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Three-dimensionally presented anti-fouling zwitterionic motifs sequester and enable high-efficiency delivery of therapeutic proteins

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Abstract

Zwitterions are well known for their anti-biofouling properties. Past investigations of zwitterionic materials for biomedical uses have been centered on exploiting their ability to inhibit non-specific adsorption of proteins. Here, we report that zwitterionic motifs, when 3-dimensionally (3D) presented (e.g. in crosslinked hydrogels), could effectively sequester osteogenic bone morphogenetic protein-2 (rhBMP-2). The ionic interactions between rhBMP-2 and the 3D zwitterionic network enabled dynamic sequestering and sustained release of the protein with preserved bioactivity. We further demonstrated that the zwitterionic hydrogel confers high-efficiency *in vivo* local delivery of rhBMP-2. It can template the functional healing of critical-size femoral segmental defects in rats with rhBMP-2 at a loading dose substantially lower than those required for current natural or synthetic polymeric carriers. These findings reveal a novel function of zwitterionic materials beyond their commonly perceived anti-biofouling property, and may establish 3D zwitterionic matrices as novel high-efficiency vehicles for protein/ionic drug therapeutic delivery.

Keywords

anti-fouling; 3-dimensional zwitterionic materials; rhBMP-2 delivery; bone tissue engineering

1. Introduction

Zwitterions, including phosphobetaine, sulfobetaine, and carboxybetaine[1]), are well-known for their anti-biofouling properties as widely demonstrated on 2-dimensional (2D) surfaces (Fig. 1a) [2–9]. The unique zwitterionic structures, simultaneously possessing cationic and anionic residues yet overall electronic neutral, exhibit strong affinity for water[10], thereby giving rise to super hydrophilic surfaces suppressing the hydrophobic

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interactions known to denature proteins. Zwitterionic motifs have also been shown to mimic the action of protein stabilizing ions[11] in stabilizing/maintaining the native conformation of proteins[6, 12, 13] and inhibiting non-specific protein adsorption[2–5, 7, 14, 15], which is known to set off undesired cascades of surface events (e.g. thrombosis, immune response) [16]. Accordingly, they have been largely exploited for constructing anti-fouling surfaces/interfaces to inhibit protein, bacterial and cellular adhesions [2–5], and as bioinert implants for reducing scar tissue formation [14].

We recently reported the use of zwitterionic sulfobetaine hydrogel to facilitate templated biomineralization, which capitalizes on the ability of the zwitterionic motifs to effectively recruit/nucleate oppositely charged mineralization precursor ions (e.g. Ca^{2+} , PO_4^{3-}) across the 3D hydrogel network[17]. Unlike non-ionic hydrogel that was only able to template the mineralization on the surface, the zwitterionic hydrogel enabled extensive mineralization throughout the 3D network, supporting the critical role of zwitterionic motifs in recruiting precursor ions (Supplementary Fig. S1). This discovery inspired us to examine the hypothesis that zwitterions, when 3D presented, can also effectively sequester ionic proteins by virtue of the oppositely charged residues to synergistically and dynamically interact with ionic protein epitopes, thereby enabling their effective retention and sustained release (Fig. 1b). If validated, such an intrinsic property could fundamentally change the current perception of zwitterionic materials as being primarily anti-biofouling and significantly broaden its potential uses for biomedical applications.

To test this hypothesis, we prepared simple crosslinked polymethacrylate hydrogels bearing zwitterionic side chains. The *in vitro* sequestration/release profile of osteogenic human recombinant bone morphogenetic protein-2 (rhBMP-2) from the zwitterionic hydrogels was investigated and compared with that of the non-ionic low-fouling poly(ethylene glycol) (PEG) hydrogel control. The efficacy of a zwitterionic sulfobetaine hydrogel in delivering rhBMP-2 *in vivo* to promote the functional healing of critical-size (5-mm) femoral segmental defects in rats and endogenous cell attachment on the otherwise low-fouling implant was investigated.

2. Experimental

2.1. Preparation of hydrogels

Zwitterionic hydrogels poly[2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (PSBMA), poly(2-Methacryloyloxyethyl phosphorylcholine) (PMPC), poly[3-((2-(methacryloyloxy)ethyl)dimethylammonio)propanoate] (PCBMA) and nonionic poly(ethylene glycol) methacrylate (PEGMA, $M_n=360$) were prepared (Supplementary Table S1). Monomers SBMA, MPC and PEGMA ($M_n=360$) and crosslinker poly(ethylene glycol) dimethacrylate (PEGDMA, $M_n=750$) were purchased from Aldrich (St. Louis, MO), while CBMA was synthesized as reported [18]. The radical inhibitors in PEGMA and PEGDMA were removed by passing through an aluminum oxide column prior to use. In a typical procedure, 2 mmol respective monomer was combined with 17.9 μL of PEGDMA, 100 μL of PBS solution of 2,2'-Azobis[2-methyl-N-(2-hydroxyethyl)propionamide] (VA-086, 2 %, w/v), and 1882.1 μL of PBS. The mixture was bath-sonicated, and sterilized by passing through 0.22- μm polyethersulfone (PES) membrane filter (Millipore). The

resulting solution was transferred to a custom-made Teflon mold with cylindrical (6 mm in diameter, 50 μL /well), square prism (5 mm $\times$ 5 mm, 50 μL /well) or rectangle (6.5 $\times$ 32.6 mm, 400 μL /well) wells and solidified under the irradiation of 365-nm light for 10 min in a sterile hood. The hydrogels were stored in sterile PBS until further uses.

2.2 Swelling ratios of the hydrogels

The swelling ratios by weight (S_w) of the hydrogels were determined in Milli-Q water or in PBS (pH = 7.4) at room temperature according to Equations 1:

$$S_w = \frac{W_h - W_d}{W_d} \quad \text{Eq - 1}$$

where W_h and W_d are the weight of the hydrogel in fully hydrated state in water/PBS and freeze-dried state, respectively.

2.3 Free water fraction in the hydrogels

The free water fraction in the hydrogels was measured by differential scanning calorimetry (DSC) on a Q200 Modulated DSC (TA Instruments). About 15 mg of hydrogel equilibrated either in water or PBS was placed in an aluminum pan. The pan was then sealed tightly to prevent water evaporation during the measurement. The testing was carried out from -40°C to 40°C at a heating rate of $2^\circ\text{C}/\text{min}$. The exothermal peak around 0°C , attributed to the melting of the free water[19], was calculated as H_{endo} , and the free water fraction (R_f) within the hydrogel was determined according equation 2:

$$R_f = \frac{\Delta H_{endo}}{\Delta H_w} \quad \text{Eq - 2}$$

where H_w is the heat fusion of pure water (332.2 mJ/mg)[6].

2.4 In vitro retention and sustained release of rhBMP-2

Recombinant protein rhBMP-2 (R&D Systems, CHO-derived) was reconstituted according to vendor specifications and diluted with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Dulbecco's phosphate-buffered saline (DPBS, pH 7.4) to a loading concentration of 30 ng/ μL . Hydrogels retrieved from the sterile stock solution were partially dried in a sterile cell culture hood (with a gel volume reduction of 50 to 100 mm^3), and then transferred into the wells of ultra-low attachment 24-well plate (Corning). Reconstituted rhBMP-2 solution (10 μL , 30 ng/ μL) was placed on each hydrogel to achieve a total loading dose of 300-ng rhBMP-2/hydrogel (cylindrical), and allowed to be incubated at 37°C for 1 h (during which rhBMP-2 solutions were fully absorbed by the hydrogels). The rhBMP-2loaded hydrogels were then incubated in 1 mL of DPBS at 37°C for 2, 4, 6, 10, and 24 h. Concentration of the released rhBMP-2 in the DPBS at various time points were determined by an enzyme-linked immuno sorbent assay (ELISA) using a rhBMP-2 Quantikine Kit (R&D Systems) and the amount of the rhBMP-2 released from hydrogels were calculated from the standard curve generated during the same experiment. A sample size of 3 was applied to each hydrogel group.

2.5 Bioactivity of the rhBMP-2 sequestered on & released from the hydrogels

The bioactivity of the rhBMP-2 retained on and subsequently released from the PEGMA and PSBMA hydrogels was evaluated by their ability to induce osteogenic trans-differentiation of murine myoblast C2C12 cells into osteoblasts [20, 21]. C2C12 cells were seeded on 24-well cell culture plate (10,000 cells/cm²) in 1 mL of Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and allowed to attach overnight. The medium was then replaced with fresh DMEM supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin, and the rhBMP-2 loaded hydrogels retrieved from prior incubation in PBS up to 6 days were placed in the adherent C2C12 culture. After 3 days, the hydrogel was removed and the cells were fixed and stained for alkaline phosphatase (ALP) using a Sigma Leukocyte Alkaline Phosphatase Kit according to the vender's protocol. C2C12 culture directly supplemented with 300-ng rhBMP-2 without any hydrogel carrier served as a positive control.

2.6 Animal surgical procedures

All animal procedures were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee. Briefly, male Charles River SASCO-SD rats (289–300 g) were sedated and maintained by 2% isoflurane–oxygen throughout the surgery. The mid-shaft of a femur was exposed by a combination of sharp and blunt dissections and the periosteum of the exposed femur was circumferentially removed to emulate a challenging clinical scenario where this important source of progenitor cells and signaling molecules is lost[22]. A radiolucent, weight bearing polyetheretherketone (PEEK) internal fixation plate was secured to the exposed femur with four bicortical screws into predrilled holes. A 5-mm mid-diaphyseal defect was then created using an oscillating Hall saw with parallel blades. The defect site was thoroughly irrigated with saline to remove bone debris and residue detached periosteum before it was press-fit with a hydrogel graft with or without 500-ng rhBMP-2 (Fig. 3a, n=4). The muscle and skin were closed with resorbable sutures and the rats were given cefazolin (20 mg/kg, once a day) and bupenorphine (0.08 mg/kg, 3 times a day) injections subcutaneously over the next 2 days. Rats were radiographed biweekly post-op to ensure proper graft positioning, and subjected to monthly longitudinal microCT (μ -CT) scans (n=4) to quantitatively monitor the mineralized callus formation until time of sacrifice at 12 weeks post-op. For end-time point analyses, the femur, with the PEEK plate fixator intact, was carefully separated from the adjacent hip and knee joints for either torsion test (n=3) or histological staining. In a second set of experiments, implants were retrieved at 2 and 7 days post-op (n=2) for examination of cellular attachment on the surface of the implant.

2.7 Longitudinal μ -CT analysis

Rats were scanned immediately post-op and every 4 weeks thereafter on a viva-CT 75 *in vivo* Micro-CT system (SCANCO Medical AG) to monitor new bone formation over time. The effective voxel size of the reconstructed images was 30×30×30 μ m³. Data were globally thresholded and 3D images of the 5-mm defect, defined as the region of interest (ROI, 167 slices, 30 μ m/slice), were reconstructed for quantification of bone volume (BV, mm³) and bone mineral density (BMD, mgHA/ccm). Two-dimensional (2D) mineral density color

mapping was generated by reconstructing the respective AIM file with a colored density gradient range of 1.5–3.5 (1/mm). An unimplanted PSBMA hydrogel was scanned to guide proper setting of the threshold (to eliminate hydrogel background) for all analyses.

2.8 Torsion test

Explanted femora were torqued to failure as previously described to assess the degree of the functional restoration of their biomechanical integrity[23]. Briefly, explant was potted in stainless steel hexanuts with poly(methyl methacrylate). The PEEK plate fixators were either carefully bisected without disturbing the underlying graft/new bone using a high-speed burr (the PSBMA group) or unscrewed and removed from the explants (rhBMP-2 treated group) before mounted on the mini-torsion tester (ADMET Inc.). Each specimen was torqued to failure at 1°/s.

2.9 Histology

The explants were fixed by 10% zinc formalin for 24 h, decalcified in 18% EDTA at 4 °C for 4 weeks, and embedded with glycol methacrylate and sectioned. The 3- μ m sections were mounted onto slides for hematoxylin & eosin (H&E) staining.

2.10 Early-stage *in vivo* cell attachment on implant surfaces

To visualize the *in vivo* cell attachment to the hydrogel scaffolds during the early stage of guided bone regeneration, the hydrogel implants with/without pre-loaded rhBMP-2 (500 ng/hydrogel) were retrieved at 2 and 7 days post-op. The explants were fixed in 3.7 % formaldehyde/DPBS solution, and the adherent cells were stained with Alexa Fluor® 488 phalloidin (for F-actin staining, red) and DAPI (for nuclei staining, blue) following the vendor's protocol, respectively, and imaged on a Leica TCS SP2 confocal microscope. Phalloidin was excited at 495 nm and observed with a 518-nm filter while DAPI was excited at 368 nm and observed with a 461-nm filter.

3. Results and Discussions

3.1. Effective sequestration of osteogenic recombinant protein rhBMP-2 by 3D zwitterionic hydrogel networks

The zwitterionic PSBMA hydrogels were prepared by photo-crosslinking sulfobetaine methacrylate (SBMA) with varying contents of crosslinker PEGDMA. And a poly(ethylene glycol) methacrylate (PEGMA) hydrogels bearing non-ionic poly(ethylene glycol) (PEG), another well-established anti-biofouling motif, were prepared at the identical crosslinker contents as controls (Supplementary Fig.S2 & Table S1). To examine the efficiency of the hydrogels for sequestering therapeutic proteins, 300 ng of rhBMP-2 (in 10 μ L PBS solution) was loaded on each partially dried hydrogel and allowed to equilibrate at 37 °C for 1 h to ensure complete absorption of the aqueous solution (Supplementary Fig. S2).

Although zwitterionic sulfobetaine and PEG surfaces are both known for resisting non-specific protein absorptions, the respective 3D networks exhibited significant differences in sequestering rhBMP-2 even with a similar swelling ratio at the identical crosslinker content of 5.33 mol% (relative to monomer; Figs. 2a & b). The non-ionic PEGMA hydrogel could

not effectively sequester rhBMP-2, with only about 10 % of the initially loaded rhBMP-2 retained on the hydrogel after 2-h incubation in PBS (Fig. 2a). This observation is consistent with previous findings that PEG hydrogels lack affinity for ionic proteins [24, 25]. By contrast, about 60 % of the initially loaded rhBMP-2 was sequestered by the zwitterionic PSBMA network of the same crosslinker content (5.33 mol%) after 2-h incubation (Fig. 2a). Given the similar swelling ratio, thus similar diffusibility of solutes across the 3D network[19], the different efficiencies of sequestering ionic proteins observed with the two identically crosslinked networks was likely due to the different ionic states of their side chains. By reducing the degree of chemical crosslinking by up to 16-fold, we showed that the zwitterionic PSBMA network could swell significantly in PBS by up to 10-fold while no significant crosslinker content-dependent changes in swelling ratio in PBS was observed with the non-ionic PEGMA network (Fig. 2b). This observation further supported that the different ionic states of side chains presented in the two 3D networks (zwitterionic vs non-ionic) can translate into significant differences in their physical properties in ionic environment, including different swelling behavior and efficiencies in sequestering/releasing ionic protein (Fig. 2a, Supplementary Fig. S3).

Furthermore, unlike the non-ionic PEGMA network that was insensitive to the presence of salts (no significant difference in swelling ratios in water vs in PBS, pH 7.4, Fig. 2c), the zwitterionic PSBMA network expanded almost 400% more in PBS than in water (Fig. 2b). Such an antipolyelectrolyte swelling behavior[26] can be attributed to the disruption of the intermolecular salt bridges formed between the anionic sulfonate and cationic ammonium residues by ionic solutes. Combined with the higher free water fractions in the equilibrated zwitterionic PSBMA hydrogel (85 % in PSBMA vs 69 % in PEGMA, Supplementary Fig. S4), this observation further supports that the ionic-sensitive nature of the zwitterionic network is beneficial to the diffusion of ionic solutes in general across the 3D network.

Taken together, these data strongly suggest that ionic interactions play an indispensable role in effectively sequestering rhBMP-2 by the zwitterionic PSBMA network. Similar rhBMP-2 retention profiles were also observed with the 3D zwitterionic networks bearing phosphobetaine (PMPC) and carboxybetaine (PCBMA) motifs (Fig. 2d), supporting effective protein retention as a novel yet generalizable feature for 3D zwitterionic matrices.

3.2 Sustained release of bioactive rhBMP-2 from zwitterionic PSBMA hydrogels

Monitoring of the rhBMP-2 release from the hydrogels within the first 24 h of incubation in PBS by ELISA revealed ~30% release of the initially loaded protein in the first 2 h, followed by a 3% of slower release in the next 22 h (Fig. 2d), leaving >65% sequestered by the zwitterionic PSBMA (1.33 mol% crosslinker content) by 24 h. To examine whether the rhBMP-2 sequestered by the PSBMA hydrogel could be continually released with retained bioactivity over a much longer period of time, an established culture model of BMP-2-induced osteogenic trans-differentiation of murine myoblast C2C12 cells was used[20, 21]. This model was chosen over BMP-2-induced osteogenesis of mesenchymal stem cells (MSCs) due to the complete lack of expression of osteogenic markers by C2C12 cells prior to BMP-2 induction (thus much cleaner background than MSCs). We showed that when the rhBMP-2-bearing PSBMA was placed in murine myoblast C2C12 culture after a 6-day pre-

incubation in PBS, the further sustainedly released rhBMP-2 (from day 7 to day 9) from the PSBMA hydrogel was able to induce robust osteogenic trans-differentiation of C2C12 cells into alkaline phosphatase (ALP)-expressing osteoblasts (Fig. 2e). The intense ALP staining, comparable to that observed with the positive control culture (Fig. 2e) where 300-ng rhBMP-2 was directly supplemented without any carrier, suggest that the bioactivity of the sequestered and subsequently released rhBMP-2 was well preserved for at least 9 days. This is in stark contrast to the minimal ALP stains detected from the C2C12 culture supplemented with the PEGMA hydrogel subjected to identical BMP-2 loading and PBS pre-incubation treatment, consistent with the much poorer initial sequestration of rhBMP-2 by the non-ionic PEGMA hydrogel. It is worth noting that the circulation half-life of rhBMP-2 and most other growth factors, when in free form, tends to be very limited (e.g. 7–16 min for rhBMP-2[27]). Here we demonstrated well-preserved bioactivity of the rhBMP-2 sequestered by the PSBMA hydrogel well over a week. This may be attributed to the superhydrophilic structural water surrounding zwitterionic residues that prevent protein denaturing[10] and the Hofmeister ions[11]-like effect of the zwitterions for stabilizing native protein conformations [13]. Overall, these observations support the zwitterionic PSBMA hydrogel as an effective carrier for the high-efficiency sequestration and sustained long-term release of therapeutic proteins such as rhBMP-2.

3.3. Robust healing of rat critical long bone defects enabled by the high-efficiency *in vivo* delivery of rhBMP-2 by PSBMA

To test the *in vivo* efficacy of the PSBMA hydrogel as a synthetic implant with rhBMP-2 delivery capability, the repair of 5-mm rat femoral segmental defect, an established critical-size non-union model [23, 28], templated by the PSBMA implant with or without pre-loaded rhBMP-2 was evaluated (Fig. 3a). Current clinical use of rhBMP-2, delivered via absorbable collagen sponge carrier (INFUSE®), to stimulate spine fusion or tibial fracture repair require exceedingly high loading doses comparable to ~1.5 mg per milliliter volume of defect (1500 ng/mm³). Such a supra-physiological dosages and their burst release from the sub-optimal collagen carrier have resulted in significant systemic and local adverse effects[29, 30]. Loading doses ranging from 2 to 50-μg rhBMP-2/scaffold (~250 to 6,250 ng/mm-defect) have been typically used to achieve adequate repair of critical-size long bone or trabecular bone defects in rats with either natural or synthetic polymeric carriers (Table 1) [28, 29, 31–39]. Loading doses of rhBMP-2 less than 2 μg (without synergistic delivery of other growth factor therapeutics) often resulted in inadequate/inconsistent repair outcomes [40]. Here, we applied a significantly lower loading dose of 500-ng rhBMP-2 to the PSBMA scaffold (equivalent to ~11 ng/mm³ or 100 ng/mm-defect) press-fit into the 5-mm rat femoral segmental defect. To our knowledge, consistent functional healing of critical rat long bone defect with such a low loading dose of rhBMP-2 alone has never been reported before.

At 2 weeks, mineralized healing callus emerged around the defects implanted with PSBMA with rhBMP-2 (Supplementary Fig. S5). Strikingly, the bony callus started to bridge over the defect by as early as 4 weeks (Fig. 3b, Supplementary Fig. S6), and by 12 weeks, mature and uniform bony callus characterized with recanalization and high bone mineral density (Figs. 3b, d, f, Supplementary S6) fully encapsulated the defect, leading to substantial restoration (~40% compared to intact age matched femur control) of the torsional rigidity of

the defect (Figs. 3e). Continued remodeling of the new bone is expected to further increase the torsional rigidity over time. In the absence of rhBMP-2, the PSBMA also led to the early onset (Supplementary Fig. S5) and steady growth of bony callus over the course of 12 weeks as characterized with increasing bone volume (Fig. 3c) and bone mineral density (Fig. 3d). However, in the absence of rhBMP-2, the calcified callus failed to bridge over the entire defects by 12 weeks (Figs. 3b, Supplementary S5, S7 & S8) to restore the biomechanical integrity of the defect (Fig. 3e). Of note, although fairly high bone volumes were detected at the regions of interest (ROI) in both treatment groups by 12 weeks (Fig. 3c, no statistically significant difference), the rhBMP-2 treated group consistently guided uniform bony callus formation across the full length of the defect whereas the new bone formation templated by the no-BMP-2 control group was primarily localized around the graft-cortical bone junctions (Fig. S8). Transverse cross-sectional view of the repaired defect (Fig. 3f) and H&E staining of the explant at 12-week post-op (Fig. 3g) revealed that the bony callus formation was tightly templated by and integrated with the PSBMA hydrogel (note that the disintegration/shrinkage of hydrogel scaffold trapped within the bony callus was a histology processing artifact as the hydrogel shrank dramatically upon dehydration). These data supported that PSBMA hydrogel implant is a highly effective carrier for the local delivery of rhBMP-2, which enabled the functional repair of rat critical-size long bone defect at a significantly reduced BMP-2 loading dose that is desired from both safety and cost-effectiveness perspectives.

3.4 rhBMP-2 sequestration promoting endogenous cell attachment & ECM deposition on the otherwise low-fouling surface of zwitterionic PSBMA hydrogel implant

The robust early bone healing enabled by PSBMA in the presence of rhBMP-2 across the entire defect suggests that a cascade of cellular events required for initiating bone healing must have occurred in a timely manner along the implant surface[41], counterintuitive to the perception that zwitterionic surfaces and scaffolds tend to reduce protein absorptions/cellular adhesion[5, 14, 42]. We hypothesize that the retention of rhBMP-2 by the PSBMA hydrogel implants shifted the microenvironment of the zwitterionic scaffolds from low-fouling to cell adhesive. To test this hypothesis, we investigated early stage *in vivo* cell attachment during the guided bone healing with and without the loading of rhBMP-2. As revealed by fluorescent microscopy and H&E staining, only limited cell attachment was observed on the surface of the PSBMA hydrogel without rhBMP-2 within the first 2 days post-implantation with no obvious increases by 7 days (Fig. 4). This is consistent with the low-fouling nature of zwitterionic surfaces as well as the recent report that zwitterionic carboxybetaine hydrogels suppressed fibrous tissue encapsulation *in vivo*[14]. In contrast, substantially more endogenous cells attached to the surface of the rhBMP-2-bearing PSBMA implant at 2 days post-implantation (Fig. 4), and these adherent cells continued to proliferate and led to more effective ECM deposition, and presumably the initiation of callus formation, at day 7 post-implantation. These observations suggest that the ionic retention of rhBMP-2 on the 3D zwitterionic scaffold not only introduced osteoinductivity, but also improved the osteoconductivity of the otherwise commonly perceived low-fouling and bioinert scaffold, enabling facile cellular attachment. As many ECM components such as fibronectin, collagen and laminin have high affinity for heparin-binding growth factors like BMPs[43], the rhBMP-2-bearing scaffold in turn could facilitate the attachment of these

ECM components and subsequent cellular adhesion and more uniform and robust bony callus formation.

4. Conclusions

Using a simple non-degradable zwitterionic hydrogel model system, we demonstrated a novel role of 3D zwitterionic matrices in effectively sequestering ionic therapeutic proteins beyond their commonly perceived anti-fouling properties. The sequestered rhBMP-2 could be sustainably released well over a week with well-preserved bioactivity, driven by the dynamic ionic interactions of rhBMP-2 with the 3-dimensionally presented zwitterionic motifs rather than by scaffold biodegradations. Such sequestration and high-efficiency delivery of rhBMP-2 translated into robust repair of critical-size rat femoral segmental defects templated by the zwitterionic hydrogel implant at an exceptionally low loading dose of 500-ng rhBMP-2. This finding establishes 3D zwitterionic matrices as novel vehicles for high-efficiency protein delivery, and significantly broadens the biomedical use of zwitterionic materials. The identified novel property of zwitterionic materials also distinguishes them from other non-ionic low-fouling bioinert materials such as PEG, and may establish them as more versatile candidates for engineering bioactive synthetic microenvironment for biomedical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Additional mineralization outcomes, hydrogel preparation scheme, cumulative rhBMP-2 release profiles, free water fraction data of hydrogels, radiographic monitoring results of implants, and μ -CT data (Figures S1–S8). Formulation of hydrogels (Table S1).

Figure 1. Schematic illustrations of a, the well-established anti-biofouling property of 2D zwitterionic surfaces vs b, hypothesized protein-sequestering property of 3D zwitterionic networks.

Figure 2. 3D zwitterionic hydrogel networks efficiently sequestered rhBMP-2 and enabled its sustained *in vitro* release

a, Sequestration of rhBMP-2 by zwitterionic PSBMA vs non-ionic PEGMA control as a function of crosslinker content ($n=3$, 0.33, 1.33 or 5.33 mol% relative to monomers) after 2-h incubation in PBS. A 300-ng rhBMP-2 initial loading dose was applied to all hydrogels and the sequestered protein content was determined after 2-h incubation in PBS. **b**, Swelling ratio by weight (S_w) of PSBMA vs PEGMA hydrogels ($n=5$) in PBS as a function of crosslinker content (0.33, 1.33 or 5.33 mol% relative to monomers). **c**, Swelling ratio by weight (S_w) of PSBMA vs PEGMA hydrogels (1.33 mol% crosslinker content; $n=5$) in water and in PBS. **d**, Cumulative release of the loaded 300-ng rhBMP-2 from three types of zwitterionic hydrogels with identical crosslinker amount of 1.33 mol% ($n=3$). **e**, Osteogenic trans-differentiation of C2C12 cells induced by the rhBMP-2 sustained-released (between day 7 to day 9) from PSBMA vs PEGMA hydrogels as shown by the expression of osteogenic marker ALP (red stains). C2C12 culture directly supplemented with 300-ng rhBMP-2 without any hydrogel carrier served as a positive control. * $p < 0.05$ (two-way ANOVA).

Figure 3. High-efficient *in vivo* local delivery of rhBMP-2 by PSBMA hydrogel implant as examined by the 5-mm rat femoral segmental defect model

a, A PSBMA hydrogel implant (5 mm × 3 mm × 3 mm) with/without rhBMP-2 press-fit within the femoral segmental defect stabilized by a radiolucent polyetheretherketone (PEEK) plate fixator. **b**, Reconstructed μ -CT 3D images & 2D bone mineral density color mapping of the center longitudinal slice of the defect treated with PSBMA hydrogel grafts with/without 500-ng rhBMP-2 at 4 and 12 weeks post-op. **c**, Bone volume & **d**, Bone mineral density of the defects (n=4) treated with PSBMA hydrogel grafts with/without 500-ng rhBMP-2 at 4, 8 and 12 weeks post-op. **p* bold > 0.05 (two-way ANOVA) **e**, Peak torque of the 12-week explants treated with PSBMA hydrogel grafts with/without rhBMP-2 (n=3) vs age-matched intact femurs (n=6). **p* < 0.05 (Student's T-test). **f**, Reconstructed μ -CT 3D image & 2D bone mineral density color mapping of the center transverse slice of the defect treated with PSBMA hydrogel graft with 500-ng rhBMP-2 at 12 weeks post-op showing mature bony callus fully encapsulating the rhBMP-2 loaded PSBMA hydrogel scaffold. **g**, H&E staining of the 12-week explant showing robust new bone (NB) fully encapsulating the rhBMP-2 loaded PSBMA scaffold and integrated with adjacent native cortical bone (CB). BM = bone marrow. Black arrows in the enlarged image denote hydrogel scaffolds integrated with the NB.

Figure 4. Temporally sequestered rhBMP-2 increased the cell attachment & ECM deposition on the low-fouling zwitterionic PSBMA hydrogel implant

a, Confocal images of *in vivo* endogenous cell attachment on the surface of PSBMA explants with/without rhBMP-2 at day 2 and 7 post-op. Actin was stained by Alexa phalloidin (red) while nuclei were stained by DAPI (blue). **b**, H&E staining of the ECM deposition on the explants with/without rhBMP-2 at day 2 and 7 post-op.

Representative literature rhBMP-2 loading doses on various natural or synthetic polymeric scaffolds used for achieving adequate healing of critical-size bone defects in rats. Literatures reporting synergistic loading of rhBMP-2 along with other growth factors/therapeutics are not included.

Table 1

Scaffold type	Scaffold materials	Defect model	rhBMP-2 loading dose			References
			µg/scaffold	µg/mm-defect	µg/mm ³	
Natural	Collagen (INFUSE®) ^a				1.5	[27]
		Gelatin	6-mm segmental, ulna	3	0.5	[29]
		Alginate	8-mm segmental, femur	2	0.25	[26]
				5	0.63	[30]
		Keratose	8-mm segmental, femur	50	6.25	[31]
			Silk	5-mm segmental, femur	2.5	0.5
Synthetic	Hyaluronic acid	5-mm cranium	5		[33]	
		PPF/TCP	5-mm segmental, femur	10	2	[36]
		PLGA & PPF	5-mm segmental, femur	6.5	1.3	[34]
			PLA-DX-PEG	4-mm, ilia	10	
		PEG-RGD	8-mm, cranium	5		[35]
<i>PSBMA^b</i>		<i>5-mm segmental, femur</i>	<i>0.5</i>	<i>0.1</i>	<i>0.01</i>	

^a commercial rhBMP-2 delivery scaffolds approved by FDA.

^b zwitterionic PSBMA hydrogel scaffold used in the current study.