Bioactive Nanoengineered Hydrogels for Bone Tissue Engineering: A Growth-Factor-Free Approach

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ABSTRACT Despite bone’s impressive ability to heal after traumatic injuries and fractures, a significant need still exists for developing strategies to promote healing of nonunion defects. To address this issue, we developed collagen-based hydrogels containing two-dimensional nanosilicates. Nanosilicates are ultrathin nanomaterials with a high degree of anisotropy and functionality that results in enhanced surface interactions with biological entities compared to their respective three-dimensional counterparts. The addition of nanosilicates resulted in a 4-fold increase in compressive modulus along with an increase in pore size compared to collagen-based hydrogels. In vitro evaluation indicated that the nanocomposite hydrogels are capable of promoting osteogenesis in the absence of any osteoinductive factors. A 3-fold increase in alkaline phosphatase activity and a 4-fold increase in the formation of a mineralized matrix were observed with the addition of the nanosilicates to the collagen-based hydrogels. Overall, these results demonstrate the multiple functions of nanosilicates conducive to the regeneration of bone in nonunion defects, including increased network stiffness and porosity, injectability, and enhanced mineralized matrix formation in a growth-factor-free microenvironment.

KEYWORDS: nanocomposite hydrogels · tissue engineering · synthetic nanosilicates · scaffolds · two-dimensional (2D) nanoparticles · bone regeneration

Nanocomposite hydrogels are emerging biomaterials used for a range of tissue engineering approaches.¹⁻⁵ Hydrogels are able to mimic some of the physical and chemical properties of the extracellular matrix (ECM) due to their structural similarity to native tissue. By adding different nanoparticles, including graphene, graphene oxide, carbon nanotubes (CNTs), hydroxyapatite (nHAp), bioactive glasses, and calcium phosphate, the mechanical and biological characteristics of nanocomposite hydrogels can be enhanced.⁶⁻⁸ Most of these nanoparticles have been shown to support cell adhesion and proliferation, and only a few have been shown to influence cell differentiation.

Recently, we reported two-dimensional (2D) nanosilicates with a high degree of anisotropy and functionality for biomedical applications.⁹⁻¹² The recent rapid advances in 2D nanoparticle technologies have raised exciting questions about their interactions with biological moieties.³⁻⁵ The addition of 2D nanosilicates to human mesenchymal stem cells (hMSCs)⁹ and adipose stem cells (ASCs)¹⁰ was shown to induce osteogenic differentiation without the use of dexamethasone, bone morphogenic protein-2 (BMP-2), or other growth factors. Nanosilicate (Laponite, Na⁺₀.₇(Mg₅.₅Li₀.₃)Si₈O₂₀₋ₐO₄₋₀.₇) is a hydrous sodium lithium magnesium silicate. The degradation products of nanosilicates are magnesium, orthosilicic acid, and lithium, which can be easily absorbed by the body and are individually shown to have properties that are potentially useful for bone tissue engineering.¹³ For example, lithium, an FDA-approved inhibitor of glycogen synthetase kinase-3β (GSK3β), activates Wnt-responsive genes by elevating cytoplasmic β-catenin.¹⁴,¹⁵ Orthosilicic acid (Si(OH)₄) promotes collagen type I synthesis and osteoblast differentiation via the Wnt/β-catenin signaling pathway. The role of

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Wnt signaling has been implicated in osteogenic differentiation of stem cells.\textsuperscript{16} Moreover, nanocomposite hydrogels loaded with nanosilicates are biocompatible and biodegradable under in vivo conditions\textsuperscript{11} and can be used for a range of biomedical and biotechnological applications.

Compared to other types of 2D nanoparticles such as graphene or graphene oxide (GO), nanosilicates have been shown to provide enhanced physical, chemical, and biological functionality due to a discotic charged surface, uniform shape, high surface-to-volume ratio, and biocompatible nature.\textsuperscript{1–5} The presence of both positive and negative charges on the nanosilicate surface results in unique anisotropic interactions between the nanoparticles that generate new unconventional phases at the nano- and microscales.\textsuperscript{17–19} This allows nanosilicates to interact with anionic, cationic, and neutral polymers to form a physically cross-linked network with shear-thinning characteristics.\textsuperscript{11,12,20} The uniform shape of nanosilicates compared to GO provides additional control over the interactions between nanoparticles and polymer chains to develop multifunctional biomaterials.\textsuperscript{3,5} Moreover, the optical transparency of nanosilicates\textsuperscript{17} in aqueous media compared to GO permits imaging of the subsurface cellular process to design complex tissue structures.

In this study, we developed an injectable osteo-inductive collagen-based nanosilicate matrix for growth-factor-free bone tissue engineering. Natural hydrogel-forming polymers such as collagen are able to mimic native tissue-like properties due to the presence of cell-binding domains Arg-Gly-Asp (RGD) and can be easily modified to obtain covalently cross-linked hydrogels with native tissue-like mechanical properties.\textsuperscript{21–24} The unique shape and surface charge of nanosilicates promote strong interactions with a collagen-based matrix (gelatin) to form self-assembled structures, which can dynamically form and break to result in shear-thinning properties. Upon UV exposure, the self-assembled structures of a collagen-based matrix and nanosilicates form a highly cross-linked and vstable network (Figure 1a). We hypothesize that incorporating nanosilicates within a collagen-based matrix will provide control over the physical properties of the hydrogel network due to enhanced interactions between the nanosilicates and gelatin backbone. Moreover, the ability of a collagen-based hydrogel loaded with nanosilicates to promote mineralized matrix formation in a growth-factor-free microenvironment for regeneration of bone is unique. Given that the treatment of bone defects remains one of the largest challenges in musculoskeletal tissue engineering, our nanocomposite hydrogel represents a new material design in the broadly interesting area of growth-factor-free strategies. Overall, these dynamic and bioactive nanocomposite gels show strong promise in bone tissue engineering applications.

RESULTS AND DISCUSSION

Nanosilicate–Gelatin Interactions. Nanosilicate (Laponite, Na\textsubscript{0.7}Li\textsubscript{0.2}Mg\textsubscript{0.3}Sl\textsubscript{c}O\textsubscript{20}(OH)\textsubscript{1.0}H\textsubscript{2}O) is a hydrous sodium lithium magnesium silicate characterized by a high aspect ratio (20–40 nm in diameter and 1 nm in thickness) (Figure 1b). Due to the unique crystal structure and charged surface of nanosilicates, a strong physical interactions between a nanosilicate and polyampholytic gelatin was expected. Gelatin is denatured collagen and mimics components of the native extracellular matrix in structure and chemical composition. To fabricate covalently cross-linked hydrogels, amine groups present on the gelatin backbone were replaced by a methacrylate group to obtain gelatin methacrylate (GelMA) according to previously published reports.\textsuperscript{21–24} The interactions between a nanosilicate and a prepolymer (GelMA) were investigated using electrophoresis and transmission electron microscopy (TEM). The zeta potential of GelMA and the nanosilicates was observed to be $-11.3 \pm 0.4$ and $-28 \pm 3$ mV, respectively. With the addition of nanosilicates to GelMA, a decrease in zeta potential was observed (Figure 1c). Specifically, the addition of 0.5, 1, and 2% nanosilicates to GelMA resulted in a decrease in the zeta potential of the nanocomposites to $-13 \pm 2.6$, $-33.4 \pm 2.9$, and $-34.4 \pm 0.9$ mV, respectively. The decrease in the zeta potential of nanocomposites indicates that nanosilicates strongly interact with gelatin at nanosilicate concentrations $\geq$ 1%. Additionally, our earlier results also indicate that nanosilicates are uniformly dispersed within the gelatin network without any aggregation.\textsuperscript{11}

The shear-thinning characteristics of the prepolymer solutions containing GelMA and GelMA–2% nanosilicates were determined by monitoring viscosity at different shear rates at 37 °C (Figure 1d). The prepolymer solution (5 wt % GelMA) was a liquid at 37 °C, and the addition of nanosilicates significantly increased the viscosity of the solution with nanosilicate concentrations higher than 2%, resulting in a semisolid that needed skilled handling with smaller diameter pipets. The unique shape and surface charge distribution on the nanosilicates provide a significantly higher surface-to-volume ratio compared to other types of nanoparticles. Moreover, due to the presence of both positive and negative charge on the nanosilicates, it results in the formation of a house-of-cards structure and exhibits a shear-thinning characteristic.\textsuperscript{17–19}

Covaletly Cross-Linked Nanocomposite Hydrogels. We fabricated a covalently cross-linked gelatin hydrogel (Gel) by exposing a prepolymer solution of GelMA to UV radiation in the presence of a photoinitiator. By adding 0.5, 1, and 2 wt % of nanosilicates to GelMA, covalently cross-linked nanocomposite hydrogels were fabricated for physical and biological characterization. The addition of nanosilicates provided a desired combination of tunable structure and bioactivity characteristics.
The presence of nanosilicates within the gelatin network after the cross-linking process was confirmed via infrared (IR) spectra, as shown in Figure 1e. The peak near 960 cm\(^{-1}\) observed in the nanosilicates was due to stretching of Si\(-\)OH and Si\(-\)O and bending of Si\(-\)O\/-\Si bonds present in the nanosilicates. Gelatin showed prominent amine peaks near 1540 and 1650 cm\(^{-1}\). The nanocomposites contained both Si\(-\)OH and amine peaks, indicating the presence of both gelatin and nanosilicates.

The hydration properties of nanocomposite hydrogels are important to investigate, as the diffusion of nutrients and waste products strongly depends on the hydration degree.\(^{25}\) Moreover, changing the hydration degree can alter cellular infiltration during in vivo implantation. The hydration degree of nanocomposites was quantified in different medium compositions, including PBS and cell culture media (DMEM and DMEM \(+\) 10% FBS) (Figure S1). The addition of nanosilicates reduced the hydration degree, likely due to strong interactions between gelatin and nanosilicates. Performing the assays with PBS at different pH levels or with culture media instead of PBS did not significantly alter the results.

The porosity of tissue-engineered scaffolds plays an important role in directing cellular behavior, as the size and distribution of pores within scaffolds dictate the cellular infiltration and distribution of cells throughout the network.\(^{25}\) Gel hydrogels showed a uniform and highly interconnected network, similar to previously published literature.\(^{21,26}\) The addition of nanosilicates to Gel resulted in an increase in pore size of the covalently cross-linked gelatin hydrogel (Gel) and a nanocomposite hydrogel indicated the presence of Si and Mg in the nanocomposite, but not in Gel.
is interesting in that it differs from the existing reports related to most other nanocomposite materials, in which physical reinforcement of the polymeric network by nanoparticles results in smaller pore size.\textsuperscript{27,28} In the nanocomposites, the increase in the pore size may be attributed to the enhanced interactions between the nanosilicates and the gelatin, which locally condense the polymer fraction to result in the formation of larger voids. The microscopic images confirmed the uniform dispersion of nanoparticles within the polymer network with no visible aggregates. A further analysis with energy-dispersive X-ray spectroscopy (EDS) also indicated the presence of silicon (Si) and magnesium (Mg) in the nanocomposites, indicating their presence in the porous structure (Figure 1f).

**Nanosilicates Enhance Mechanical Stiffness.** Nanosilicates interacted with the polymeric matrix to increase the mechanical stiffness. Figure 2a shows that the Gel hydrogel readily broke when compressed with a sharp object, whereas the addition of 2D nanosilicates significantly improved the network stability under stress. To quantify the effects of nanosilicates on the mechanical stiffness, hydrogel samples were subjected to uniaxial compression testing. The Gel hydrogel showed an increase in stress with an increase in strain (Figure 2b). The modulus calculated from the initial linear region (0.1–0.2 mm/mm strain) of the Gel stress–strain curve was observed to be 3.3 ± 0.4 kPa, which is comparable to previously published reports.\textsuperscript{26,29} The addition of small amounts of nanosilicates showed a significant increase in compressive modulus. For example, the addition of 0.5, 1, and 2% nanosilicates resulted in an increase in modulus to 4.7 ± 0.9, 8.9 ± 2.1, and 12.9 ± 1.3 kPa, respectively. The 4-fold increase in compressive modulus due to the addition of 2% nanosilicates can be attributed to enhanced nanoparticle–polymer interactions. A 10-fold increase in peak tensile stress at 90% strain was also observed upon
addition of nanosilicates with the Gel displaying a stress of $16 \pm 2.9$ kPa and the nanocomposite containing 2% nanosilicates displaying a stress of $164 \pm 30$ kPa.

To further investigate the effect of nanosilicates on the elastomeric properties of hydrogels, cyclic compression testing was performed consisting of five cycles of loading and unloading. The energy dissipated during cyclic loading was calculated from the area under the stress–strain curves over a range of strain amplitudes (Figure 2c). Hysteresis became apparent for strains above 0.40, indicating absorption of energy during deformation. Consistent with the results from a single loading (Figure 2b), the maximum stress at each strain amplitude was significantly higher for nanocomposites compared to the Gel hydrogels. The energy absorbed during cycling (90% cyclic compression) of the Gel and nanocomposite containing 2% nanosilicates was $1.5 \pm 0.4$ and $10.4 \pm 2.3$ kJ/m$^3$, respectively (Figure 2d). The addition of 2% nanosilicates to the Gel resulted in a greater than 6-fold increase in energy absorbed by the network. The increased energy dissipation is due to the relatively high stress generated with the stiff nanocomposite. Furthermore, the curves were repeatable after multiple cycles of loading and unloading, demonstrating that no plastic deformation was detected (Figure 2d). Thus, nanocomposites are each highly elastic under high compressive strains.

The effects of the addition of nanosilicates to the Gel network on gel rheology were investigated by subjecting the hydrogels to a range of amplitudes of oscillatory shear stress to calculate the elastic storage ($G'$) and loss ($G''$) moduli (Figure 2e). Each hydrogel showed significantly higher $G'$ compared to $G''$ at all shear stress amplitudes tested. Consistent with the results of the compression testing, the addition of nanosilicates increased $G'$.

In contrast, other types of nanoparticles incorporated within Gel hydrogels display minimal increases in modulus. For example, Shin et al. incorporated 5 mg/mL of multiwalled carbon nanotubes functionalized with carbboxyl groups within Gel hydrogels, and no significant increase in compressive modulus was observed. In a similar study, the addition of graphene oxide into Gel hydrogels did not significantly change the compressive modulus for a given UV exposure time. These studies indicated that these nanoparticles act simply as fillers that did not increase the mechanical stiffness of the hydrogel network. However, covalent cross-linking of nanoparticles to polymer chains can significantly increase stiffness. For example, Cha et al. observed that 3 mg/mL of methacrylated graphene oxide conjugated to the Gel resulted in a 2- to 3-fold increase in stiffness. In the current study, the addition of nanosilicates to the Gel increased the stiffness by more than 4-fold through noncovalent interactions between the nanosilicates and the Gel. Although the exact interactions between the Gel and nanosilicates have yet to be fully established, it can be expected that free amine and carboxylic groups present on the Gel backbone interact with the charged surface of the nanosilicates.

**Nanosilicates Enhance in Vitro Biomineralization and Physiological Stability.** in vitro biomineralization, which involves the deposition of hydroxyapatite in the presence of simulated body fluid (SBF), can be used to evaluate the in vitro osteogenic bioactivity of the hydrogel network. When a biomaterial’s surface is submerged in SBF, the formation of apatite-like deposits on the biomaterial surface indicates the bone-bonding capability of the hydrogel network. Collagen is one of the major components of bone and induces the nucleation of hydroxyapatite crystals. In our study, scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) data clearly indicated the formation of mineralized deposits over the surface of both the Gel and nanocomposites (Figure S3a). Images of these materials stained with Alizarin Red S to identify calcium deposition indicated staining intensity increased with increasing nanosilicate concentration (Figure S3b). Alizarin Red S was extracted with acetic acid and quantified by colorimetric detection. The results indicate a nanosilicate dose-dependent increase of more than 50% in Alizarin Red S staining. These results suggest that nanosilicates provide additional sites for hydroxyapatite nucleation and growth.

Gelatin is a biodegradable polymer and is highly susceptible to enzymatic degradation. The enzymatic degradation rates of nanosilicate-reinforced Gel hydrogels were evaluated in vitro under accelerated degradation conditions by using 2.5 units/mL of a collagenase solution (at 37 °C), Gel hydrogels readily degraded in the presence of collagenase, with full dissolution occurring within 10–12 h (Figure S4a and b), consistent with previous reports. Due to the highly interconnected and porous network (Figure S2), the collagenase solution can easily permeate the hydrogel network and degrade the entire network uniformly and rapidly. The addition of nanosilicates to the gelatin network significantly improved the stability of the hydrogel network under enzymatic conditions. The nanocomposite hydrogels containing 0.5, 1, and 2% nanosilicates degraded completely in approximately 50, 70, and 90 h, respectively. By fitting a first-order degradation (one-phase decay) curve to these results, the half-life of the Gel was observed to be 3.4 h, and the addition of 0.5, 1, and 2% nanosilicates improved the high-life of the network to 9.1, 18.5, and 22.9 h (Figure S4c). The more than 6-fold increase in the physiological stability of Gel hydrogels upon addition of 2% nanosilicates is likely due to the strong interactions between the polymer and nanoparticles.
The nanosilicate may act as a multifunctional cross-linker that interacts with multiple gelatin chains to potentially mask cleavage sites from collagenases.

**Nanocomposites Support Cellular Adhesion and Enhance in Vitro Mineralization.** The ability to support initial cell adhesion and subsequent proliferation is an important requirement of a tissue-engineered scaffold. Gelatin is a denatured protein that contains RGD groups, which play a significant role in cell adhesion via integrins. MC3T3 preosteoblasts were used to investigate the effect of nanosilicates on initial cell adhesion and proliferation. The cells readily adhered to Gel, and the addition of nanosilicates did not result in any significant influence in initial cell adhesion (Figures 3a and S5a). The cellular morphology indicated that the nanocomposites are cytocompatible and did not elicit any cytotoxic effects. The metabolic activity of preosteoblasts on the scaffold was quantified as a measure of cell proliferation over a period of 14 days in both normal and osteoconductive media (Figures 3a and S5b). All nanocomposites supported cellular proliferation, and cells showed metabolic activity over a period of 14 days similar to cells cultured on conventional tissue culture polystyrene (TCPS) dishes. These results support our previous published results indicating that nanosilicates are cytocompatible. Moreover, subcutaneous implantation of nanosilicate–gelatin showed a locally restricted inflammatory reaction in the host and complete degradation occurred within 28 days under in vivo conditions. On the basis of these studies, nanosilicate-based hydrogels can be easily used for biomedical applications.

Specific biochemical and physical cues in the ECM play a critical role in regulating cellular processes and their fate. By controlling these cues in the engineered ECM, it is possible to regulate or enhance outcomes
that facilitate tissue regeneration. To this end, the effects of nanosilicates on early osteogenic responses were monitored by qualitative and quantitative assessment of the presence and activity of ALP as an early marker of differentiation. We investigated the effect of nanosilicates on intracellular ALP production on days 3, 7, and 14 in both growth and osteoconductive media (growth media supplemented with 10 mM β-glycerophosphate and 50 μM ascorbic acid phosphate) (Figure S6). In growth media, cells seeded on the nanocomposites displayed a higher level of ALP staining (purple stain) compared to Gel and TCPS on days 3, 7, and 14. A significantly higher amount of ALP staining was observed using the osteoconductive media. The increase in nanosilicate concentration was also evident on ALP staining, indicating the key role of nanosilicate in the upregulation of osteogenic differentiation.

The ALP activity was then quantified with a colorimetric assay to quantify the ability of nanosilicates to promote osteogenic differentiation (Figure 3b). The results indicated a nanoparticle dose-dependent increase in ALP activity, with the addition of 2% nanosilicates causing a greater than 2.5-fold increase in ALP activity on day 14 compared to cells seeded on the Gel. There was no difference in the ALP activity of cells seeded on the Gel and TCPS on day 14, indicating that these materials are not osteoinductive. Importantly, the addition of 1% and 2% nanosilicates was able to significantly enhance the ALP activity in the growth media in the absence of any supplements (beta glycerol phosphates and ascorbic acid). A similar nanosilicate dose-dependent increase in ALP activity was detected in the presence of osteoconductive media, with the absolute ALP activities increased relative to that in growth media.

The production of bone-like inorganic calcium deposits, a hallmark for late-stage osteogenic differentiation, was determined by Alizarin Red S staining (Figures 3c and S7). While red staining was observed in differentiation, was determined by Alizarin Red S staining composite hydrogels was constructive media. The deposition of hydroxyapatite in nano-

clearly greater calcium deposition in the osteoconductive media after printing. This indicates that the bioactive nanosilicate-based hydrogel construct can be used to encapsulate cells without adversely affecting cellular viability.

A major challenge for 3D printing of biopolymers is maintaining mechanical integrity and preventing pore collapse while building the construct. Reasonable improvements in printing quality have been made by including hyaluronan or using precise control of the deposition head and substrate temperatures. However, these constructs required high concentrations (10–20 wt %) of gelatin to create viable scaffolds. In contrast, the shear-thinning characteristic of the Gel–nanosilicate microgels enables the printing of precisely designed scaffolds (Figure 4c). Solutions without nanosilicates did not retain dimensional features after printing. This indicates that the nanosilicate-based hydrogels can be used as bioinks for printing complex structures for tissue repair and organ replacement. Our future work in this direction will involve the encapsulation of human bone marrow derived stem cells within the 3D nanosilicate-based hydrogels for functional tissue and organ replacement strategy.

CONCLUSION

We engineered bioactive nanocomposite hydrogels loaded with nanosilicates within collagen-based polymer networks. Although the use of nanosilicates in the life sciences is still in its infancy, the promising bioactive effects promoted by interactions with nanosilicates has opened a wide range of biomedical and biological applications, including therapeutics, imaging, and disease-related diagnostics for regenerative medicine. When incorporated into a gelatin matrix, the nanosilicates likely interact with polymeric chains through strong electrostatic interactions and were shown to enhance several physical, chemical, and biological properties of the hydrogel. The addition of silicate nanoparticles increased stiffness and in vitro enzymatic stability, thus improving the tunable mechanical properties and degradation profile. In vitro data indicated that silicate-based bioactive nanocomposites
support cell adhesion and proliferation and promote osteogenic differentiation of preosteoblasts in the absence of any osteoinductive factor. Overall, these nanosilicate-based nanocomposites are highly bioactive and show strong promise for use in a range of bone tissue engineering applications.

METHODS

Synthesis of Methacrylated Gelatin and Gelatin–Nanosilicate Nanocomposites. Gelatin (type A, from porcine skin) and methacrylic anhydride (MA) were purchased from Sigma-Aldrich, USA. Gelatin methacrylate was synthesized using a previously published method. Briefly, a 10% gelatin (w/v) solution in Dulbecco’s phosphate-buffered saline (PBS) was prepared at 60 °C. To induce the acrylation of pendent amine groups of the gelatin, methacrylic anhydride (8 mL/10 g gelatin) was added dropwise into the solution while vigorously stirring for 3 h and maintained at 60 °C. The reaction was eventually terminated by addition of excess preheated PBS at 40 °C into the solution. The as-prepared GelMA solution was dialyzed against ultrapure water (18.2 Ω cm) for a week at 40 °C to remove unreacted methacrylic anhydride and other impurities using a dialysis membrane (mol wt cut off ~ 12–14 kDa). The solution was then frozen at ~80 °C and lyophilized for further characterizations. Nanocomposite hydrogels from GelMA and nanosilicates (also known as Laponite) were prepared at room temperature. The nanosilicates (Laponite XLG) were obtained from Southern Clay Products Inc., USA, and were 25–30 nm in diameter and 1 nm thick. To prepare nanocomposite hydrogels, first the nanosilicates (0.5, 1, and 2 wt %) were dispersed in a photoinitiator solution (0.5 wt % Ciba IRGACURE 2959, Ciba Specialty Chemical, USA) and mixed vigorously for 15 min. Then, GelMA (5 wt %) was added to the nanosilicate solution and was allowed to disperse for an additional 15 min by vortexing. The prepolymer solution (GelMA and nanosilicates) was cross-linked using UV exposure (320–500 nm) (Omnicure S200, Lumen Dynamics, Canada) for 60 s at an intensity of 6.9 mW/cm². Nanocomposite hydrogels were then soaked in PBS to remove noncross-linked prepolymer segments.

Physical and Structural Characterization of Nanocomposite Hydrogels. Fourier transform infrared spectra (FT-IR) of the hydrogel were characterized using a Bruker Vector-22 FTIR spectrophotometer (PIKE Technologies, USA). The zeta potential and electrophoretic mobility of the gelatin, nanosilicates, and GelMA–silicate prepolymer solutions were measured at 25 °C using a Zetasizer (Malvern Instruments, UK) equipped with a He–Ne laser. Transmission electron microscopy was performed using a JEOL-JEM 2010 (Japan) at an accelerating voltage of 200 kV on a carbon grid to confirm the size of nanosilicates and interactions between GelMA and nanosilicates. The surface morphology of the nanocomposite hydrogels was evaluated using scanning electron microscopy (FEI Quanta 600 FE-SEM, USA, fitted with an Oxford EDS system) at an accelerating voltage of 20 kV. In brief, to prepare samples for SEM the nanocomposite hydrogels were frozen using liquid nitrogen and then lyophilized for 3 days to obtain dried samples. The dried nanocomposite samples were sputter coated with Au/Pd up to a thickness of 8 nm before Figure 4. Microfabricated structures from nanocomposite hydrogels. (a) Schematic representation of fabrication of nanosilicate-loaded microgels. Cells readily adhered, elongated, and formed interconnected networks on these microgels, as shown by phalloidin/DAPI images stained for actin filaments and nuclei. (b) In the 3D encapsulation of cells in nanocomposite hydrogels, all scaffolds supported cellular viability, as represented with live/dead images on day 1, day 2, and day 4. (c) The addition of nanosilicate to GelMA (prepolymer) results in a shear-thinning characteristic and can be printed to design complex structures. The printed hydrogels were covalently cross-linked using UV to obtain mechanically stiff hydrogels.
ammonium hydroxide. The calibration curve was obtained from di- 

two moles of calcium in an Alizarin Red S-calcium complex. A 

Life Technologies, USA), and 1% penicillin/streptomycin (100 U/100 

μL) were cultured in normal growth media (Alpha MEM, 

USA), the amount of Alizarin Red S was quanti-

fied and neutralizing with 10% (v/v) ammonium hydroxide (Sigma, 

acid solution to dissolve the Alizarin Red S. After centrifuging 

hydrogels. Later, the hydrogels were dissolved in a 0.01 M acetic 

acid solution. The obtained SBF-coated gels were stained with a 0.5% 

fluorescent microscopy (Amoscope, USA). The mineral-

ized matrix produced by preosteoblast cells on the nanocompo-

sites was analyzed on days 7 and 14 using Alizarin Red S staining. The 

cells on the nanocomposite samples were fixed with 2% 
gluteraldehyde for 15 min and then added with 0.5% Alizarin 

Red S (pH 4.2; Sigma-Aldrich, USA). After 15 min of incubation, the 
samples were washed with distilled water to remove the weakly 

adhered Alizarin Red S, and then imaging was performed. The 

chemical characteristics of deposited mineral were confirmed by 

Raman spectra (R200-L, SENTERRA Raman microscope) using 
excitation: 785 and 532 nm (He–Ne laser).

Microfabricated Structures and 3D Encapsulation. To fabricate 
nanocomposite microgels, 20 μL of prepolymer solution con-
taining GelMA, nanosilicates, and 0.25% photoinitiator was placed 
between glass slides and UV cross-linked using 6.9 mW/cm² for 
20 s. After cross-linking, the microgels were washed with PBS, 
seeded with preosteoblasts cells, and incubated in normal 
media conditions. After 4 days of postseeding the normal media 
was removed, microgels were washed with PBS, and the cells 
were fixed using 2% glutaraldehyde solution. The Alexa Fluor 
488 Phalloidin (Life Technologies, USA) working solution was 
used to visualize the cellular structures. 

The wet weight (Mw) of the samples was measured prior to their 
lyophilization for 24 h to obtain the dried weight (Md). The 
hydration degrees of the nanocomposite hydrogels are calcu-
ulated using eq 1:

\[
\text{Hydration degree} = \frac{(M_w - M_d)}{M_d} \times 100
\] (1)

Hydrogels were subjected to enzymatic degradation by incubating the hydrogels in a collagenase enzyme solution at 37 °C. The enzyme solution was freshly prepared on the day of the experiment using phosphate-buff-

ered saline. The working solution of collagenase (2.5 units/mL) was prepared in PBS from the 300 units/mg collagenase type II 

enzyme (Worthington Biochemicals, USA). Weight loss was monitored for 4 days, and the percent mass remaining of the 
nanocomposites was calculated. The ability of nanocomposites to facilitate biomineralization was determined by using 10× 
stained on a laboratory glass slide. Nanocomposites prepared by photo-

polymerization with varying nanosilicate concentrations were 
immersed in 10× SBF solution for 30 min at 37 °C. Then gels 
were removed from the SBF solution and washed 3–5 times in 
distilled water to remove the weakly bound serum from the gel surface. The obtained SBF-coated gels were stained with a 0.5% 
Alizarin Red S (Sigma-Aldrich, USA) staining for calcium depos-
its. A stereomicroscope was used to take the images of the 
hydrogels. Later, the hydrogels were dissolved in a 0.01 M acetic 
solution to dissolve the Alizarin Red S. After centrifuging and 
neutralizing with 10% (v/v) ammonium hydroxide (Sigma, 
USA), the amount of Alizarin Red S was quantified by 
determining the absorbance at 405 nm. One mole of Alizarin Red binds to 
two moles of calcium in an Alizarin Red-Ca complex. A 
calibration curve was obtained from different concentrations of 
Alizarin Red S in distilled water at pH 4.2, adjusted with 10% (v/v) 
ammonium hydroxide.

In Vitro Cell Studies. Preosteoblast cell lines (NIH MC3T3 E1–4, 
ATCC, USA) were cultured in normal growth media (Alpha MEM, 
Life Technologies, USA), supplemented with 10% FBS (Life Tech-

nologies, USA) and 1% penicillin/streptomycin (100 U/100 
μL). 

In Vitro Cell Studies. Preosteoblast cell lines (NIH MC3T3 E1–4, 
ATCC, USA) were cultured in normal growth media (Alpha MEM, 
Life Technologies, USA) at 37 °C with 5% CO₂. The nanocomposite 
hydrogels of 6 mm diameter and 400 μm thickness were 
prepared in 96-well plates. Then, preosteoblast cells were tryp-
tsinated 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 ethidium homodimer (Santa Cruz Biotech-

ology Inc., USA) were added to the nanocomposites and 
icubated for 30 min at 37 °C. The samples were washed with PBS 
times and imaged using an epifluorescence micro-

scope (TE2000-S, Nikon, USA). The in vitro studies were performed 
in normal media and osteoconductive media. The osteoconduc-
tive media was prepared by supplementing the normal growth 
media with 10 mM β-glycerophosphate and 50 μM ascorbic acid 
phosphate (Sigma-Aldrich, USA). The metabolic activity of these 
cells was analyzed using the Alamar Blue assay (Thermo Scientific, 
USA) in both normal and osteoconductive media on days 0, 1, 3, 7, 
10, and 14 using the manufacturer’s protocol. The absorbance 
was recorded using a fluorescence microplate reader (Infinite 
M200PRO, TECAN, Europe) to obtain the percent reduction in 
Alamar Blue. The expression of intercellular alkaline phosphatase in 
preosteoblast cells seeded on the nanocomposite hydrogels in 
a 48-well plate was evaluated using nitro-blue tetrazolium/indolyl 
phosphate (NBT/BCIP) staining (Thermo Scientific, USA). The 
nanocomposites were washed with PBS and then incubated with 
0.25 mL of NBT/BCIP at 37 °C for 30 min. After incubation, 
samples were washed with PBS and imaged under bright-field 
mode using a stereomicroscope (Amoscope, USA). The mineral-
ized matrix produced by preosteoblast cells on the nanocompo-
sites was analyzed on days 7 and 14 using Alizarin Red S staining. The 
cells on the nanocomposite samples were fixed with 2% 
gluteraldehyde for 15 min and then added with 0.5% Alizarin 
Red S (pH 4.2; Sigma-Aldrich, USA). After 15 min of incubation, the 
samples were washed with distilled water to remove the weakly 
adhered Alizarin Red S, and then imaging was performed. The 
chemical characteristics of deposited mineral were confirmed by 
Raman spectra (R200-L, SENTERRA Raman microscope) using 
excitation: 785 and 532 nm (He–Ne laser).

3D-printed nanocomposite gels were fabricated with fused 
deposition modeling. Prepolymer solutions containing 8% 
GelMA (80% methacrylation degree), 0.25% photoinitiator, and 
2% nanosilicates, by weight, were mixed at elevated 
temperature and allowed to cool to room temperature before 
printing. Solutions were loaded into a HYRELE Engine 3D printer 
equipped with an EMO-25 emulsifiable Snyder and UV 
blocking, 22 AWG (200 μm) tapered. A cylinder model, h = 5 mm, diameter = 20 mm, was used as the 
physical template for printing. SLIC3r v1.1.7 was used to create 
the g-code for printing. The following settings were used for printing: printing speeds of 5 mm/s, nonprinting speeds of 
25 mm/s, layer thickness of 200 μm, extrusion width of 1.0 mm, and no perimeters, top layers, or bottom layers. Constructs were subjected to 
UV light for the duration of the print (6 min and 50 s). The 
UV source consists of four UV LEDs (365 nm, 500 mW radiant flux) 
positioned at a 5 cm vertical distance and 2 cm radial distance from 
the printing nozzle. After printing, gels were subjected to 
an additional 5 min of cross-linking under the UV printing 
source. 

Statistical Analysis. All the data presented are means and 
standard deviations of the experiments (n = 5 or 6). Statistical analysis was performed using GraphPad Prism 5 (San Diego, 
CA, USA). The significant differences between sample groups were 
determined using the nonparametric test, one-way analysis of 
variance (ANOVA), and Tukey’s post-hoc analysis for pairwise 
mean comparisons. The statistical significance was defined as 
p < 0.05, **p < 0.01, ***p < 0.005.
Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Additional data (structural, chemical, and biological characterization of nanocomposite hydrogels) are available free of charge via the Internet at http://pubs.acs.org.

REFERENCES AND NOTES


