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Fracture-Targeted Delivery of β -Catenin Agonists via Peptide-Functionalized Nanoparticles Augments Fracture Healing

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Abstract

Despite several decades of progress, bone-specific drug delivery is still a major challenge. Current bone-acting drugs require high-dose systemic administration which decreases therapeutic efficacy and increases off-target tissue effects. Here, a bone-targeted nanoparticle (NP) delivery system for a β -catenin agonist, 3-amino-6-(4-((4-methylpiperazin-1-yl)-sulfonyl)phenyl)-*N*-(pyridin-3-yl)pyrazine-2-carboxamide, a glycogen synthase kinase 3 beta (GSK-3 β) inhibitor, was developed to enhance fracture healing. The GSK-3 β inhibitor loading capacity was found to be 15 wt % within highly stable poly(styrene-*alt*-maleic anhydride)-*b*-poly(styrene) NPs, resulting in ~50 nm particles with ~ -30 mV surface charge. A peptide with high affinity for tartrate-resistant acid phosphatase (TRAP), a protein deposited by osteoclasts on bone resorptive surfaces, was introduced to the NP corona to achieve preferential delivery to fractured bone. Targeted NPs showed improved pharmacokinetic profiles with greater accumulation at fractured bone, accompanied by significant uptake in regenerative cell types (mesenchymal stem cells (MSCs) and osteoblasts). MSCs treated with drug-loaded NPs *in vitro* exhibited 2-fold greater β -catenin

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Author Contributions

Y.W. and D.B. designed the experiments. M. N. contributed to peptide synthesis, M.A.F. contributed to NP characterization and histology; M.B. contributed to synthesis of PSMA-*b*-PS NPs, and T.J.S. contributed to animal management and animal studies. Y.W. and M.A.F. collected the data. Y.W. and D.B. wrote the manuscript. All authors edited and revised the manuscript.

Supporting Information

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Figure S1: Transmission electron microscope (TEM) images of plain PSMA-*b*-PS NPs (NP), NP conjugated with TRAP-binding peptide (TBP-NP) and scrambled peptide (SCP-NP). Figure S2: Live animal imaging of tissue distribution (A) 24 h and (B) 48 h after injection of IR780 delivered *via* different carriers including NP alone, SCP-NP, and TBP-NP. Figure S3: Ratio of NP-IR780 accumulation between fractured bone to liver and spleen. Figure S4: *In vivo* uptake of injected FITC-labeled NPs in cells located in (A) unfractured bone and (B) unfractured bone marrow quantified by flow cytometry. Figure S5: Percentage of each cell types isolated from fracture bone and marrow Figure S6: Percentage NP positive cells determined by flow cytometry. Figure S7: NP-delivered β -catenin agonists significantly increase MSC β -catenin level with less cytotoxicity (PDF)

signaling than free drug that was sustained for 5 days. To verify similar activity *in vivo*, TOPGAL reporter mice bearing fractures were treated with targeted GSK-3 β inhibitor-loaded NPs. Robust β -galactosidase activity was observed in fracture callus and periosteum treated with targeted carriers *versus* controls, indicating potent β -catenin activation during the healing process. Enhanced bone formation and microarchitecture were observed in mice treated with GSK-3 β inhibitor delivered *via* TRAP-binding peptide-targeted NPs. Specifically, increased bone bridging, ~4-fold greater torsional rigidity, and greater volumes of newly deposited bone were observed 28 days after treatment, indicating expedited fracture healing.

Graphical Abstract

Keywords

nanoparticles; fracture healing; peptide; drug delivery; small molecule drug

Fractures are a significant clinical problem, with predicted associated financial costs of \$474 billion by 2020 in the United States.¹ Despite advances in bone realignment and immobilization techniques, a high percentage (~10%) of fractures result in non-unions,^{2,3} leading to prolonged hospitalization and secondary interventions as well as significant morbidity and healthcare costs.

Growth factors critical for fracture healing have been investigated to enhance bone repair. To treat long bone fracture non-unions, bone morphogenetic protein-2 (BMP-2) has been approved clinically.⁴ Though only approved for osteoporosis, parathyroid hormone (PTH) may also be adaptable as a treatment for fracture non-unions.^{5–7} However, growth factor therapies are limited by their pleiotropic nature, high manufacturing costs, poor stability, and short half-lives (~1 h).⁸ Furthermore, delivery of supra-physiological doses is often required to achieve therapeutic levels at fracture sites, leading to adverse side effects and clinical complications.^{6,7,9,10} Thus, development of alternative bone therapeutics that can safely enhance fracture healing has great potential.

Novel bone regeneration strategies involving small molecule compounds have been explored to circumvent challenges of biologics. These small molecules, including statins,^{11,12} steroid hormones and prostaglandin agonists,¹³ and Wnt/β-catenin agonists,^{13–16} are more stable, affordable, and non-immunogenic and thus overcome many of the challenges associated with growth factors.¹⁷ In particular, Wnt/β-catenin signaling agonists have been widely explored because of their crucial role in all events of bone healing, including cell fate determination, migration, proliferation, as well as matrix deposition.^{16,18,19}

Despite significant promise of Wnt/ β -catenin agonists and other small molecule drugs for bone regeneration, achieving therapeutic concentrations in bone while limiting off-target effects remains a challenge. Without targeting strategies, small molecule drugs exhibit poor bone biodistribution *in vivo*, with <1% of injected drug accumulated at bone due to rapid drug degradation in the liver and renal clearance.²⁰ The lack of bone specificity requires administration of high doses to reach therapeutic concentrations in bone, resulting in myriad off-target effects. Small molecule glycogen synthase kinase 3 beta (GSK-3 β) inhibitors have been investigated to activate the Wnt/ β -catenin pathway and tested preclinically, such as 603281-31-8 (Eli Lilly), which increased bone formation markers in ovariectomized rats,²¹ and AZD2858 (AstraZeneca), which enhanced osteoblastic activity and fracture healing in rats.²² However, the major concern of Wnt agonists is oncogenic side-effects resulting from poorly controlled doses at bone and off-target tissues.^{23–25} In fact, AZD2858 resulted in similar changes in bone mineral density in both unfractured and fractured bone.²² Due to the widespread expression of GSK-3 β and myriad roles of Wnt/ β -catenin biologically, highly localized and controlled GSK-3 β inhibition would be advantageous from a fracture healing perspective to reduce risk of off-target effects.^{22,25}

To address challenges associated with suboptimal pharmacokinetics and biodistribution of bone-acting compounds, drug delivery systems have been developed. Commonly, nanoparticles (NPs) are employed, as they increase small molecule drug solubility, stability, and circulation time.^{26–28} Efficient NP extravasation into bone can be achieved by exploiting NPs that are smaller than bone sinusoids (80–100 nm).^{29,30} To further enhance bone biodistribution, NPs can be functionalized with cell/tissue targeting groups,³¹ including bisphosphonates,³² acidic oligopeptides,^{30,33,34} and aptamers.³⁵ While these have shown promise in targeting drugs to bone, they target bone mineral or osteoblasts in general, with no precise control over sites of cellular activity during bone turnover events.

Here, a fracture-targeted poly(styrene-*alt*-maleic anhydride)-*b*-poly(styrene) (PSMA-*b*-PS) NP delivery system was developed for the β -catenin agonist, 3-amino-6-(4-((4-methylpiperazin-1-yl)sulfonyl)phenyl)-*N*-(pyridin-3-yl)pyrazine-2 carboxamide (AZD2858, a GSK-3 β inhibitor). PSMA-*b*-PS-based NPs, which have shown excellent loading and sustained release of multiple hydrophobic drugs,^{36–39} offer vast chemical versatility for functionalization with various targeting ligands for tissue/cell-specific affinity due to myriad maleic anhydride carboxylic acid groups.^{38,40,41} To enhance drug accumulation at sites of bone remodeling, a peptide with high affinity for tartrate-resistant acid phosphate (TRAP),⁴² a protein deposited on bone resorptive surfaces, was introduced to NPs. *In vivo* biodistribution was studied using an *in vivo* imaging system (IVIS), and flow cytometry was used to identify cell types that are targeted by the NPs. *In vitro* NP uptake and activation of Wnt/ β -catenin of MSCs, which was identified to be the major regenerative cell target of NPs at fractures, were investigated. Moreover, β -catenin activation at fracture sites was investigated *in vivo* in a murine femoral fracture model. Fracture healing efficiency after treatment with NP-drug was studied using histology, microcomputed tomography analysis, as well as bone mechanical testing.

RESULTS AND DISCUSSION

Design, Synthesis, and Characterization of Peptide-Functionalized PSMA-*b*-PS NPs

To synthesize PSMA-*b*-PS copolymers, reversible addition–fragmentation chain transfer (RAFT) polymerization was used (Scheme 1). As shown by our lab and others, RAFT is ideal for synthesis of block copolymers with well-controlled molecular weight and hydrophobicity.^{37,43–46} In this study, amphiphilic diblock polymers were synthesized by adding an excess amount of styrene monomer in the reaction (Scheme 1A) with 4-cyano-4-dodecylsulfanyltrithiocarbonyl sulfanyl pentanoic acid (DCT) as the RAFT chain transfer agent. Polymers were analyzed using gel permeation chromatography (GPC) for molecular weight and polydispersity (PDI), which were 54,000 Da and 1.03, respectively. Self-assembled NPs (NPs) were formed from PSMA-*b*-PS polymers *via* solvent exchange from dimethylformamide (DMF) to water using a syringe pump (Scheme 1B).^{37,47,48}

To synthesize for bone-targeting NPs, NPs were functionalized with a peptide that exhibits high affinity for TRAP.⁴² TRAP is deposited by osteoclasts during resorption and is specifically in remodeling bone, such as within fracture milieu.^{49–51} After systemic injection, TRAP-binding peptide functionalized NPs (TBP-NPs) were designed to accumulate preferentially at fractures (Scheme 2). The TBP-NP conjugation was performed *via* carbodiimide chemistry, exploiting the conjugation versatility provided by hydrolyzed maleic anhydride units within the corona of PSMA-*b*-PS NPs (Scheme 1C). A scrambled control peptide (SCP), which has the same amino acids as TBP but in random order, was also introduced as a control. Furthermore, introduction of peptide increased NP size slightly but did not alter morphology, where NP, TBP-NP (Scheme 2), and SCP-NP had similar appearance *via* transmission electron microscope (TEM) (Figure S1).

NP hydrodynamic diameter (measured by dynamic light scattering (DLS)) increased from ~42 nm to ~70 nm with peptide functionalization, which is in agreement with TEM (Table 1). Peptide functionalization also modestly altered zeta potential from –33 mV for NPs to ~–26 mV for peptide-NPs, likely due to conversion of anionic carboxylic acid groups to amide bonds during the peptide conjugation. The size (~40–70 nm) and negative charge of NPs are consistent with highly stable carriers that provide long drug circulation half-lives due to bypassing renal and hepatic filtration which occurs with NPs < 10 nm and reducing clearance by opsonization which occurs with NPs > 100 nm.^{27,52,53}

Previous studies showed increased drug loading efficacy with NPs that have greater hydrophobic PS core and greater hydrophobic:hydrophilic molecular weight (Mn) ratios.^{37,38} The specific polymer used here, with a hydrophobic:hydrophilic Mn ratio of 0.7, exhibited 65% loading efficiency (loaded drug/ initial drug) and 6% loading capacity (drug mass/micelle mass) for GSK-3 β inhibitor. TBP-NPs and SCP-NPs provided a loading efficiency of 97% and 51% and loading capacity of 14% and 7%, respectively (Table 1). The higher loading efficiency observed in TBP-NP might be due to the more homogeneous micellar structure as indicated by the low PDI of TBP-NP, which leads to greater interaction between the drug and NP cores. The number of peptide functionalities on each polymer was quantified by calculating the ratio between peptide per NP and polymer per NP, while the number of NPs was acquired using a Malvern Nanosight analysis. TBP and SCP

functionalization resulted in similar peptide conjugation efficiency of ~ 6.9 and 5.5 peptide groups per polymer.

***In Vivo* Biodistribution of Peptide-Targeted NPs**

After fracture, osteoclast activity, including TRAP deposition, increases and continues over the entire healing time frame,^{49,51,54} thus enabling TBP-NPs to preferentially target fracture sites irrespective of dosing regimen relative to injury. IVIS imaging was used to examine fracture targeting by TRAP-binding peptide-targeted NPs (TBP-NPs) (Figure 1A) at a representative injection time of 3 days postfracture. Saline controls, NP alone, SCP-NP, and TBP-NP loaded with IR780 (a near-IR model drug for visualization) were injected retro-orbitally to analyze fracture localization. Figure 1A shows representative images of mice at specific time points after NP injections. Quantitative analysis of IR780 accumulation was performed by measuring the total radiant efficiency of regions of interest (left femurs) and normalizing to saline controls (Figure 1B). In all groups tested, accumulation of drug peaked at ~24–48 h post-injection. Untargeted NPs also accumulated at fracture sites, which is likely due to enhanced permeation and retention (EPR) that occurs concomitant with inflammation.^{55,56} However, targeting *via* TBP showed a 2-fold greater accumulation over untargeted NPs, presumably due to specific binding to TRAP deposited by osteoclasts. Tissue distribution was also analyzed by IVIS 24 h after injection by measuring IR780 signal in organs (Figure S2A). Signal was nearly undetectable in heart, lungs, and kidneys in all treatment groups, with exception to limited liver signal in mice treated with NP-IR780 and SCP-NP-IR780. Similar results were found at 48 h post-injection (Figure S2B).

NP delivery systems were first found to passively accumulate in tumor tissue *via* the EPR effect,^{57,58} where NPs are able to penetrate through leaky vasculature that forms due to perfusion demands of rapidly growing tumors. Similar passive targeting behavior of NPs has been found in inflamed joints, bone lesions, and fractures, where leaky vasculature is also present.^{55,56,59,60} Consistent with these findings, untargeted PSMA-*b*-PS localized at fracture sites after systemic delivery. As inflammation occurs at initial stages of fracture healing, preferential accumulation of untargeted NPs herein is likely due to this tumor-like EPR mechanism.^{55,61} NP net negative charge makes them less susceptible to nonspecific accumulation in liver and spleen.^{27,52} Although it has been shown that these NPs bind with serum protein (bovine serum albumin (BSA)) readily, greater stability and longer circulation times were observed *in vivo*.^{39,62} Results herein showed homogeneous spherical structures of NPs after systemic injection (Figure 1C) using TEM, indicating that the stability of NP was retained in systemic circulation.

To evaluate *in vivo* distribution more closely, femurs were harvested from fractured mice injected with NP-IR780, SCP-NP-IR780, and TBP-NP-IR780. Specimens were frozen, sectioned, and counterstained with DAPI. All NPs were present at liver and spleen, with the spleen showing greater accumulation. SCP-NP-IR780 also showed significant accumulation in lung, which might be due to different SCP secondary structures compared with TBP,⁶³ as both peptides share identical amino acids. Heart and kidney showed minimal signal, while NP and SCP-NP accumulated at both unfractured and fractured bone. TBP-NP accumulated preferentially at fractured bone (Figure 1D(u)), with minimal signal from normal,

unfractured bones (Figure 1D(t)), which is consistent with IVIS results. Quantification of IR780 signal (Figure 1E) showed ~6-fold increase of IR780 when delivered *via* TBP-NP *versus* nontargeted NP at fractured bone, as well as significantly decreased accumulation within liver, spleen, and lung. Since NPs generally accumulate within the reticuloendothelial system, including liver and spleen, the ratio of IR780 signal in fractured bone *versus* liver and spleen was calculated (Figure S3), where fracture to liver ratio was 20× and fracture to spleen ratio was 16× in the TBP-NP treatment group *versus* NP treated group.

Bisphosphonates, such as zoledronate⁶⁴ and alendronate,^{65,66} have been widely explored as an effective strategy to target bone due to their high affinity to hydroxyapatite (HAp), which is the main mineral component of bone.^{67–69} However, the lack of fracture specificity and inhibitory effects on osteoclast function limits the utility of bisphosphonates for bone targeting.^{29,69,70} An osteoblast-specific aptamer, CH6, which was screened by exponential enrichment (Cell-SELEX), was used for cell-specific siRNA delivery *via* lipid NPs.³⁵ Aspartic acid (Asp) peptide sequence has been applied previously by several groups to target drugs to the bone tissue, due to its affinity to HAp with higher crystallinity, which is characteristic of bone resorption surfaces.^{71,72} Oligopeptide (AspSerSer)₆ functionalized liposomes have shown efficient targeting to osteoblast-mediated mineralizing nodules at bone formation surface and enhanced bone formation.³⁰ Though biodistribution was altered to favor bone delivery,^{30,43,73,74} the liver still remains as a major organ of biodistribution for these targeted carriers. A simvastatin prodrug micelle system was designed to target fracture *via* aforementioned EPR effect, or “extravasation through leaky vasculature and inflammatory cell-mediated sequestration” (ELVIS) mechanism.^{55,56,75} The passive targeting of NPs exhibited localization at the fracture for <7 days, which might not be ideal for late stage fractures where inflammation is resolved. In contrast, the PSMA-*b*-PS NPs herein take advantage of EPR effect and are also functionalized with a peptide identified by phage display that has specific affinity to TRAP,⁴² which is presented at bone-resorption surfaces, including fractures.^{49,51} Histology showed enhanced TBP-NP-IR780 accumulation at fracture callus compared with SCP-NP-IR780, indicating that localization is peptide sequence specific. Although NP, SCP-NP, and TBP-NP all showed minimal accumulation in the liver, which is a characteristic of NPs with size of 50–100 nm, TBP-NP showed ~5-fold higher accumulation at a fractured femur compared with SCP-NP. With the combination of passive and active targeting of NPs, off-target effects in nonfractured bone and nonskeletal tissue are reduced.

Characterization of *in Vivo* Cellular Uptake at Fracture Sites

To investigate *in vivo* NP uptake at a cellular level within the fracture microenvironment, mice were injected with fluorescently labeled PSMA-*b*-PS NPs (FITC-NP, FITC-TBP-NP, FITC-SCP-NP) and saline 3 days after fractures, followed by cell isolation from bone and marrow for flow cytometry analysis (Figure 2). Cells isolated from fractured bone (Figure 2A) treated with TBP-NP exhibited a 4-fold increase in and 7-fold increase in percentage positive cells compared to NP and SCP-NP, respectively. In contrast, cells isolated from bone marrow (Figure 2B) did not show significant differences in NP positive cell population between treatments, indicating TBP biases NP accumulation in the bone. Cells isolated from unfractured bone (Figure S4A) and marrow (Figure S4B) also showed no significant

differences between treatments, confirming fracture-targeting efficiency of NPs. To dissect the cell types positive for NPs, marrow and bone cells were incubated with specific cell markers to identify major cell types at fractures, such as endothelial cells (CD45⁺/Ter119⁺/CD31⁺), osteoblasts (CD45⁺/Ter119⁺/CD31⁺/Sca-1⁺/CD51⁺), macrophages (CD45⁺/F4/80⁺/Gr-1⁺), neutrophils (CD45⁺/F4/80⁺/Gr-1⁺), and MSCs (CD45⁺/Ter119⁺/CD31⁺/Sca-1⁺/CD51⁺) (Figures 2C and S5). Endothelial cells (Figure 2D), macrophages (Figure 2F), as well as neutrophils (Figure 2G) from bone tissue were all positive for NPs, indicating cellular uptake. Although regenerative MSCs were found to be rare cell types at the fracture microenvironment, constituting ~0.19% in total isolated cells, 95% and 77% of MSCs identified within marrow and bone tissue were positive for NPs (Figure 2C,H). Osteoblasts also exhibited high positivity (83% and 37% of the population) for NPs (Figure 2C,E). Representative flow cytometry histograms of these cell types from marrow and fractures are shown in Figure S4C–G.

***In Vitro* Cellular Uptake and Biocompatibility of PSMA-*b*-PS NPs**

To investigate the intracellular trafficking of PSMA-*b*-PS NPs, NP, TBP-NP, and SCP-NP were labeled with Texas Red cadaverine and incubated with mMSCs, which is a relevant regenerative cell type and target for NP, as assessed *via* flow cytometry. Cellular uptake was visualized using fluorescence microscopy (Figure 3A), where NP fluoresces red and cell nuclei fluoresce blue (DAPI staining). Successful and robust cellular uptake was observed in cells treated with all NPs. To quantitatively analyze uptake over time, flow cytometry was used to evaluate median fluorescence intensity (Figure 3B) and percentage of NP positive cells (Figure S6) 1, 4, and 24 h after treatment. NP, SCP-NP, and TBP-NP treatments showed 90%, 97%, and 100% positive cells after just 1 h of treatment. At 4 and 24 h, the uptake increased to ~100% for all treatment groups (Figure S6). The median fluorescence intensity (MFI) of cells was not significantly different between NPs after 1 h of treatment. However, cells treated with SCP-NP and TBP-NP showed ~1.3- and ~1.5-fold increases in MFI at 4 h over cells treated with NP, respectively. After 24 h, SCP-NP and TBP-NP treated cells showed further ~1.8- and ~1.6-fold increases in MFI, indicating greater uptake.

To assess NP delivery system biocompatibility, MSCs were treated with unloaded or drug-loaded PSMA-*b*-PS NPs at 100 μ g/mL for 24 h at 37 °C. There was no significant decrease in cell viability (Figure S1). These data show that PSMA-*b*-PS NPs are able to accumulate intracellularly without compromising MSC viability. In addition, peptide functionalization does not significantly impact NP physicochemical properties, realizing robust cellular uptake. To confirm the presence of NPs intracellularly, lysosomes were stained using LysoTracker (Invitrogen) (Figure 3C). After 4 h of treatment, fluorescein-labeled NPs (FITC-NP, green) showed excellent co-localization with lysosomes, indicating that the endolysosomal pathway is involved in the cellular uptake of these NPs.

Relationship between *in Vitro* Drug Efficacy and Drug Release Kinetics

Activation of Wnt/ β -catenin signaling can be achieved at many steps in the pathway. Most commonly, though, is the inhibition of GSK-3 β , which is part of the multiprotein complex which degrades β -catenin and prevents its translocation to nucleus. A small molecule GSK-3 β inhibitor, 3-amino-6-(4-((4-methylpiperazin-1-yl)sulfonyl)phenyl)-*N*-(pyridin-3-

yl)pyrazine-2 carboxamide (AZD2858), was loaded successfully in NPs. Drug release from micelles was examined at pH 4.5 and 7.4 to represent the pH present in the endolysosomal compartment consistent with typical NP trafficking after uptake and physiological pH, respectively. Free drug released immediately, while drug-loaded NPs released only 20% at physiological pH in 9 days and 60% release over 48 h at endolysosomal pH (Figure 3D).

To evaluate *in vitro* efficacy of NP-drug, upregulation of β -catenin was quantified using a luminescent reporter plasmid for active nuclear β -catenin (TOPFlash/FOPFlash).¹⁶ C3H10T1/2 cells were used as surrogate for MSCs due to poor MSC transfection reported in literature.^{16,76,77} Cells were transfected with TOPFlash or FOPFlash reporter plasmid, followed by treatment with GSK-3 β inhibitor (free drug) or drug-loaded NPs at various concentrations for 24 h. Active nuclear β -catenin levels were represented by the ratio of TOPFlash to FOPFlash signal normalized to cellular DNA concentration (Figure 3E). Continuous treatment for 5 days with 10 μ M of drug resulted in sustained up-regulation of β -catenin in both free and NP-drug groups, with NP-drug showing more robust β -catenin activation than free drug. The enhanced β -catenin agonism is likely due to a sustained intracellular release of drug *via* the NP delivery system, which prevented acute cellular exposure to high drug concentrations and subsequent cytotoxicity (Figure S7).

Drug release from PSMA-*b*-PS-based micellar NPs is highly dependent on the NP structure (core:shell ratio, block molecular weight, *etc.*) and relative hydrophobicity of drug investigated, ranging from 8 h to several days.^{36,37,45,47,62,78} Our results show that NP-mediated release of GSK-3 β inhibitor is pH-responsive, where <20% release occurs over 7 days under neutral pH, while ~80% was released when the pH drops to 4.5. This acidic pH was investigated, as it is consistent with the pH in lysosomes where NPs are trafficked after endocytosis. The pH responsiveness is likely due to the protonation of –COOH groups in maleic anhydride units within the polymer chain and decreased electrostatic interactions with positively charged primary amines of the GSK-3 β inhibitor. In fact, this pH-responsive behavior is not unique to the GSK-3 β inhibitor, since it has been shown in previous studies that release of doxorubicin, which also has amines, was pH dependent.^{37,39} Nile red, which has no protonatable groups, did not show any pH-dependent change in release.^{37,39} Co-localization of NPs and lysosomes was observed, together with enhanced drug efficacy when delivered with NPs, and indicates that a sustained intracellular release of drug from lysosomes *via* the NP delivery system likely occurs, as lipophilic small molecules that are <700 Da molecular weight are freely permeable across the cell membrane.⁷⁹ This pH-responsive release is beneficial for the design of NPs since it provides a maximum release of drug at endosomal pH, reducing the possibility of premature drug release during systemic circulation.⁸⁰ These observations reveal further insights into the NP/drug interaction, and although not necessary for bone fracture healing, it provides further application of NPs where pH-responsive release is beneficial, such as drug delivery for cancer and acidic milieus of biofilms.⁸¹

NP-Mediated Bone-Localized β -Catenin Upregulation Results in Expedited Fracture Healing

Fracture β -galactosidase activity was examined following retro-orbital injection of TBP-NPs loaded with GSK-3 β inhibitor 3 days after fractures using TOPGAL reporter mice (Tg(Fos-LacZ)-34Efu/J). Greater β -galactosidase activity (arrows) was observed in callus and periosteum in fractures treated with GSK-3 β inhibitor-loaded TBP-NPs compared to untreated controls and untargeted NPs 2 and 4 weeks after injection (Figure 4A). These data indicate that TBP functionalization results in preferential NP accumulation at fracture sites and upregulated β -catenin signaling. Histological analysis of cartilage and bone formation showed increased cartilage (blue) at week 2, indicating effective endochondral bone formation in all treatment groups (Figure 4B). By week 4, greater bone formation (orange to red) and less soft callus were observed at fractures treated with GSK-3 β inhibitor-loaded TBP-NPs, indicating more rapid cartilage turnover and expedited fracture healing.

To further examine bone healing, microcomputed tomography (μ CT) was employed to evaluate bone formation 2 and 4 weeks after treatment (Figure 5A,B). Fractures treated with TBP-NP-drug exhibited greater callus formation than controls at week 2 and formation of completely bridged bone callus by week 4 (Figure 5A). Saline, free drug, and SCP-NP-drug treated fractures showed an increase in callus volume at week 4 compared to week 2, but incomplete bridging was observed (Figure 5A). Quantitatively, saline, free drug, and SCP-NP-drug showed 2.0-, 1.9-, and 1.5-fold decreased bone volume compared to TBP-NP-drug at week 2, and 1.4-, 1.9-, and 1.4-fold decreased bone volume compared to TBP-NP-drug at week 4, respectively (Figure 5B(i)). Bone volume fraction (BV/TV) of femurs treated with TBP-NP-drug also showed 1.6-fold increase compared to saline control (Figure 5B(ii)), indicating significantly improved bone mineralization. As the ultimate outcome of bone healing, biomechanical properties of fractures were investigated *via* torsion testing at week 4. Femurs treated with TBP-NP-drug showed significantly increased maximum torque (Figure 5C) and yield torque compared to saline controls (Figure 5D). TBP-NP-drug treated fractures also showed torsional rigidities (Figure 5E) that were 4.4-, 2.4-, and 2.4-fold higher than saline, free drug, and SCP-NP-drug treated fractures, respectively. Greater bone volume and strength, together with enhanced ossification observed in histological analysis, indicate that Wnt/ β -catenin agonism *via* GSK-3 β inhibition improved bone healing by improving osteogenesis, which is consistent with the previously described osteogenic activity of GSK-3 β inhibitors.^{21,22,82,83}

Fracture non-union is a major challenge in orthopedics. As a promising alternative to traditional growth factor therapy, small molecules with osteoinductive potential are advantageous due to low immunogenicity, high stability *versus* growth factors, and cost effectiveness.^{84–86} The need for anabolic drug development for orthopedic approaches is critical, as 85% of anabolic use is off-label.⁸⁷ This limitation necessitates a clinically relevant approach: a therapeutic administered systemically, but that targets the fracture site. Local delivery of therapeutics shows positive results, where localized delivery is achieved *via* drug loading into a sustained release device.^{88–90} Local delivery offers the advantages of reducing systemic side effects and provides a physical substrate for cell growth, which could be beneficial for bone regeneration.⁹⁰ However, local delivery does not allow for dose

adjustment after implantation, and the rate of drug release typically decreases with time. Systemic delivery, in contrast, offers the flexibility of controlling dose and is non-invasive, which is beneficial when delivering various drugs at different stages of bone healing. Therefore, a fracture-targeted PSMA-*b*-PS NP system was explored for its ability to provide β -catenin activation to enhance fracture healing. NPs showed uniform spherical morphology and negative net surface charge and exhibited high β -catenin agonist loading efficiencies and pH-responsive release behavior. NPs exhibited preferential accumulation at fracture sites after systemic injection, which was dramatically improved *via* conjugation of the bone targeting peptide TBP. Greater β -galactosidase activity was observed in callus and periosteum in TOPGAL mice treated with TBP-NP- β -catenin agonist *versus* untreated controls, indicative of successful β -catenin activation during healing. Although not investigated here, GSK-3 β inhibition also has been shown to increase capillary density and blood flow as a mechanism by which fracture healing is expedited.^{91–93} Finally, significantly improved bone mechanical properties, along with enhanced bone bridging and greater volume of newly formed bone were observed 28 days after treatment *versus* controls, indicating expedited fracture healing.

Despite the exciting development of NP systems for drug delivery, translation of these therapies from the bench into clinical practice remains a major issue. In fact, multiple NP systems are currently in clinical trials for efficacy and safety for cancer therapy.⁹⁴ Considering a growing number of small molecules have been discovered with osteogenic capabilities, a platform that provides safe, efficacious, and preferential fracture-targeted delivery may represent a critical feature for the next generation of bone regenerative medicine.⁸⁶

CONCLUSION

Numerous molecules have been developed to treat bone-related disease.⁶⁷ However, their use is challenging due to poor stability, short half-life, and poor bone biodistribution of drugs. The significance of this work is the development of NPs-mediated targeted delivery of small molecule drugs to fractures with promising healing outcomes, which leverages the specificity of targeting to resorption sites. Overall, targeted NPs efficiently load small molecule drugs, resulting in preferential localization to fracture sites following systemic injection. More specifically, GSK-3 β inhibitor loaded TBP-NPs and subsequent targeted delivery to fractures will allow for enhanced bone regeneration with reduced off-target side effects, which is not currently possible *via* systemic delivery of small molecule drugs. The versatile drug loading chemistry, along with the formation of uniform spherical NPs with favorable physicochemical properties and size make these NPs an excellent platform for delivering small molecule drugs. Furthermore, β -catenin agonist loaded TBP-NPs and subsequent targeted delivery to fractures improved bone regeneration, which is attributed to target specific upregulation of Wnt/ β -catenin pathway.

MATERIALS AND METHODS

Poly(styrene-*alt*-maleic anhydride)-*b*-poly(styrene) (PSMA-*b*-PS) Synthesis

Reversible addition–fragmentation chain transfer (RAFT) polymerization were used to synthesize amphiphilic diblock copolymers of PSMA-*b*-PS, as described previously.³⁷ Briefly, 4-cyano-4-dodecylsulfanyltrithiocarbonyl sulfanyl pentanoic acid (DCT) was synthesized as a RAFT chain transfer agent (CTA).⁹⁵ Styrene (99%, ACS grade) was purified by distillation. Maleic anhydride (MA) was recrystallized from chloroform. 2,2'-Azo-bis(isobutyronitrile) (AIBN) was used as initiator and recrystallized from methanol. To make amphiphilic PSMA-*b*-PS polymers, styrene (Sty) and excess of maleic anhydride (MA) ([Sty]:[MA] = 4:1) were added in DCT ([monomer]:[CTA] = 100:1) and AIBN ([AIBN]:[CTA] = 1:10) in dioxane (50% w/w).³⁷ After purging with N₂ on ice for 45 min, the solution was placed in a 60 °C oil bath for 3 days for polymerization. The reaction was then terminated by exposure to air. The reaction product was diluted with acetone and then precipitated in petroleum ether. The final product was placed in vacuum to dry. Gel permeation chromatography (GPC) was used to determine the polymer molecular weight as described previously.³⁷

NPs Self-Assembly and Characterization

Amphiphilic PSMA-*b*-PS copolymers self-assemble into core–shell NPs *via* solvent exchange.³⁷ Briefly, 200 mg of copolymers was dissolved in 30 mL of dimethylformamide (DMF). Thirty mL ddH₂O was added into stirring polymer solution using a syringe pump with 29.2 mm diameter at 24.4 μ L/min. After completion of pumping, NPs were formed, and DMF was removed by dialysis against water using MWCO 6–8 kDa dialysis tubing for 3 days. NP concentrations were determined gravimetrically after lyophilization. Both NP size and surface zeta potential were measured at concentrations of 0.1 mg/mL using dynamic light scattering (DLS, Malvern Instruments, Worcestershire, UK).³⁷ Transmission electron microscopy (TEM) was used to investigate the morphology of NPs and to confirm size measurement from DLS. Diluted NPs (0.1 mg/mL in PBS) were incubated with 2% (v/v) phosphotungstic acid negative stain at 1:1 ratio on coated grids for 5 min. Upon completion of staining, excess solution was removed from the grids using filter paper. The grids were imaged using a Hitachi 7650 transmission electron microscope.³⁷ NP quantification was performed using a NanoSight NS300 following the manufacturer's protocols (Malvern Instruments, Malvern, UK).

Peptide Synthesis and NP Functionalization

TRAP-binding peptide (TBP, sequence: TPLSYLKGLVTVG) and scrambled control peptide (SCP, sequence: VPVGTL SYLKLTG) were synthesized using microwave-assisted solid-phase peptide synthesis (CEM Corp, Liberty1 synthesizer). Peptides were synthesized at a 0.5 mmol scale on Fmoc-Gly-Wang resin (Millipore) with Fmoc-protected amino acids (AAPPTec and Peptides International) using microwave-assisted solid-phase peptide synthesis (CEM Corp, Liberty1 synthesizer). Coupling was achieved with 0.5 M *O*-benzotriazole-*N,N,N,N*-tetramethyl-uronium-hexafluoro-phosphate (HBTU) in DMF and 2 M *N,N*-diisopropylethylamine (DIEA) in *N*-methyl-2-pyrrolidone (NMP), and deprotection was achieved with 5% piperazine in DMF. Peptides were cleaved in a cocktail of 92.5%

trifluoroacetic acid (TFA)/2.5% H₂O/2.5% triisopropylsilane (TIPS)/2.5% 3,6-dioxo-1,8-octanedithiol (DOTD) for 2.5 h and precipitated in ice-cold diethyl ether. Peptides were validated using mass spectrometry and absorbance and then were conjugated to NP *via* standard carbodiimide chemistry. Briefly, 0.5 M sodium phosphate buffer (pH 7.4) was prepared by mixing dibasic sodium phosphate and monobasic sodium phosphate. The PSMA-*b*-PS NP solution was diluted in 0.5 M sodium phosphate buffer, yielding a final concentration of 0.1 M sodium phosphate. Excess amounts of 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC, Thermo) ([EDC]:[polymer] = 10:1 molar ratio), 5 mM hydroxysulfosuccinimide (sulfo-NHS, Thermo), and peptide ([peptide]:[polymer] = 10:1 molar ratio) were added into NP in sodium phosphate buffer. The reaction was stirred overnight and purified by dialysis (MWCO 6–8 kDa). Conjugation efficiency was quantified using the Fluoraldehyde o-Phthaldialdehyde (OPA, Thermo Scientific) assay (Ex/Em = 360 nm/455 nm). For *in vitro* flow cytometry assays, NP, TBP-NP, and SCP-NP were further labeled with Texas Red Cadaverine (Texas Red C5, ThermoFisher) *via* carbodiimide chemistry.⁹⁶

Drug Loading into NPs

The GSK-3 β inhibitor, 3-amino-6-(4-((4-methylpiperazin-1-yl)sulfonyl)phenyl)-*N*-(pyridin-3-yl)pyrazine-2 carboxamide (Calbiochem), was loaded into a NP, which was subsequently characterized for size, surface charge, drug loading, and release. NPs, TBP-NP, or SCP-NP were diluted to 1.7 mg/mL. 1.2 mg of GSK-3 β inhibitor was dissolved in 100 μ L of dimethyl sulfoxide (DMSO) and then diluted in 400 μ L of chloroform. The 500 μ L of drug solution was added quickly into the stirring NPs solution. The reaction was stirred overnight open to atmosphere in a chemical fume hood to allow evaporation of chloroform and protected from light as the drug is light sensitive. Loaded drug NPs were centrifuged twice at 4000 rpm for 10 min to remove insoluble drug precipitates and then further purified thrice using centrifugal filters (100,000 MWCO Amicon, Millipore) at 2000 rpm for 10 min to remove unloaded drug and free polymers. Drug-loaded NPs were diluted in water, yielding a final volume of 5 mL and stored at 4 °C. GSK-3 β inhibitor loading was quantified *via* high-performance liquid chromatography (HPLC). HPLC analysis was performed using a Kromasil C18 column (50 mm $\times$ 4.6 mm, 5 μ m particle size, 100 Å pore size) with a variable wavelength UV–vis detector (Shimadzu) using the following parameters: flow rate = 0.5 mL/min, from 90% to 30% A (0.1% TFA in HPLC-grade water) and 10% to 70% B (HPLC-grade acetonitrile) over 6 min. The retention time for the GSK-3 β inhibitor was 3 min at 302 nm. Drug loading efficiency was calculated as loaded drug/total drug $\times$ 100%. Drug loading capacity was calculated as loaded drug mass/NP mass $\times$ 100%.³⁷

NP, SCP-NP, and TBP-NP were loaded with IR780 (a near-IR hydrophobic model drug for visualization) for analysis of biodistribution. Instead of using DMSO and chloroform, acetone was used to make 500 μ L of drug solution prior to loading, and ~90% loading efficiency was achieved in all NPs. NP, SCP-NP, and TBP-NP loaded IR780 were concentrated to 2 mg/mL polymer concentration and stored in the dark at 4 °C.

Mid-Diaphyseal Femur Fracture Model

All animal experiments were approved by the Institutional Animal Care and University Committee of Animal Resources (UCAR). *In vivo* studies were performed using a previously developed mid-diaphyseal femur fracture model in female 6–8 week old Balb/c mice (Harlan Laboratories, Indianapolis, IN). After 1 week of acclimatization, mice were anesthetized with 60 mg/kg of ketamine and 4 mg/kg of xylazine intraperitoneally (IP). An 8 mm long skin incision was made on the femur, and blunt dissection of muscle was used to expose the midshaft of the femur. Mid-diaphyseal femur fractures were created using a rotary Dremel with a diamond blade attachment. A 25-gauge needle was inserted into the medullary canal of the femur from the distal end. A Faxitron system (Faxitron X-ray, Wheeling, IL) was used to take X-ray images at the time of surgery and every week following surgery until sacrifice. Buprenorphine (0.05 mg/kg IP) was given 6–12 h prior, at the time of surgery, and every 6–12 h for 3 days postfracture.

In Vivo Biodistribution Studies using IVIS Imaging

Three days after surgery, mice were injected with NP-loaded IR780 retro-orbitally (0.7 mg/kg IR780, 5 mg/kg of NPs).⁹⁷ Mice were monitored *via* XENOGEN/IVIS imaging system (PerkinElmer) longitudinally (780 nm/820 nm for IR780). IVIS images were taken at multiple time points for 2 weeks after injection using 2% isoflurane gas for anesthesia. Quantification of NP biodistribution from IVIS imaging was achieved by measuring the total radiant efficiency of regions of interest (right femurs) and normalizing to saline and day 0. Tissue distribution was also analyzed by IVIS 24 and 48 h after injection by measuring IR780 signal in dissected organs. Specimens were also embedded and cut using a cryostat and imaged at 40× magnification using an Olympus VS110 Virtual Microscopy System for whole-slide scanning. Visiopharm software was utilized to determine the area of cells (DAPI) and NP-loaded IR780.⁹⁸

In Vivo Cellular Uptake Assay by Flow Cytometry

Fluorescein-labeled PSMA-*b*-PS NPs (FITC-NP, FITC-TBP-NP, FITC-SCP-NP at 10 mg/kg) and saline were injected retro-orbitally on day 3 postfracture. Mice were sacrificed 24 h post-injection, and bone and marrow tissue were harvested from fracture sites for cell isolation. Bone marrow tissue was incubated with red blood cell lysis buffer for 5 min at room temperature, followed by digestion in 1 mg/ mL collagenase type IV (Sigma), 2 mg/mL dispase (Sigma), and 10 U/mL DNase (Thermo Scientific) for 45 min at 37 °C. Bone tissue was crushed into small pieces and digested in 0.7 mg/mL collagenase type I (Sigma) for 1.5 h. Both bone marrow and bone associated cells were passed through 100 μ m cell strainers (Fisher) to achieve single cell suspensions. Cells were suspended in flow buffer at 1×10^6 cells/ ml containing 2% FBS, 3 μ M DAPI (Thermo Fisher), and 0.01% trypan blue. For evaluation of cell markers, samples were incubated with rat antimouse antibodies (PE-Cy7-labeled Ter119 (BD Biosciences), PE-labeled CD51 (eBioscience), APC-labeled CD31 (BD Biosciences), Alexa 700-labeled Gr-1 (eBioscience), PE-CF594-labeled F4/80 (BD Biosciences), PerCP-Cy5.5-labeled Sca-1 (eBio-science), and PE-Cy7-labeled CD45 (BD Biosciences)) for 30 min on ice in the dark. Cells were analyzed using BD LSR II Flow Cytometer (BD Biosciences).

GSK-3 β Inhibitor Release from Nanoparticles

Drug-loaded NPs were dialyzed at physiological pH (PBS, pH 7.4) and acidic pH (pH 4.5) using MWCO 6–8 kDa dialysis membrane (Spectrum). Release media was changed twice daily, and 200 μ L samples were taken over 9 days. Drug release was quantified using HPLC as described in the previously.

Cell Culture

Mouse mesenchymal stem cells (mMSCs) (Cyagen) isolated from C57BL/6 mice were cultured at 37 °C and 5% CO₂ in growth media consisting of low-glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin-streptomycin (Gibco). Mouse embryonic fibroblasts (C3H10T1/2) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown at 37 °C and 5% CO₂ in Basal Medium Eagle (Cellgro) supplemented with 10% FBS and 100 units/mL penicillin-streptomycin (Gibco). C3H10T1/2s were used as surrogate of MSCs for transfection experiments due too poor transfection reported in MSCs.^{16,76,77}

Flow Cytometry and Fluorescent Microscopy for *in Vitro* Cellular Uptake Assays

To evaluate uptake quantitatively, flow cytometry was used. mMSCs were seeded in 24-well plates at 20,000 cells/cm² 1 day prior to treatment. Texas Red-labeled NPs were used to treat cells at 100 μ g/mL for 1, 4, and 24 h. Cells were washed 3 times with PBS, trypsinized, and then suspended in flow buffer (0.5% bovine serum albumin (BSA) in PBS). Cellular uptake was measured by flow cytometry using an Accuri C6 flow cytometer (BD Biosciences).

Fluorescent microscopy was used to qualitatively cellular uptake. In this assay, mMSCs were seeded in chamber slides (Lab-Tek) at 15,000 cells/cm² 1 day prior to treatment. Texas Red-labeled NPs were used to treat cells at 100 μ g/mL for 1, 4, and 24 h. mMSCs were washed 3 times with PBS and fixed with paraformaldehyde (PFA; 4%) for 10 min. mMSCs were then washed with deionized water and coverslipped with the addition of ProLong Gold Antifade Mountant (Thermo Fisher Scientific) to prevent quenching. mMSCs were imaged using a Nikon E600 fluorescence microscope.

NP *in Vitro* Biocompatibility Assay

To assess NP biocompatibility, mMSCs were seeded at 10,000 cells/cm² and treated with unloaded PSMA-*b*-PS NPs at 0, 1, 10, 50, 100, and 200 μ g/mL for 24 h at 37 °C and 5% CO₂. Cells were washed with PBS for 5 times and lysed using cell lysis buffer (Promega). Cell viability was measured using Quant-iT PicoGreen DNA quantification kit (Invitrogen) on a plate reader (Tecan M200 Infinite).

Luciferase Reporter Assay for Analysis of β -Catenin Activity in C3H10T1/2 Cells

C3H10T1/2 cells were used as surrogate for MSCs due to poor MSC transfection reported in literature.^{16,76,77} C3H10T1/2 cells were seeded at 10,000 cells/cm² in 48-well plates 1 day prior to transfection. Cells were treated separately with 0.4 μ g/well TOPFlash and FOPFlash reporter plasmid (Addgene Plasmid #12456 and #12457 (Biechele and Moon, 2008)) using

Lipofectamine LTX and PLUS reagents (Invitrogen) and high-glucose DMEM (without FBS or PSF) at 37 °C and 5% CO₂ for 3 h.¹⁶ Transfection media was aspirated, and BME cell culture media (described in the previous section) with NP-loaded or free GSK-3 β inhibitor (10 μ M) was added to wells. Total luminescence was measured at varied time points post-treatment using a Luciferase Assay Kit (Promega) on a BioTek Synergy Mx plate reader. Cellular DNA concentration was obtained in parallel using a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). Active nuclear β -catenin levels represented by the ratio of TOPFlash to FOPFlash luminescence normalized to cellular DNA concentration.

Histological Analysis of Local β -Catenin Activation and Fracture Healing Efficiency

Free GSK-3 β inhibitor and GSK-3 β inhibitor loaded in NPs, TBP-NPs, and SCP-NPs were injected retro-orbitally 3 days after fractures. Fractured femurs were harvested at weeks 2 and 4 and processed for histological analyses. Excess soft tissue and skin were removed. Specimens were fixed in 4% neutral buffered formalin (NBF) for 3 days before decalcification in 10% ethylenediaminetetraacetic acid (EDTA) for 10 days at room temperature. The intramedullary stainless-steel pins were removed carefully. Then, specimens were embedded in paraffin and cut into 5 μ m sections. Alcian blue (blue, glycosaminoglycan/proteoglycan) and Orange G (pink, bone/soft tissue) staining was used to identify soft callus and new bone. Histological investigation of fracture localized β -catenin upregulation was analyzed *via* LacZ reporter mice (Tg(Fos-LacZ)^{34Efu/J}) by staining for β -galactosidase. These mice contain a LacZ gene inserted downstream of seven consensus binding sites for the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors, which are activated specifically upon nuclear (activated) β -catenin binding. This results in β -galactosidase expression when β -catenin/TCF/LEF-driven transcription is activated through canonical Wnt stimulation. The femurs were fixed in glutaraldehyde fixative for 3 days before decalcification in 10% EDTA for 10 days at room temperature and then placed in tubes containing X-Gal staining solution, consisting of 2 mM MgCl₂, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg/mL X-gal (Fermentas Inc., Glen Burnie, MD) for 36 h. Three nonconsecutive sections were obtained from each specimen and imaged at 40 $\times$ magnification using an Olympus VS110 Virtual Microscopy System for whole-slide scanning.

Microcomputed Tomography

Mice were sacrificed in a CO₂ chamber, followed by cervical dislocation, and femurs were disarticulated from the hip and knee joints. After 3 days of fixation in 10% paraformaldehyde (PFA), the intramedullary stainless-steel pins were removed carefully, and samples were transferred to PBS and scanned in a Scanco Medical VivaCT 40 at high resolution (10.5 μ m voxel size), with X-ray energy of 55 kVp and intensity of 145 μ amps, integration time 300 ms. Analysis was performed with Scanco's proprietary evaluation software. Each sample was contoured around the external callus and along the edge of the cortical bone. To measure new bone callus volume, fracture contouring was performed to exclude cortical bone from all bone space. Bone volume (BV), mineralization density, trabecular thickness, trabecular number, and trabecular bone volume fraction (bone volume (BV)/total volume (TV)) were analyzed in a volume of interest (VOI) including 1 mm of the proximal and distal region of the femur fracture.⁹⁹

Biomechanical Testing

Mice were sacrificed at week 2 and 4 postfracture. Femurs were harvested after sacrifice, and the intra-medullary stainless-steel pins were removed carefully. Specimens were wrapped in gauze and stored in saline at -20°C until thawing for biomechanical testing. Torsional loading was used to assess healed bone strength under nearly pure shear conditions.¹⁰⁰ The proximal and distal ends of harvested femur specimens were cemented into 6.35 mm^2 aluminum tubes using bone cement prepared to the manufacturer's specifications (DePuy Endurance; Warsaw, IN). Specimens were bathed in PBS at room temperature for 2 h after potting to allow for rehydration of the tissue and hardening of the bone cement. Samples were mounted on an EnduraTec TestBench system (200 N mm torque cell; Bose Corp., Minnetonka, MN) and tested in torsion until failure at a rate of $1^{\circ}/\text{s}$ to determine ultimate torques.⁹⁹

Statistical Analyses

Data are expressed as mean $\pm$ standard deviation with sample sizes indicated in figure legends. Differences between groups were compared using unpaired Student's *t* tests or one-way or two-way analysis of variance (ANOVA) with Tukey's or Dunnett's posthoc testing, as indicated in figure legends. For *in vivo* studies, 6–12 mice per condition were used based on an *a priori* power analysis and preliminary *in vivo* gene expression data. A *p*-value 0.05 was used to define statistical significance. Statistics were assessed with GraphPad Prism 6 Software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

TBP functionalization results in preferential NP accumulation at fracture sites. Fractures were created in mice 3 days prior to 5 mg/kg of NPs retro-orbital injection. Live animal imaging showed qualitatively (A) and quantitatively (B) increased fracture targeting of TBP-NPs *versus* untargeted NPs and SCP-NPs. $N = 6$, mean $\pm$ SD, *, #, and \$ indicate significant difference ($p < 0.05$) *versus* saline, NP-IR780, and SCP-NP-IR780. (C) Transmission electron micrographs of femur fracture (i–ii) and liver histology sections (iii–iv) showed NP internalization. (ii and iv) Zoomed in images of (i) and (iii), respectively. Triangles indicate presence of NPs in vesicles intracellularly. Arrows indicate endolysosomal compartment. (D) NP (a–g), SCP-NP (h–n), and TBP-NP (o–u) treated mice were sacrificed 24 h after injection. Fractured bone, unfractured bone, as well as organs were harvested and processed for cryosectioning. Nuclei are stained with DAPI (blue) and IR780 loaded NPs fluoresce pink. Scale bar = 200 μ m. (E) The signal from IR780, which indicates NP presence, was quantified using Visiopharm software. * $p < 0.05$ indicates significance evaluated using two-way ANOVA with Dunnett's posthoc analysis.

Figure 2.

TBP functionalization results in preferential NP accumulation in regenerative cell types. (A) *In vivo* uptake of injected FITC-labeled NPs in cells located in fractured bone and (B) fractured marrow quantified by flow cytometry. $N = 3$, mean $\pm$ SD. $*p < 0.05$ indicates significance evaluated using two-way ANOVA with Dunnett's posthoc analysis. (C) Flow cytometry analysis of TBP-NP % positive cells from each cell type isolated from bone marrow and bone tissue at the fracture. Live cells are identified by DAPI negative gate, and cell types are identified by cell-specific markers: endothelial cells (CD45 $^-$ /Ter119 $^-$ /CD31 $^+$), osteoblasts (CD45 $^-$ /Ter119 $^-$ /CD31 $^-$ /Sca-1 $^-$ /CD51 $^+$), macrophages (CD45 $^+$ /F4/80 $^+$ /Gr-1 $^-$), neutrophil (CD45 $^+$ /F4/80 $^-$ /Gr-1 $^+$), and MSCs (CD45 $^-$ /Ter119 $^-$ /CD31 $^-$ /Sca-1 $^+$ /CD51 $^+$). $N = 3$, mean $\pm$ SD. $*p < 0.05$ indicates significance evaluated using two-way ANOVA with Dunnett's posthoc analysis. Representative histograms from flow cytometry analysis of (D) endothelial cells, (E) osteoblasts, (F) macrophages, (G) neutrophils, and (H) MSCs isolated from fractured bone tissue. White and gray peaks indicate cells isolated from saline and TBP-NP treated mice, respectively.

Figure 3.

NP-delivered GSK-3 β inhibitor increases β -catenin level and exhibited pH-responsive release, which enhances intracellular release of drug in the endolysosomal compartment. (A) NPs, TBP-NPs, and SCP-NPs were labeled with Texas Red fluorophore and exhibited robust cellular uptake. Cells were incubated with NPs at 0.1 mg/mL for 1, 4, and 24 h and stained with DAPI (blue) for cell nuclei. (B) Median fluorescent intensity quantified 1, 4, and 24 h after treatment *via flow cytometry* assay. $N=3$, Mean $\pm$ SD. * $p < 0.05$ versus NP, all NP treated groups, and significantly different versus untreated control (2-way ANOVA and Tukey's posthoc analysis). (C) Co-localization of FITC-labeled NPs (green) and lysosomes stained with Lysotracker (red). White triangles indicate areas of robust co-localization. (D) GSK-3 β inhibitor release from PSMA-*b*-PS NPs. Loaded NPs were dialyzed against PBS at 37 $^{\circ}$ C with pH of 7.4 or 4.5. Mean $\pm$ SD, $n=3$. (E) Active nuclear β -catenin level is represented by the ratio of TOPFlash to FOPFlash, normalized to cellular DNA concentration. Mean $\pm$ SD, $n=3$, * $p < 0.01$ versus untreated control, # $p < 0.0001$ versus free drug (2-Way ANOVA with Tukey's posthoc analysis).

Figure 4. Delivery of GSK-3 β inhibitor *via* targeted NPs improves β -catenin agonism for fracture healing. (A) LacZ staining shows localized activation of β -catenin (blue) 14 and 28 days after injection of GSK-3 β inhibitor-loaded NPs. Scale bar = 100 μ m. (B) Histological analysis of bone formation using Alcian Blue (blue; glycosaminoglycans and proteoglycans) and Orange G staining (pink; bone and surrounding soft tissue). Scale bar = 200 μ m.

Figure 5.

Fractures treated with TBP-NP-GSK-3 β exhibited increased *in vivo* bone callus formation. (A) Microcomputed tomography (μ CT) scans were taken 2 and 4 weeks post-treatment to assess *in vivo* bone callus formation. Subsequent quantification showed TBP-NP-Drug significantly increased bone callus volume (B(i)) and (ii) bone volume fraction (bone volume/total volume). Mean $\pm$ SD, $n = 6$. Statistical significance was evaluated using two-way ANOVA with Tukey's posthoc analysis. $*p < 0.05$ indicates significance between treatment groups. Scale bar = 1 mm. Biomechanical strength of fractured femurs were enhanced 28 days after targeted drug treatment. (C) Maximum torque and (D) yield torque of TBP-NP-Drug treated fractures were significantly increased over untreated controls 4 weeks after treatment. (E) Torsional rigidity of TBP-NP-Drug treated fractures was significantly improved compared to saline, free drug, and SCP-NP-Drug controls. Mean $\pm$ SD, $n = 6$. Statistical significance was evaluated using one-way ANOVA with Tukey's posthoc analysis. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$, and $****p < 0.001$ indicates significance between treatment groups.

Scheme 1. (A) Synthesis of PSMA-*b*-PS via RAFT Method with DCT as the RAFT Chain Transfer Agent, (B) Self-Assembly of PSMA-*b*-PS Diblock Copolymers into Micelle NPs via Solvent Exchange Method, and (C) Functionalization of PSMA-*b*-PS with Peptide Targeting Moieties via Carbodiimide Chemistry^a

^aTBP: TRAP-binding peptide; SCP: scrambled control peptide.

Scheme 2. Schematic Representation of TRAP-Binding Peptide-Functionalized NPs for Targeted Delivery of β -Catenin Agonist for Fracture Healing^a

^a β -catenin agonists were loaded into highly stable NPs. A peptide with high affinity for TRAP was introduced to the spherical NP to enhance fracture specificity.

Table 1

Characterization of NP Physicochemical Properties

NP characterization	NP	TBP-NP	SCP-NP
size (nm) ^a	42 ± 3	61 ± 2	68 ± 2
PDI ^a	0.2	0.1	0.2
zeta potential (mV) ^a	-33 ± 2	-26 ± 1	-29 ± 2
loading efficiency ^b (mass _{loaded drug} /mass _{feed})	65%	97%	51%
loading capacity ^b (mass _{drug} /mass _{NP})	6%	14%	7%
peptides/polymer ^c	NA ^d	6.9	5.5

^aDLS using a Malvern Zetasizer Nano ZS.^bHPLC analysis of drug loaded NP solution.^cMalvern Nanosight NS300 analysis of NP numbers by acquiring 60 s NP tracking analysis videos.^dNA: not applicable.