs within the cross-linked samples (Figure S5). The presence of MNPs in the prepolymer solution before photo-cross-linking was analyzed by TEM, which displayed the homogeneous distribution, indicating ultrastable suspension in prepolymer solution (Figure 2d). The nanoparticles are crystalline and hence they appear dark and can be seen easily in the lighter polymer background.

The morphological features of the Gel and Gel-MNP hydrogels were analyzed using scanning electron microscopy (SEM), and the results indicated a porous and interconnected network with pore sizes of 8–10 μm (Figure 2e), similar to previously published reports. The addition of MNPs to Gel significantly reduced the pore size of the nanocomposite hydrogels, indicating an increase in cross-linking density. Thus, an increase in mechanical stiffness due to addition of MNPs can be expected. However, all the hydrogel showed highly porous and interconnected network, indicating their ability to support

![Figure 3](image-url)

Figure 3. Effect of MNPs on the mechanical properties of covalently cross-linked hydrogel networks. (a) Representative stress—strain curves for nanocomposites loaded with 8 nm MNPs (0.5 and 5 μg/mL of Fe). Box plot of nanocomposites reinforced with different concentrations of 4, 8, and 12 nm MNPs for (b) compressive modulus, (c) ultimate stress and toughness. (d) The effect of polymer concentration (2.5, 5, 10 and 15%) on compressive modulus of nanocomposite reinforced with 8 nm MNPs (5 μg/mL Fe). (Error bars not visible in some plots; they are too small). (e) The comparative chart of the moduli of various nanocomposite hydrogels: Gel-CNTs, Gel/Graphene, Gel/Silicate, Gelatin+Genipin, Gel/PEG, Gel/hHAp, Gel/Cloisite, Gel-GellanGum(GGMA), Acrylamide/MBAA, Alginate/CaCl₂, Hyaluronan/Au.
nutrient and waste transfer. Furthermore, we investigated the effect of MNPs addition to Gel on hydration and degradation characteristics of hydrogel network. The addition of MNPs to the Gel hydrogels did not have a significant effect on the hydration and degradation profile (Figure S6).

Physical and Structural Characterization of Mechanically Stiff Nanocomposite Hydrogels. To investigate the influence of MNPs on mechanical properties, an unconfined compression test was performed on the nanocomposite hydrogels and compressive modulus was calculated from engineering stress–strain curve. The modulus calculated from the initial linear region (0.1–0.2 mm/mm strain) of the stress–strain curve of nonreinforced Gel hydrogels was observed to be 3.2 ± 0.5 kPa (Figure 3a), which is comparable to previously published reports. The addition of nitro-dopamine functionalized MNPs to gelatin hydrogels resulted in a significant increase in mechanical stiffness compared to the Gel hydrogels (Figure 3b); the addition of 0.1, 0.5, 1, 2.5, and 5 μg/mL (Fe) of MNPs (8 nm) to Gel hydrogels increased the modulus to 4 ± 0.2, 16.9 ± 4.1, 17.3 ± 6.6, 22.2 ± 2.2, and 26.7 ± 4.7 kPa, respectively. A more than 8-fold increase in mechanical stiffness was observed by adding just 0.5 μg/mL of MNPs, which corresponded to a 10 000-fold lower concentration compared to the polymer concentration (50 mg/mL). It should be appreciated that the unreacted MNPs were exhausted at higher GelMA concentration. This result indicates that by changing the concentration of polymer and nanoparticles, a range of mechanical stiffness can be obtained.

Compared to other reported stiff hydrogels, the amount of nanoparticles (or cross-linker) used here is 2–3 orders of magnitude lower. Even at a 10 000-fold lower concentration of nanoparticles compared to polymer, we were able to achieve more than a 10-fold increase in mechanical stiffness. This is mainly due to the fact that each nanoparticle acts as cross-link epicenter, and the nanoparticle surface covalently interacts with multiple polymeric chains to form highly cross-linked network. Furthermore, we believe this concept can easily be generalized for different shaped nanoparticles such as one-dimensional (1D), two-dimensional (2D), and three-dimensional (3D) nanoparticles to further enhance the stiffness of polymeric networks. As the proposed approach is surface-mediated, by increasing surface roughness or features, it is possible to fine-tune the mechanical properties of nanocomposites. Notably, some of the other cross-linking techniques that have been used to achieve mechanically stiff hydrogels (e.g., double-network hydrogels, supramolecular or topological hydrogels, microsphere-cross-linked hydrogels, nanoclay-cross-linked hydrogels, and hybrid physical/chemical cross-linked hydrogels) have also not been able to provide a 10-fold increase in stiffness with such low concentrations of reinforcing agents.

To illustrate the significance of this result, the comparative plot Figure 3e shows the percentage increase in moduli with respect to nanofiller/reinforcing agent/cross-linker concentration in Gel hydrogel systems including Gel-CNTs, Gel/Graphene, Gel/Silicate, Gelatin + Genipin, Gel/PFG, Gel/nHAp, Gel/Cloisite, Gel-GellanGum (GGMA), Acrylamide/MBAA, Alginate/CaCl2, Hyaluronic Acid. For example, the addition of CNTs to Gel hydrogels resulted in merely 3-fold increase in hydrogel modulus. 2D nanomaterials such as graphene did not significantly change the compressive modulus when incorporated within Gel, and instead simply acted as a filler. When the surface of graphene oxide was modified with methacrylate groups, its interactions with Gel were increased and resulted in a 2- to 3-fold increase in stiffness. Another type of 2D nanomaterial that has a charged surface (synthetic silicates) resulted in a 4-fold increase in stiffness due to the enhanced nanoparticle–polymer interactions. Some other attempts to increase the mechanical stiffness of Gel hydrogels include the formation of semi-interpenetrating networks using Gellan Gum and PEG; these approaches also resulted in moderate increases in stiffness. Comparing to the existing literature, the present study clearly highlights that dopamine functionalized MNPs are efficient in increasing the mechanical robustness by 10-fold at ultralow (10 000-fold less) nanoparticle concentrations, which is superior to many alternatives at similar relative concentrations.

Chemical Characterization of Reinforced Hydrogel Network. The increase in mechanical stiffness can be attributed to covalent cross-linking between gelatin backbones and surface functionalized MNPs. Being photoactive, nitro-dopamine (λabs = 310 nm) present on MNPs might act as a cross-link epicenter. The role of surface functionalized MNPs in reinforcing the hydrogel network was probed using XPS. The change in binding energies (B.E.) for nitrogen (N 1s), carbon (C 1s) and oxygen

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Figure 4. Chemical interactions between nanoparticle and polymer. (a) High-resolution X-ray photoelectron spectra of Gelatin, GelMA (prepolymer), Gel (i.e., cross-linked GelMA), MNPs, GelMA/MNPs (i.e., prepolymer solution), and Gel/MNPs (cross-linked nanocomposite). The peaks for nitrogen (N 1s), oxygen (O 1s) and carbon (C 1s) atoms have been deconvoluted into curve components, which are shown underneath the experimental data points (dotted lines). (b) Proposed cross-linking mechanism between surface modified MNPs and polymer backbone (GelMA).

(O 1s) for gelatin, GelMA, Gel (i.e., cross-linked gelatin), MNPs, GelMA/MNPs and Gel/MNPs are shown in Figure 4a and Table S1. The N 1s peak for gelatin appeared at around 402.5 and 402.8 eV due to presence of free amine (NH$_3^+$) on polymer backbone. The methacrylation of gelatin gives rise to amide (399.8 eV) along with primary amine (397 eV) peaks in PBS. After photo-
cross-linking of the methacrylate groups, the associated N 1s peak was shifted marginally toward lower binding energy due to the change in vicinity. The N 1s peaks at 398.4 and 403.8 eV in MNPs are attributed to the amide and nitro groups, respectively, of the nitro-dopamine-PEG. In the prepolymer solution consisting of GelMA and MNPs; amide and amine peaks would be expected to appear at 399.8 and 397 eV, respectively (due to GelMA). Instead, a new peak with considerable shift in the primary amine position, at 398.4 eV emerged, indicating a strong carboxylate-amine interaction in the prepolymer solution of GelMA and MNPs. After UV cross-linking, peak splitting was observed in Gel/MNPs, suggesting the shift in carboxylate-amine cross-linking (399.1 eV) and the formation of imide linkages (399.7 eV), due to interactions between the amide groups of the methacrylated polymer chains and the carboxylates of the MNPs. The peak associated with nitro groups of the MNPs was not detected in the prepolymer solution (GelMA+MNPs), likely due to the ultralow concentration.

Comparison of the O 1s peaks for gelatin and GelMA indicated the presence of noncarbonyl oxygen (534 eV) that was transformed into carbonyl (531.5 eV) and anhydride (532.1 eV) after methacrylation. After UV cross-linking, the carbonyl and anhydride peaks in Gel were shifted due to change in vicinity in the neighboring atoms. In MNPs, the carbon positions corresponding to COOH and amide were observed at 282.7 and 286.8 eV, respectively. In the prepolymer solution (GelMA + MNPs), all the peaks corresponding to GelMA and MNPs were observed. After UV cross-linking, peaks at 288.1 and 285.8 eV indicate the formation of carboxylate-amine and imide interactions.

Overall, the XPS data confirmed that interactions occurred between the MNPs and polymer in the nanocomposite hydrogels and provided further evidence for MNPs acting as focal points from which polymeric cross-linking originated. The cross-linking mechanism obtained from XPS data indicates the formation of "imide" bonds as well as the presence of "carboxylate-amine" interactions between nanoparticles and polymer (Figure 4b). The ability of MNPs to provide multiple cross-linking points and its ability to covalently interact with polymer chains at multiple location are believed to be the

Figure 5. Effect of mechanically stiff nanocomposite hydrogels on cellular behavior. (a) The addition of nanoparticles result in significant increase in mechanical stiffness of hydrogel matrix and it is expected to influence cell morphology. (b) Due to facile fabrication process, encapsulated cells showed high viability after 24 and 72 h. (c) Cell shape was significantly influenced due to matrix stiffness. Cells encapsulated within Gel showed circular morphology, while cells in nanocomposite showed elongated and spread morphology, indicating that cells sense and respond to external stimuli. (d) Cells seeded on nanocomposite hydrogel readily proliferated as determined by Alamar blue assay. (e) Cells were synchronized via serum reduction for a 24 h period. No significant effect of stiffness was observed in cell cycle.

The iron oxide, nitro-dopamine, and carboxylate peaks were shifted and peaks corresponding carboxylate-amine interactions (532.6 eV) appeared, confirming the strong interactions of GelMA and MNPs. Upon UV exposure, a strong peak corresponding to the imide formation between the amides of GelMA and carboxylate groups of MNPs was observed.

The C 1s peaks for gelatin show the presence of carbonyl C=O and carboxyl (O−C=O) peaks at 287.5 and 288.8 eV, respectively. Methacrylation of the primary amines of gelatin resulted in the formation of C=CC=O (283.8 eV) and amide (288.2 eV). After UV cross-linking of GelMA, a peak appeared at 284.4 eV, corresponding to the C−C bond formed by polymerization. In MNPs the carbon positions corresponding to COOH and amide were observed at 282.7 and 286.8 eV, respectively. In the prepolymer solution (GelMA + MNPs), all the peaks corresponding to GelMA and MNPs were observed. After UV cross-linking, peaks at 288.1 and 285.8 eV indicate the formation of carboxylate-amine and imide interactions.

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underlying mechanism for the observed increases in mechanical stiffness.

**Effect of Mechanically Stiff Nanocomposite Hydrogels on Cellular Behavior.** The ability to encapsulate cells and subsequent support proliferation is an important requirement of a tissue engineered scaffold. Cellular behavior of encapsulated cells is regulated via multiple microenvironmental cues including mechanical stiffness, presence of cell adhesion ligands, ECM proteins and soluble factors. Among these external signals, mechanical stiffness of the hydrogel scaffolds plays an important role in governing cells shape (Figure 5a). Various reports demonstrated that cell shape profoundly impacts the cell fate via intracellular signaling. This ability of cells to sense and respond to external mechanical stimuli is extensively investigated for regenerative engineering. Moreover, ECM stiffness can also direct the differentiation of encapsulated stem cells toward certain tissue lineages. For example, soft hydrogels (<5 kPa) support circular morphology of encapsulated cells and support adipogenic/chondrogenic differentiation, while stiff hydrogels (>20 kPa) facilitate cells spreading and formation of strong cytoskeleton and induces osteogenic differentiation. Here, we demonstrated that surface-modified MNP's can reinforce GelMA by up to 10-fold at far lower concentrations (10,000-fold) nanoparticle concentrations. These ultralow nanoparticle concentrations modify matrix stiffness (~3–30 kPa) with minimal direct interactions with cells. Thus, it is expected that this nanocomposite hydrogel system can be used to delineate the effect of matrix stiffness and engineer cell-matrix interactions.

Due to facile cross-linking process, we can encapsulate human bone marrow stem cells (hMSCs) and murine-derived preosteoblasts (MC3T3s) within the nanocomposite hydrogel network. Cells survive the gelation process and maintain high viability after 24, 48, and 72 h, as evident by high number of live (green) cells compared to dead cells (red) (Figures 5b, S8 and S9). The effect of matrix stiffness was observed on cell spreading. After 24 h, encapsulated cells showing circular morphology were observed in Gel and nanocomposite hydrogels. After 72 h, no significant change in cell spreading was observed (Figure 5c) in Gel. The addition of 4, 8, and 12 nm MNPs to Gel results in significant increase in cell spreading. The projected cell area was calculated from the confocal images, quantitatively demonstrating the effect of matrix stiffness on cells spreading (Figure 5c).

The effect of matrix stiffness was also investigated by determining the circularity index (1 Circular and <1 elongated) of encapsulated cells. Cells encapsulated within Gel (0.6 ± 0.2) and Gel/MNPs (12 nm) (0.3 ± 0.1) showed significant difference in circularity index. In addition, well-organized actin cytoskeleton was observed in nanocomposite hydrogel with high stiffness (>20 kPa).

The metabolic activity of encapsulated cells was investigated using Alamar Blue assay and cell cycle analysis. The addition of nanoparticles does not result in any significant change in metabolic activities of cells, indicating high cytocompatibility of cross-linking process (Figure 5d). To investigate proliferative capacities of the nanocomposite hydrogels, cell cycle analysis via flow cytometry was performed. Initially, cells were synchronized via serum reduction for a 24 h period, enabling presentation of a more robust material effect after cell seeding. Incubation on the materials for 24 and 72 h provided ample time for trends to emerge among cell populations (Figure 5e). Cells seeded on the nanocomposite hydrogels began to display enhanced proliferation after 24 and 72 h. A significant increase in the G2/M population was observed, signifying a shift into a higher proliferative capacity. As expected, this was accompanied by a decrease in the G1/G0 population as cells entering a mitotic state. RGD sequences within the natural polymer promote cellular adhesion and subsequent cellular spreading, likely leading to the augmentation of cell numbers. Overall, the presented data suggest a promising future for the use of epicenter cross-linked nanocomposite hydrogels in cell and tissue engineering, and ex vivo cell culture models. Future investigations will focus on understanding the effect of mechanical stiffness of nanocomposite hydrogels on cellular behavior.

**CONCLUSIONS**

We have successfully synthesized mechanically stiff collagen-based hydrogels by incorporating a 10,000-fold lower concentration of nanoparticles without sacrificing pore morphology and interior hydrogel architecture. The chemical reinforcement with nitro-dopamine-PEG functionalized nanoparticles resulted in a 10-fold increase in mechanical stiffness and a 20-fold increase in toughness. The increase in mechanical stiffness of the nanocomposite hydrogels is due to the multifunctional nature of the nanoparticles, which act as cross-link epicenters. The mechanical stiffness of the nanoengineered hydrogel can be tailored between 0.2 and 200 kPa simply by manipulating the size of the nanoparticles (4, 8, and 12 nm), as well as the concentrations of the nanoparticles and polymer. Moreover these nanoengineered hydrogels are highly elastomeric and can sustain more than 90% compressive strain with minimal plastic deformation. Due to facile cross-linking process, high viability of encapsulated cells was observed. In addition, encapsulated cells were able to sense and respond to external mechanical stimuli. The ultralow nanoparticle concentrations required to modify the matrix stiffness can be used to understand and direct cellular fate. Overall, these engineered hydrogels can be used to engineer mechanically stiff networks for biomedical applications including tissue-engineered scaffolds, drug delivery vehicles, bioactuators, and sensors.

**METHODS**

**Synthesis of Magnetite Nanocrystals.** For the synthesis of 4 nm magnetite nanocrystals, the protocol established by Sun et al. was used.

In a typical reaction, the precursors iron(III) acetylacetonate (ca. 1.41 g), lauric acid (ca. 2.40 g), hexadecanediol (ca. 5.17 g), dodecylamine (2.76 mL) (all procured from Sigma) were dissolved altogether in 40 mL of benzyl ether (bp 298 °C) and heated to 180 °C for about 4 h under N2 blanket. The reaction chamber was raised to 295 °C slowly and maintained for 10 min. The formed nanocrystals were of the size approximately 4 nm with oleic acid coated onto it.

For the synthesis of 8 and 12 magnetite nanocrystals, we followed the standard methodology based on the high temperature decomposition of iron oleate complex (Figure S1). In brief, 40 mmol of ferric chloride hexahydrate (FeCl3·6H2O, procured from Sigma) and 120 mmol of sodium oleate (procured from Fluka) dissolved in the mixture solution of ethanol, water and hexane was heated at 70 °C for about 4 h under N2 blanket. The thus obtained complex was properly washed with ultrapure water and the excess of hexane was evaporated to get wax-like solid product. In the next step, the calculated amount of the obtained complex (5.815 g) was dissolved in 40 mL of 1-octadecane (bp 317 °C) and 1.08 mL of oleic acid and raised to 315 °C at controlled rate of 3.3 °C/min and maintained for 10 (or 15) min to achieve hydrophobic oleic acid functionalized Fe3O4 nanocrystals of sizes 8 (or 12 nm) respectively.

**Hydrophilic Fe3O4 MNPs by Nitro-Dopamine PEG.** The oleic acid coated hydrophobic Fe3O4 nanocrystals were made aqueous stable by replacing the long aliphatic chain molecules from the surface and stabilizing them by functionalizing with PEG diacid (procured from Millipore, USA) via strong anchoring ligand of nitro-dopamine. The
dual hydroxyl groups of the catechol part of nitro-dopamine get oxidized easily and bind with the Fe³⁺ onto the nanoparticle surfaces and hence establish a robust and highly sustainable nanoparticles under physiological conditions.²⁻¹⁴ The presence of additional nitro group (−NO₂) facilitates electron deficiency in the benzene ring and thus helps easy oxidation of hydroxyl groups. Furthermore, the surface was modified with PEG-diacid using simple EDC-NHS chemistry wherein the one end carboxylate group of PEG undergoes esterification and binds with nitro-dopamine, while the other end extends in water and provides ultrastable aqueous suspension. The detail mechanisms of this chemistry is described in the Supporting Information (Figure S2). In typical laboratory procedure for the ligand solutions, 55.8 mg of nitro-dopamine was dispersed in 15 mL of typical laboratory procedure for the ligand solutions, 55.8 mg of nitro-dopamine was dispersed in 15 mL of PBS. The prepared solution was subsequently mixed with 144 mg of PEG diacid were dissolved in 30 mL of PBS. The entire mixture was stirred for about 24 h to get the nitro-dopamine-PEG ligand solution. The prepared oleic acid coated Fe₃O₄ nanocrystals were then added into this prepared solution and the mixture was sonicated for another 24 h to get an aqueous stable MNPs.

**Synthesis of Methacrylated GelMA.** Gelatin has abundance of free amine groups in the form of lysine which provide easy acylation sites. Gelatin methacrylate (GelMA) with 80% methacrylated degree was prepared by acrylation of pendent amine groups initiated by 2-methacrylic anhydride (MA) in PBS at elevated temperature, according to previously published protocols.³⁻¹⁴ In a typical experiment, 10 g of gelatin (extracted from porcine skin, Sigma) was dissolved in 100 mL of PBS at 60 °C for an hour under vigorous stirring using magnetic bar. Eight milliliters of MA was added dropwise thereafter, and the solution was allowed to stir for another 3 h. The reaction was finally terminated by adding excess of preheated PBS into the solution. The prepared sample was collected and dialyzed against ultrapure water (18.2 MΩ-cm) at 40 °C in a cellulose dialysis bag (Mol. wt. cutoff ~12 kDa) for a week before freeze-drying and lyophilizing to get the GelMA in white powder form. The GelMA prepared this way was roughly 80% acrylated.

**Preparation of UV Cross-Linked Gel Discs.** Photo-cross-linked 5% (w/v) GelMA and GelMA incorporated with MNPs (GelMA + MNPs) with varied MNPs concentrations from 0.1 to 5 μg/mL. Fe of different sizes (4, 8, and 12 nm) were prepared using 0.5% irgacure 2959 (Sigma) as photoinitiator (PI). Typically, 100 μL of GelMA (5 mg) solution with PI (0.5 mg) in aqueous medium was used to expose UV light (30 mW/cm²) for 60 s to get a disc-shaped gel of the dimensions 7 mm (diameter) × 2 mm (thickness) and maintained in 1 mL of DPBS overnight before using them for further experiments and measurements. All the measurements were carried out using five replicates unless otherwise stated.

**Physical and Chemical Characterizations.** The zeta potential and electrophoretic mobility of the GelMA and GelMA/MNPs prepolymer solutions were measured at 25 °C using a Zetasizer (Malvern Instrument, U.K.) equipped with a He–Ne laser. Transmission electron microscopy (TEM) was performed using JEOL-JEM 2010 (Japan) at an accelerating voltage of 200 kV on carbon grid. The surface morphology of the nanocomposite hydrogels was evaluated using scanning electron microscopy (SEM, FEI Quanta 600 FE-SEM, USA, fitted with Oxford EDS system) at an accelerating voltage of 20 kV. In brief, to prepare sample for SEM, the nanocomposite hydrogels were frozen using liquid nitrogen and then lyophilized for 3 days to obtain dried sample. The dried nanocomposite samples were sputter coated with Au/Pd up to a thickness of 8 nm before being mounted onto the specimen stage for imaging. The pore diameters were analyzed using ImageJ software (National Institutes of Health, USA).

The mechanical properties of the Gel and nanocomposite hydrogels were determined using a mechanical tester (Xpert 7600, ADMET, USA). Hydrogels (n = 6) were incubated in PBS for 24 h before testing. A uniaxial compression test was performed on the cylindrical samples (6 mm × 2 mm) at a strain rate of 1 mm/min. The compressive modulus was calculated from the slope in the toe region corresponding to 0.10–0.20 strain in engineering stress–strain curve (force divided by original cross-section area). The viscoelastic behavior of the hydrogels was evaluated using MCR Rheometer (Anton Paar, USA) equipped with 50 mm flat geometry and a gap of 50 μm.

The swelling behavior (or hydration degree) of the nanocomposites were also studied and calculated according to the formula mentioned below. The samples were soaked in 1× PBS (pH 7.4) and the wet weight (M₂) was measured; then the samples were lyophilized for 24 h to measure the dry weight (M₁).

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\text{Hydration degree} = \frac{M₂ - M₁}{M₁} \times 100
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For enzymatic degradation of the samples, gel discs were placed in 1 mL freshly prepared 2.5 units/mL collagenase type II (procured from Worthington Biochemicals, USA) solution in PBS at 37 °C. Weight loss was monitored until the gels were completely decomposed (Figure S6).

The formation of dual cross-linked network between gelatin and MNPs coated with nitro-dopamine-PEG diacid was analyzed using X-ray photoelectron spectroscopy (XPS) (Omicron XPS/UPS system with Argus detector). The changes in binding energies (B.E.) for nitrogen (N 1s), carbon (C 1s) and oxygen (O 1s) and gelatin, GelMA, Gel (cross-linked gelatin), MNPs, GelMA/MNPs and Gel/MNPs were recorded and all the raw data were processed and deconvoluted by CasaXPS multiple peak fit software version 2.3.15. The references for XPS interpretations are listed in Table S1. Furthermore, the semicrystalline property of Gelatin was utilized to confirm the cross-linking of Gel/MNPs nanocomposites. X-ray diffraction pattern (Bruker, USA) of both gelatin and nanocomposite recorded showed the forward angle shift in the characteristic peak positions as shown in Figure S5.

**In Vitro Cell Studies.** Human Mesenchymal Stem Cells (hMSCs) were obtained from Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott & White. Murine-derived preosteoblasts (NIH MC3T3 E1–4, ATCC, USA) and hMSCs were cultured in normal growth media (Alpha MEM, Life Technologies, USA) supplemented with 10% FBS (Life Technologies, USA) and 1% penicillin/streptomycin (100 U/100 μg/mL; Life Technologies, USA) at 37 °C with 5% CO₂. The nanocomposite hydrogels of 6 mm diameter and 400 μm thickness were prepared in a 96-well plates. Then, hMSCs were trypsinized and seeded onto the hydrogels at a density of 10 000 cells/hydrogel in normal growth media. After 24 and 48 h of cell seeding, the normal media were removed and the hydrogels were washed with PBS. The prepared Live/Dead assay reagent from Calcein AM and Ethdium Homodimer (Santa Cruz Biotechnology, Inc., USA) were added to the nanocomposites and incubated for 30 min at 37 °C. The samples were washed with PBS thrice, and imaged using the epifluorescence microscope (TE2000-S, Nikon, USA). Cell cycle analysis was performed using the BD Accuri C6 Flow Cytometer and propidium iodine (PI) stain following manufacturer’s protocol. Prior to seeding, hMSCs were starved for 24 h and then seeded on nanocomposite hydrogels. Cells seeded on each composition were trypsinized at two separate time points (24 and 72 h) and fixed in cold 70% ethanol. Cell pellets were formed and washed in PBS, followed by incubation in a PI staining solution at 37 °C for 30 min. Cells were stored at 4 °C until analysis. For 3D encapsulation, trypsinized MC3T3s were pelleted and subsequently resuspended within the nanocomposite prepolymer solution, which was maintained at 37 °C, and gels were formed on glass coverslips under previously described UV conditions. Coverslips with gels were stored in a 24-well plate with enough media to completely cover the gels for the required time points. Both live/dead
and actin cytoskeletal staining was performed according to manu-
facture’s protocol.

Statistical Analysis. The experimental data were presented as mean
and standard deviations (n = 5 or 6). GraphPad Prism 5 was used to
perform statistical analysis using nonparametric tests and one-way
analysis of variance (ANOVA). Tukey’s posthoc analysis was used for
pairwise comparisons and the statistical significance was defined as *p <
0.05, **p < 0.01, ***p < 0.005.

ASSOCIATED CONTENT

1 Supporting Information

The Supporting Information is available free of charge on the
ACS Publications website at DOI: 10.1021/acsnano.5b03918.

Physical and chemical characterizations; Stress and
frequency sweeps of nanocomposites; in vitro cytocompat-
ibility and encapsulation of hMSCs and preosteoblast
within nanocomposite hydrogels (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: gaharwar@tamu.edu.

Author Contributions

M.K.J. and A.K.G. designed the experiments and wrote the
manuscript. J.R.X. and P.D. contributed to materials synthesis
and mechanical characterizations. J.K.C. contributed in the cell
culture work and editing the manuscript. D.A. contributed to the
rheology, the interpretation of chemical interactions with XPS
data and manuscript preparation.

Notes

The authors declare no competing financial interest.

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REFERENCES

Delivery Rev. 2012, 64, 18–33.
(2) Langer, R.; Tirrell, D. A. Designing Materials for Biology and
(3) Ludolf, M.; Hubbell, J. Synthetic Biomaterials as Instructive
Extracellular Microenvironments for Morphogenesis in Tissue En-
(4) Burdick, J. A.; Murphy, W. L. Moving from Static to Dynamic
(5) Kerativitayanan, P.; Carrow, J. K.; Gaharwar, A. K. Nanomaterials
2015, 4, 1600−1627.
(6) Gaharwar, A. K.; Peppas, N. A.; Khademhosseini, A. Nano-
2014, 111, 441−453.
(7) Seliktar, D. Designing Cell-Compatible Hydrogels for Biomedical
(8) Purcell, B. P.; Dob, D.; Charati, M. B.; Dorsey, S. M.; Wade, R. J.;
Zellers, K. N.; Doviak, H.; Pettaway, S.; Logdon, C. B.; Shuman, J. A.
Injectable and Bioresponsive Hydrogels for On-demand Matrix
Commun. 2013, 4, 2226.
(10) Gaharwar, A. K.; Arpanaei, A.; Andreessen, T. L.; Dolatshahi-Pirouz,
A. 3D Biomaterial Microarrays for Regenerative Medicine: Current
2016, DOI: 10.1002/adma.201503918.
(11) Gong, J. P.; Katsuyama, Y.; Kurokawa, T.; Osada, Y. Double-
Network Hydrogels with Extremely High Mechanical Strength. Adv.
Mater. 2003, 15, 1155−1158.
(12) Okumura, Y.; Ito, K. The Polyrotaxane Gel: A Topological Gel by
(13) Huang, T.; Xu, H. G.; Jiao, K. X.; Zhu, L. P.; Brown, H. R.; Wang,
H. L. A Novel Hydrogel with High Mechanical Strength: A
Macromolecular Microsphere Composite Hydrogel. Adv. Mater. 2007,
19, 1622−1626.
Kimbara, K.; Aida, T. High-water-content Mouldable Hydrogels by
343.
(15) Haraguchi, K.; Takehisa, T. Nanocomposite Hydrogels: A Unique
Organic−Inorganic Network Structure with Extraordinary Mechanical,
1120−1124.
(16) Carrow, J. K.; Gaharwar, A. K. Bioinspired Polymeric Nano-
composites for Regenerative Medicine. Macromol. Chem. Phys. 2015,
216, 248.
(17) Sun, J.-Y.; Zhao, X.; Illerupema, W. R. K.; Chaudhuri, O.; Oh, K.
H.; Mooney, D. J.; Vlassak, J. J.; Suo, Z. Highly Stretchable and Tough
Khademhosseini, A. Cell-laden Microengineered Gelatin Methacrylate
(19) Shin, S. R.; Aghaei-Ghareh-Bolahag, B.; Dang, T. T.; Topkaya, S.
et al. Cell-laden Microengineered and Mechanically Tunable Hybrid
6391.
S. B.; Nikkhah, M.; Kabir, M.; Azize, M.; Kong.; et al. A. Carbon-
Nanotube-Embedded Hydrogel Sheets for Engineering Cardiac
Conducts and Bioactuators. ACS Nano 2013, 7, 2369−2380.
(21) Shin, S. R.; Bae, H.; Cha, J. M.; Mun, J. Y.; Chen, Y.-C.; Tekin, H.;
Nanotube Reinforced Hybrid Microgels as Scaffold Materials for Cell
(22) Xavier, J. R.; Thakur, T.; Desai, P.; Jaiswal, M. K.; Sears, N.;
Cosgriff-Hernandez, E.; Kaunas, R.; Gaharwar, A. K. Bioactive
Nanoengineered Hydrogels for Bone Tissue Engineering: A Growth-
(23) Jana, N. B.; Chen, Y.; Peng, X. Size- and Shape-Controlled
Magnetic (Cr, Mn, Fe, Co, Ni) Oxide Nanocrystals via a Simple and
(24) Park, J.; An, K.; Hwang, Y.; Park, J.-G.; Noh, H.-J.; Kim, J.-Y.; Park,
J.-H.; Hwang, N.-M.; Hyeon, T. Ultra-large Scales Syntheses of
Prasad, P. V.; Bahadur, D.; Dravid, V. P. Thermoresponsive Magnetic
Hydrogels as Theranostic Nanoconstructs. ACS Appl. Mater. Interfaces
2014, 6, 6237−6247.
(26) Amstad, E.; Gillich, T.; Bilecka, I.; Textor, M.; Reimhult, E.
Ultrastable Iron Oxide Nanoparticle Colloidal Suspensions Using
Dispersants with Catechol-derived Anchor Groups. Nano Lett. 2009,
9, 4042−4048.
(27) Viola, K. L.; Sbarborio, J.; Sureka, R.; De, M.; Bicca, M. A.; Wang,
J.; Vasavada, S.; Satpathy, S.; Wu, S.; Joshi, H.; et al. Towards Non-
Invasive Diagnostic Imaging of Early-stage Alzheimer’s Disease. Nat.
Nanotechnol. 2015, 10, 91−98.
(28) Xie, J.; Xu, C.; Kohler, N.; Hou, Y.; Sun, S. Controlled PEGylation
of Monodisperse Fe3O4 Nanoparticles for Reduced Non-specific
(29) Damasceno, P. F.; Engel, M.; Glotzer, S. C. Predictive Self-
assembly of Polyhedra into Complex Structures. Science 2012, 337,
453−457.


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