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Thiol-Epoxy “Click” Chemistry to Engineer Cytocompatible PEG-Based Hydrogel for siRNA-Mediated Osteogenesis of hMSCs

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Abstract

Thiol-epoxy “click” chemistry is employed for the first time to engineer a new cytocompatible PEG-based hydrogel system in aqueous media with the ability to encapsulate human mesenchymal stem cells (hMSCs) and control their fate for tissue regeneration. Cells were easily encapsulated into the hydrogels and exhibited high cell viability over 4 weeks of culture regardless of the presence of siRNA, complexed with polyethylenimine (PEI) in the form of siRNA/PEI nanocomplexes, indicating the biocompatibility of the developed hydrogel. Loading pro-osteogenic siNoggin in the hydrogel significantly enhanced the osteogenesis of encapsulated hMSCs, demonstrating the potential application of this system in tissue engineering.

Graphical Abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information

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Detailed procedure for hydrogel preparation, reaction monitoring, gelation time measurement, hydrogel swelling measurement, stem cells encapsulation, and differentiation experiment ([PDF](#))

The authors declare no competing financial interest.

Keywords

biomaterial; click chemistry; hydrogel; RNAi; stem cell differentiation; tissue regeneration

Hydrogel biomaterials are three-dimensional (3D) networks of cross-linked hydrophilic polymers that contain a large fraction of water and have been widely explored as biomaterial matrices for use in applications, such as therapeutic delivery, wound dressing and tissue engineering.^{1–12} Hydrogel biomaterials play an important role in the field of regenerative medicine and tissue engineering by offering the potential to encapsulate cells with minimal cytotoxicity.^{10–13} Additionally, hydrogel biomaterials possess a highly hydrated, porous structure that may provide the capacity for localized, sustained, or controlled release of bioactive molecules and facilitate the transport of nutrients and oxygen to incorporated cells and the removal of waste.^{10–13} A wide range of natural and synthetic polymers including alginate,¹⁴ collagen,^{15,16} chitosan,¹⁷ gelatin,¹⁶ polypeptides,¹² and poly(ethylene glycol) (PEG),^{4,18–20} and a variety of mechanisms, such as self-assembly,^{7–9} ionic cross-linking^{14,21} and covalent cross-linking,^{1–4,22} have been utilized to fabricate hydrogel biomaterials. Among these chemistries, covalent cross-linking including free radical photopolymerization, Michael-type addition, click reactions, Schiff base reaction, and enzyme-mediated reactions^{1–4,10–12,16,22,23} has been extensively used to fabricate hydrogels for tissue engineering. “Click” reaction between thiol and epoxy groups is an efficacious chemistry for organic/polymer synthesis, and polymer/material chemistry.^{24–32} This chemistry has also been used to fabricate hydrogels in organic solvents^{32,33} and in aqueous media.³⁴ Although it offers many advantages for hydrogel formation such as high reaction efficiency, controllable in situ gelation time at ambient temperature for concurrent injection, and fabrication of hydrophilic hydrogel networks with abundant functionalizable hydroxyl groups,^{32–34} this chemistry has never been exploited to fabricate a hydrogel system for

bioactive factor delivery, cell-encapsulation, or use in tissue regeneration. Therefore, employing thiol-epoxy chemistry to fabricate a cytocompatible hydrogel system capable of encapsulation of cells or bioactive molecules while maintaining high cell viability may provide an important new strategy for biomaterial design for tissue engineering applications.

Human mesenchymal stem cells (hMSCs) are a promising cell source for cell-based therapies such as tissue regeneration applications due to their ability to produce trophic factors, modulate the immune system and differentiate into a variety of cell types under proper conditions, such as osteoblasts, chondrocytes, adipocytes, and myoblasts.^{2-4,35-43} hMSCs have been encapsulated into polymeric hydrogel biomaterials to regenerate tissues, such as bone,^{4,22,44,45} cartilage,^{46,47} and fat.⁴⁵ Bioactive molecules, such as growth factors (e.g., bone morphogenetic protein-2 (BMP-2)^{2-4,22,35} and transforming growth factors³⁵⁻³⁷) and genetic material (e.g., plasmid DNA and RNA),^{2-4,48,49} have been shown to play an important role in guiding the differentiation of hMSCs in regenerative medicine and tissue engineering strategies. RNA interference (RNAi) in particular, which includes both small interfering RNAs (siRNAs) and microRNAs (miRNAs), is a powerful tool for post-transcriptionally silencing cell gene expression to modulate cell behavior to regenerate tissues or treat diseases.^{2-4,49-51} RNAi-molecule delivery provides an effective approach for controlling the differentiation of hMSCs down the osteoblastic,^{2-4,22,38,49} chondrogenic,⁵² and adipogenic^{38,49} lineages via regulating the expression of specific genes. For example, siRNA targeting noggin (siNog) has been used for enhancing osteogenic differentiation of hMSCs by silencing the expression of noggin, a protein that negatively affects the osteogenesis of hMSCs by binding to BMPs, preventing their interactions with receptors on the cell surface that mediate downstream signaling pathways.^{2-4,53} Co-encapsulation of hMSCs and siNog into 3D hydrogel networks has been reported to enhance hMSC osteogenesis to different extents^{4,22,54} and offers a potential strategy for engineering bone tissue.

This study reports for the first time a novel cytocompatible PEG-based hydrogel system formed via thiol-epoxy “click” chemistry in aqueous media that was prepared under mild conditions directly from commercially available macromers and possessed the capability of encapsulating hMSCs, maintaining high cell viability, and supporting cell differentiation. In addition, a pro-osteogenic siNog (sequence 5'-AAC ACU UAC ACU CGG AAA UGA UGG G-3') was loaded into the hydrogels to demonstrate the potential to guide the differentiation of hMSCs encapsulated in this hydrogel system.

The hydrogels were fabricated by mixing solutions of 8-arm thiolated PEG (PEG(-SH)₈) and PEG-diepoxy (PEG-DE) (Figure 1 A and B, respectively) at basic pH. A detailed schematic of hydrogel preparation is shown in Scheme S1. In aqueous media at basic pH, the reaction between epoxy and thiol groups occurred rapidly, as demonstrated by the appearance and increase in intensity over time of methylene protons of the formed β -hydroxyl thioether groups in NMR spectra (Figure 1C). Consistent data was also recorded via carbon NMR (Figure 1D). However, the reaction did not occur at neutral pH, as shown via monitoring the change of chemical groups in the macromer mixture solution (Figure S1). In addition, the NMR confirmed that self-reaction of thiol or epoxy groups in the individual macromer

solutions did not occur (Figure S1). These NMR results confirm that the reaction of thiol and epoxy groups in aqueous media at basic pH can be used to prepare a hydrogel network.

After confirming the reactivity between epoxy and thiol groups, the hydrogels were fabricated and the gelation times were measured using the tube inversion method^{2,4} and rheological measurements.² It was observed in both methods that the gelation time increased with decreasing macromer concentration. The hydrogels formed between 9.4 to 16.9 min after formulation, depending on the final macromer concentration (Figure 2A) and the number of arms present in the PEG-thiol (Figure S2). Similar results were observed in rheological measurements, in which the gelation point was determined as the time at which G' and G'' crossed each other (Figure 2B). This gelation time frame offers a suitable window, which ranges from minutes to an hour,^{55,56} for cell encapsulation within the hydrogel and subsequent injection into the body, which will be valuable for tissue engineering applications. Increasing the hydrogel concentration also led to increased hydrogel stiffness, as illustrated by increased G' . In addition, the swelling ratio of the hydrogels increased slightly over the course of 4 weeks in both PBS and DMEM-HG media (Figure S3). A slightly higher swelling ratio in DMEM-HG group compared to those in PBS was observed which may be a result of the differences in composition between the two media, such as glucose content. Decreasing cross-linking density via lowering hydrogel concentration led to the increase in swelling ratio (Figure S3).⁵⁷

Biocompatibility is a very important factor to consider in the engineering of biomaterial systems for cell encapsulation-based tissue regeneration.^{2,4,12,22} To determine the potential cytotoxicity of this hydrogel system, hMSCs were encapsulated in the hydrogels with or without the loading of siRNA against luciferase (siLuc) complexed with polyethylenimine (PEI), in the form of siRNA/PEI nanocomplexes. The cell viability over time was examined using live/dead staining. The encapsulated hMSCs remained highly viable over 4 weeks of culture, regardless of the absence or presence of siRNA/PEI nanocomplexes (Figure 2C, D), indicating the cytocompatibility of the fabricated hydrogel system. In addition, using Fiji software to quantify the cell viability in live/dead stained images, no significant differences was observed between experimental time points or between groups with and without loaded RNA/PEI nanocomplexes in the hydrogels (Figure S4). Although a hydrogel fabricated via thiol-epoxy chemistry in aqueous media was recently confirmed to be biocompatible in the presence of monolayer cultured cells for up to 24 h,³⁴ longer culture times and, importantly, cell encapsulation were not examined. The developed hydrogel system in this study offers the capacity to encapsulate cells with long-term viability, demonstrating its potential for 3D cell culture.

After confirming cytocompatibility of the hydrogel system, hMSCs were then encapsulated within hydrogels loaded with and without siNog, a pro-osteogenic siRNA,²⁻⁴ complexed with PEI to examine the ability of the developed hydrogel and/or siNog to support hMSC osteogenesis (Figure 3). Calcium (Ca) is a major component of hydroxyapatite, the inorganic portion of bone extracellular matrix, and thus, was used to evaluate the degree of encapsulated hMSC osteogenic differentiation. It was hypothesized that hMSCs in the constructs cultured in growth media (GM) would survive but not differentiate (Figure 3C), while encapsulated hMSCs cultured in osteogenic media (OM) would differentiate and

deposit Ca (Figure 3D). Additionally, it was anticipated that encapsulated siNog in the hydrogels would enhance hMSCs differentiation rate and extent, and, therefore, increase the amount of deposited Ca (Figure 3E).

To validate the hypothesis, hMSCs from two different donors were separately encapsulated in the hydrogel constructs, which were then cultured in GM or OM, and harvested at predetermined time points to assay DNA and Ca content (Figure 4). DNA content was quantified as an indirect measure of construct cellularity, and Ca content served as a late differentiation marker for osteogenesis. Detailed experimental protocols are provided in the Supporting Information (SI). In GM, no decrease in DNA content in RNA-free constructs was observed for either donor after 4 weeks of culture (Figure 4A1 and B1), indicating the cytocompatibility of the developed hydrogel system. When the RNA-free constructs were cultured in OM, DNA significantly decreased from 2 to 4 weeks in donor 2 constructs (Figure 4B1), while a significant decrease in DNA was observed in both donors at 3 or 4 weeks compared to culturing in GM (Figure 4A1 and B1). There are reports in the literature that suggested osteoblasts have a limited life span and tend to become lining cells or osteocytes or undergo apoptosis.^{58–60} Therefore, the decrease in DNA content observed may be attributed to a degree of apoptosis that occurred during the osteogenic differentiation of the encapsulated hMSCs. hMSCs encapsulated in hydrogels cultured in OM showed significantly more Ca deposition compared to those cultured in GM, both in the absolute amount and after normalizing to DNA content (Figure 4A2,3 and B2,3), demonstrating that this hydrogel system supports the osteogenesis of encapsulated hMSCs.

When cultured in OM, siRNA loading within hydrogels did not affect the DNA content of constructs from either donor (Figures 4A1 and B1 and S5A), indicating low cytotoxicity with siRNA treatment. Importantly, loaded siNog significantly enhanced the osteogenesis of encapsulated hMSCs, as demonstrated by a significant increase in Ca deposition compared to hydrogels cultured in OM without incorporated genetic material (Figure 4A2,3 and B2,3) or loaded with nontargeting control siRNA (siCT; Figure S5B and C). siNog was previously reported to enhance the osteogenesis of hMSCs with approximately 3.5-fold and 1.5-to-2.2-fold increase in Ca deposition when cultured in monolayer^{2,3} or encapsulated in 3D hydrogel networks,^{4,22} respectively. Construct sections stained for calcium with Alizarin Red S (ARS) also exhibited more intense staining in siNog/OM constructs compared to the other groups (Figure 4C), which is consistent with biochemical results. No Ca was found biochemically or histologically in hydrogels without cells and siRNA cultured in either GM or OM for 4 weeks (Figure S6), indicating that the detected Ca in the previously described experiments was produced by differentiated hMSCs. These results confirm that the developed hydrogel system is a promising biomaterial candidate for 3D cell culture and bone tissue regeneration.

In summary, a novel PEG-based hydrogel formed via thiol-epoxy “click” reaction has been engineered. The hydrogel supports 3D cell culture and the osteogenesis of encapsulated hMSCs. Specifically, loading the developed hydrogels with siNog, a pro-osteogenic siRNA, significantly enhanced the osteogenic differentiation of hMSCs. This cross-linking strategy has great utility in forming hydrogels for cell encapsulation, tissue engineering, bioactive factor delivery, and other biomedical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- (1). Huynh CT; Nguyen MK; Tonga GY; Longe L; Rotello VM; Alsberg E Photocleavable Hydrogels for Light-Triggered siRNA Release. *Adv. Healthcare Mater* 2016, 5, 305–310.
- (2). Huynh CT; Zheng Z; Nguyen MK; McMillan A; Yesilbag Tonga G; Rotello VM; Alsberg E Cytocompatible Catalyst-Free Photodegradable Hydrogels for Light-Mediated RNA Release to Induce hMSC Osteogenesis. *ACS Biomater. Sci. Eng* 2017, 3, 2011–2023.
- (3). Huynh CT; Nguyen MK; Naris M; Tonga GY; Rotello VM; Alsberg E Light-Triggered RNA Release and Induction of hMSC Osteogenesis Via Photodegradable, Dual-Crosslinked Hydrogels. *Nanomedicine* 2016, 11, 1535–1550. [PubMed: 27246686]
- (4). Nguyen MK; Jeon O; Krebs MD; Schapira D; Alsberg E Sustained Localized Presentation of RNA Interfering Molecules from in Situ Forming Hydrogels to Guide Stem Cell Osteogenic Differentiation. *Biomaterials* 2014, 35, 6278–6286. [PubMed: 24831973]
- (5). Nguyen KT; West JL Photopolymerizable Hydrogels for Tissue Engineering Applications. *Biomaterials* 2002, 23, 4307–4314. [PubMed: 12219820]
- (6). Wu J; Zhu J; He C; Xiao Z; Ye J; Li Y; Chen A; Zhang H; Li X; Lin L; Zhao Y; Zheng J; Xiao J Comparative Study of Heparin-Poloxamer Hydrogel Modified bFGF and aFGF for in Vivo Wound Healing Efficiency. *ACS Appl. Mater. Interfaces* 2016, 8, 18710–18721. [PubMed: 27384134]
- (7). Huynh CT; Nguyen MK; Lee DS Injectable Block Copolymer Hydrogels: Achievements and Future Challenges for Biomedical Applications. *Macromolecules* 2011, 44, 6629–6636.
- (8). Nguyen QV; Lym JS; Huynh CT; Kim BS; Jae HJ; Kim YI; Lee DS A Novel Sulfamethazine-Based pH-Sensitive Copolymer for Injectable Radiopaque Embolic Hydrogels with Potential Application in Hepatocellular Carcinoma Therapy. *Polym. Chem* 2016, 7, 5805–5818.
- (9). Lym JS; Nguyen QV; Ahn DW; Huynh CT; Jae HJ; Kim YI; Lee DS Sulfamethazine-Based pH-Sensitive Hydrogels with Potential Application for Transcatheter Arterial Chemoembolization Therapy. *Acta Biomater* 2016, 41, 253–263. [PubMed: 27184404]
- (10). Drury JL; Mooney DJ Hydrogels for Tissue Engineering: Scaffold Design Variables and Applications. *Biomaterials* 2003, 24, 4337–4351. [PubMed: 12922147]
- (11). Hoffman AS Hydrogels for Biomedical Applications. *Adv. Drug Delivery Rev* 2012, 64, 18–23.
- (12). Nguyen MK; Alsberg E Bioactive Factor Delivery Strategies from Engineered Polymer Hydrogels for Therapeutic Medicine. *Prog. Polym. Sci* 2014, 39, 1235–1265.
- (13). Hubbell JA Biomaterials in Tissue Engineering. *Nat. Biotechnol* 1995, 13, 565–576.
- (14). Krebs MD; Jeon O; Alsberg E Localized and Sustained Delivery of Silencing RNA from Macroscopic Biopolymer Hydrogels. *J. Am. Chem. Soc* 2009, 131, 9204–9206. [PubMed: 19530653]
- (15). Nguyen K; Das B; Dobrowolski C; Karn J Multiple Histone Lysine Methyltransferases Are Required for the Establishment and Maintenance of HIV-1 Latency. *mBio* 2017, 8, e00133–17. [PubMed: 28246360]
- (16). Harding JL; Osmond MJ; Krebs MD Engineering Osteoinductive Biomaterials by Bioinspired Synthesis of Apatite Coatings on Collagen Hydrogels with Varied Pore Microarchitectures. *Tissue Eng., Part A* 2017, 23, 1452–1465. [PubMed: 28622088]

- (17). Han HD; Mora EM; Roh JW; Nishimura M; Lee SJ; Stone RL; Bar-Eli M; Lopez-Berestein G; Sood AK Chitosan Hydrogel for Localized Gene Silencing. *Cancer Biol. Ther* 2011, 11, 839–845. [PubMed: 21358280]
- (18). Wang Y; Malcolm DW; Benoit DSW Controlled and Sustained Delivery of siRNA/NPs from Hydrogels Expedites Bone Fracture Healing. *Biomaterials* 2017, 139, 127–138. [PubMed: 28601703]
- (19). Huynh CT; Nguyen QV; Lym JS; Kim BS; Huynh DP; Jae HJ; Kim YI; Lee DS Intraarterial Gelation of Injectable Cationic pH/Temperature-Sensitive Radiopaque Embolic Hydrogels in a Rabbit Hepatic Tumor Model and Their Potential Application for Liver Cancer Treatment. *RSC Adv.* 2016, 6, 47687–47697.
- (20). Huynh CT; Nguyen QV; Kang SW; Lee DS Synthesis and Characterization of Poly(Amino Urea Urethane)-Based Block Copolymer and Its Potential Application as Injectable pH/Temperature-Sensitive Hydrogel for Protein Carrier. *Polymer* 2012, 53, 4069–4075.
- (21). Krebs MD; Alsberg E Localized, Targeted, and Sustained siRNA Delivery. *Chem. - Eur. J* 2011, 17, 3054–3062. [PubMed: 21341332]
- (22). Nguyen MK; McMillan A; Huynh CT; Schapira DS; Alsberg E Photocrosslinkable, Biodegradable Hydrogels with Controlled Cell Adhesivity for Prolonged siRNA Delivery to hMSCs to Enhance Their Osteogenic Differentiation. *J. Mater. Chem. B* 2017, 5, 485–495. [PubMed: 28652917]
- (23). Dong D; Li J; Cui M; Wang J; Zhou Y; Luo L; Wei Y; Ye L; Sun H; Yao F In Situ “Clickable” Zwitterionic Starch-Based Hydrogel for 3D Cell Encapsulation. *ACS Appl. Mater. Interfaces* 2016, 8, 4442–4455. [PubMed: 26817499]
- (24). Stuparu MC; Khan A Thiol-Epoxy “Click” Chemistry: Application in Preparation and Postpolymerization Modification of Polymers. *J. Polym. Sci., Part A: Polym. Chem* 2016, 54, 3057–3070.
- (25). Gadwal I; Binder S; Stuparu MC; Khan A Dual-Reactive Hyperbranched Polymer Synthesis through Proton Transfer Polymerization of Thiol and Epoxide Groups. *Macromolecules* 2014, 47, 5070–5080.
- (26). Gadwal I; Khan A Protecting-Group-Free Synthesis of Chain-End Multifunctional Polymers by Combining ATRP with Thiol-Epoxy ‘Click’ Chemistry. *Polym. Chem* 2013, 4, 2440.
- (27). Brändle A; Khan A Thiol-Epoxy ‘Click’ Polymerization: Efficient Construction of Reactive and Functional Polymers. *Polym. Chem* 2012, 3, 3224.
- (28). Lin H; Ou J; Liu Z; Wang H; Dong J; Zou H Thiol-Epoxy Click Polymerization for Preparation of Polymeric Monoliths with Well-Defined 3D Framework for Capillary Liquid Chromatography. *Anal. Chem* 2015, 87, 3476–3483. [PubMed: 25679546]
- (29). Jin K; Wilmot N; Heath WH; Torkelson JM Phase-Separated Thiol-Epoxy-Acrylate Hybrid Polymer Networks with Controlled Cross-Link Density Synthesized by Simultaneous Thiol-Acrylate and Thiol-Epoxy Click Reactions. *Macromolecules* 2016, 49, 4115–4123.
- (30). Nair DP; Podgórski M; Chatani S; Gong T; Xi W; Fenoli CR; Bowman CN The Thiol-Michael Addition Click Reaction: A Powerful and Widely Used Tool in Materials Chemistry. *Chem. Mater* 2014, 26, 724–744.
- (31). Hoyle CE; Lowe AB; Bowman CN Thiol-Click Chemistry: A Multifaceted Toolbox for Small Molecule and Polymer Synthesis. *Chem. Soc. Rev* 2010, 39, 1355–1387. [PubMed: 20309491]
- (32). Mommer S; Truong K.-N. i.; Keul H; Möller M An Epoxy Thiolactone on Stage: Four Component Reactions, Synthesis of Poly(Thioether Urethane)S and the Respective Hydrogels. *Polym. Chem* 2016, 7, 2291–2298.
- (33). Cengiz N; Rao J; Sanyal A; Khan A Designing Functionalizable Hydrogels through Thiol-Epoxy Coupling Chemistry. *Chem. Commun* 2013, 49, 11191–11193.
- (34). Gao L; Li X; Wang Y; Zhu W; Shen Z; Li X Injectable Thiol-Epoxy “Click” Hydrogels. *J. Polym. Sci., Part A: Polym. Chem* 2016, 54, 2651–2655.
- (35). Dang PN; Dwivedi N; Yu X; Phillips L; Bowerman C; Murphy WL; Alsberg E Guiding Chondrogenesis and Osteogenesis with Mineral-Coated Hydroxyapatite and BMP-2 Incorporated within High-Density hMSC Aggregates for Bone Regeneration. *ACS Biomater. Sci. Eng* 2016, 2, 30–42.

- (36). Dikina AD; Strobel HA; Lai BP; Rolle MW; Alsberg E Engineered Cartilaginous Tubes for Tracheal Tissue Replacement Via Self-Assembly and Fusion of Human Mesenchymal Stem Cell Constructs. *Biomaterials* 2015, 52, 452–462. [PubMed: 25818451]
- (37). McMillan A; Nguyen MK; Gonzalez-Fernandez T; Ge P; Yu X; Murphy WL; Kelly DJ; Alsberg E Dual Non-Viral Gene Delivery from Microparticles within 3D High-Density Stem Cell Constructs for Enhanced Bone Tissue Engineering. *Biomaterials* 2018, 161, 240–255. [PubMed: 29421560]
- (38). Hemming S; Cakouros D; Isenmann S; Cooper L; Menicanin D; Zannettino A; Gronthos S EZH2 and KDM6A Act as an Epigenetic Switch to Regulate Mesenchymal Stem Cell Lineage Specification. *Stem Cells* 2014, 32, 802–815. [PubMed: 24123378]
- (39). Pirjali T; Azarpira N; Ayatollahi M; Aghdaie MH; Geramizadeh B; Talai T Isolation and Characterization of Human Mesenchymal Stem Cells Derived from Human Umbilical Cord Wharton's Jelly and Amniotic Membrane. *Int. J. Organ Transplant Med* 2013, 4, 111–116. [PubMed: 25013662]
- (40). Amado LC; Saliaris AP; Schuleri KH St; John St.; Xie JS; Cattaneo S; Durand DJ; Fitton T; Kuang JQ; Stewart G; Lehrke S; Baumgartner WW; Martin BJ; Heldman AW; Hare JM Cardiac Repair with Intramyocardial Injection of Allogeneic Mesenchymal Stem Cells after Myocardial Infarction. *Proc. Natl. Acad. Sci. U. S. A* 2005, 102, 11474–11479. [PubMed: 16061805]
- (41). Pittenger MF; Mackay AM; Beck SC; Jaiswal RK; Douglas R; Mosca JD; Moorman MA; Simonetti DW; Craig S; Marshak DR Multilineage Potential of Adult Human Mesenchymal Stem Cells. *Science* 1999, 284, 143–147. [PubMed: 10102814]
- (42). Hofer HR; Tuan RS Secreted Trophic Factors of Mesenchymal Stem Cells Support Neurovascular and Musculoskeletal Therapies. *Stem Cell Res. Ther* 2016, 7, 131. [PubMed: 27612948]
- (43). Ma S; Xie N; Li W; Yuan B; Shi Y; Wang Y Immunobiology of Mesenchymal Stem Cells. *Cell Death Differ.* 2014, 21, 216–225. [PubMed: 24185619]
- (44). Zhu M; Lin S; Sun Y; Feng Q; Li G; Bian L Hydrogels Functionalized with N-Cadherin Mimetic Peptide Enhance Osteogenesis of hMSCs by Emulating the Osteogenic Niche. *Biomaterials* 2016, 77, 44–52. [PubMed: 26580785]
- (45). Benoit DS; Schwartz MP; Durney AR; Anseth KS Small Functional Groups for Controlled Differentiation of Hydrogel-Encapsulated Human Mesenchymal Stem Cells. *Nat. Mater* 2008, 7, 816–823. [PubMed: 18724374]
- (46). Parmar PA; Chow LW; St-Pierre JP; Horejs CM; Peng YY; Werkmeister JA; Ramshaw JA; Stevens MM Collagen-Mimetic Peptide-Modifiable Hydrogels for Articular Cartilage Regeneration. *Biomaterials* 2015, 54, 213–225. [PubMed: 25907054]
- (47). Parmar PA; St-Pierre JP; Chow LW; Spicer CD; Stoichevska V; Peng YY; Werkmeister JA; Ramshaw JAM; Stevens MM Enhanced Articular Cartilage by Human Mesenchymal Stem Cells in Enzymatically Mediated Transiently RGDS-Functionalized Collagen-Mimetic Hydrogels. *Acta Biomater.* 2017, 51, 75–88. [PubMed: 28087486]
- (48). Martin JR; Nelson CE; Gupta MK; Yu F; Sarett SM; Hocking KM; Pollins AC; Nanney LB; Davidson JM; Guelcher SA; Duvall CL Local Delivery of PHD2 siRNA from ROS-Degradable Scaffolds to Promote Diabetic Wound Healing. *Adv. Healthcare Mater* 2016, 5, 2751–2757.
- (49). Andersen MO; Nygaard JV; Burns JS; Raarup MK; Nyengaard JR; Bunger C; Besenbacher F; Howard KA; Kassem M; Kjems J siRNA Nanoparticle Functionalization of Nanostructured Scaffolds Enables Controlled Multilineage Differentiation of Stem Cells. *Mol. Ther* 2010, 18, 2018–2027. [PubMed: 20808289]
- (50). Parvani JG; Jackson MW Silencing the Roadblocks to Effective Triple-Negative Breast Cancer Treatments by siRNA Nanoparticles. *Endocr.-Relat. Cancer* 2017, 24, R81–R97.
- (51). Peng H; Yang H; Song L; Zhou Z; Sun J; Du Y; Lu K; Li T; Yin A; Xu J; Wei S Sustained Delivery of siRNA/PEI Complex from in Situ Forming Hydrogels Potently Inhibits the Proliferation of Gastric Cancer. *J. Exp. Clin. Cancer Res* 2016, 35, 57. [PubMed: 27029190]
- (52). Kondo M; Yamaoka K; Sakata K; Sonomoto K; Lin L; Nakano K; Tanaka Y Contribution of the Interleukin-6/STAT-3 Signaling Pathway to Chondrogenic Differentiation of Human Mesenchymal Stem Cells. *Arthritis Rheumatol.* 2015, 67, 1250–1260. [PubMed: 25604648]

- (53). Ferreira Bassit AC; Moffatt P; Gaumond M-H; Hamdy R The Potential Use of Nanoparticles for Noggin siRNA Delivery to Accelerate Bone Formation in Distraction Osteogenesis. *J. Nanomed. Nanotechnol* 2015, 6, 257.
- (54). Nguyen MK; Jeon O; Dang PN; Huynh CT; Varghai D; Riazi H; McMillan A; Herberg S; Alsberg E RNA Interfering Molecule Delivery from in Situ Forming Biodegradable Hydrogels for Enhancement of Bone Formation in Rat Calvarial Bone Defects. *Acta Biomater.* 2018, 75, 105. [PubMed: 29885529]
- (55). Yu F; Cao X; Li Y; Zeng L; Zhu J; Wang G; Chen X Diels–Alder Crosslinked HA/PEG Hydrogels with High Elasticity and Fatigue Resistance for Cell Encapsulation and Articular Cartilage Tissue Repair. *Polym. Chem* 2014, 5, 5116–5123.
- (56). Zheng Shu X; Liu Y; Palumbo FS; Luo Y; Prestwich GD In Situ Crosslinkable Hyaluronan Hydrogels for Tissue Engineering. *Biomaterials* 2004, 25, 1339–1348. [PubMed: 14643608]
- (57). Nguyen K; Dang PN; Alsberg E Functionalized, Biodegradable Hydrogels for Control over Sustained and Localized siRNA Delivery to Incorporated and Surrounding Cells. *Acta Biomater.* 2013, 9, 4487–4495. [PubMed: 22902819]
- (58). Westhrin M; Xie M; Olderoy MO; Sikorski P; Strand BL; Standal T Osteogenic Differentiation of Human Mesenchymal Stem Cells in Mineralized Alginate Matrices. *PLoS One* 2015, 10, e0120374. [PubMed: 25769043]
- (59). Manolagas SC Birth and Death of Bone Cells: Basic Regulatory Mechanisms and Implications for the Pathogenesis and Treatment of Osteoporosis. *Endocr. Rev* 2000, 21, 115–137. [PubMed: 10782361]
- (60). Jilka RL; Weinstein RS; Bellido T; Parfitt AM; Manolagas SC Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. *J. Bone Miner. Res* 1998, 13, 793–802. [PubMed: 9610743]

Figure 1.

Chemical structure of (A) PEG-diepoxy (PEG-DE) and (B) 8-arm PEG-thiol (PEG(-SH)₈) macromers. (C) Proton NMR with assigned protons of the macromer mixture at different time points. (D) ^{13}C NMR with assigned carbons of (1) PEG-DE, (2) PEG(-SH)₈, and their mixture at (3) neutral and (4) basic pH. NMR data shows the appearance of proton (C) and carbon (D) peaks in the newly formed β -hydroxyl thioether groups.

Figure 2.

(A) Gelation time determined by tube inversion method. * $p < 0.05$. (B) Rheology of combined macromer solutions over time at 25 °C. (C, D) Live (green)/dead (red) staining of hMSCs encapsulated in the hydrogels (C) without and (D) with RNA/PEI nanocomplexes cultured in growth media.

Figure 3.

Summarized procedure for preparation of hMSC-encapsulated hydrogels via thiol-epoxy “click” chemistry in the (A) absence or (B) presence of siNog/PEI nanocomplexes for regulating hMSC osteogenic differentiation (C–E). It is hypothesized that hMSCs in the siNog-free hydrogels do not differentiate when cultured in growth media (C) but are induced to differentiate into osteoblasts and deposited Ca when cultured in osteogenic media (D). When siNog is coencapsulated into the hydrogels, it enhances the hMSC differentiation rate and extent, as evidenced by significantly increased Ca deposition (E).

Figure 4.

Biochemical assay for osteogenesis of hMSCs of (A) Donor 1 and (B) Donor 2 showing DNA (1) and Ca (2) per gel volume unit, and DNA normalized Ca amount (3). (C) Histology images of the sectioned gels (Donor 1) stained with ARS for Ca visualization showing consistent results with much more Ca in siNog/OM compare to others condition. * $p < 0.05$; # $p < 0.05$ compared to the same group at D7; & $p < 0.05$ compared to the same group at other time points; + $p < 0.05$ compared to the same group at D14.