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

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Supporting Information

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 polyethyleneimine (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.

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),18−20 and a variety of mechanisms, such as self-assembly,−8 ionic cross-linking4,21 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 reactions1−4,10−12,16,22,23 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.24−35 This chemistry has also been used to fabricate hydrogels in organic solvents52,53 and in aqueous media.54 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,52−54 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.2−4,43−45 hMSCs have been encapsulated into polymeric hydrogel biomaterials to regenerate tissues, such as bone,2−4,35−37 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. RNAi-molecule delivery provides an effective approach for controlling the differentiation of hMSCs down the osteoblastic, chondrogenic, and adipogenic 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. Co-encapsulation of hMSCs and siNog into 3D hydrogel networks has been reported to enhance hMSC osteogenesis to different extents 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 1A 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 and rheological measurements. 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.

![Figure 2](image-url)

**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](image-url)

**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).
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, 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 increased swelling ratio (Figure S3). 

Biocompatibility is a very important factor to consider in the engineering of biomaterial systems for cell encapsulation-based tissue regeneration. 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 polyethyleneimine (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 were 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; 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, 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...
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 3E). 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 suggest osteoblasts have a limited life span and tend to become lining cells or osteocytes or undergo apoptosis. 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 SSA), 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 encapsulated 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.

**REFERENCES**


