APPLIED SCIENCES AND ENGINEERING

Smoothened agonist sterosome immobilized hybrid scaffold for bone regeneration

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Biomaterial delivery of bioactive agents and manipulation of stem cell fate are an attractive approach to promote tissue regeneration. Here, smoothened agonist sterosome is developed using small-molecule activators [205-hydroxycholesterol (OHC) and purmorphamine (PUR)] of the smoothened protein in the hedgehog pathway as carrier and cargo. Sterosome presents inherent osteoinductive property even without drug loading. Sterosome is covalently immobilized onto three-dimensional scaffolds via a bioinspired polydopamine intermediate to fabricate a hybrid scaffold for bone regeneration. Sterosome-immobilized hybrid scaffold not only provides a favorable substrate for cell adhesion and proliferation but also delivers bioactive agents in a sustained and spatially targeted manner. Furthermore, this scaffold significantly improves osteogenic differentiation of bone marrow stem cells through OHC/PUR-mediated synergistic activation of the hedgehog pathway and also enhances bone repair in a mouse calvarial defect model. This system serves as a versatile biomaterial platform for many applications, including therapeutic delivery and endogenous regenerative medicine.

INTRODUCTION

Bone regeneration is an important clinical issue accompanying population aging and increased life expectancy (1, 2). Readily available and reliable bone graft devices are required for craniofacial and orthopedic surgeries. Tissue engineering scaffolds can be fabricated by well-defined procedures, and their physical and chemical properties are easily tunable to apply in various therapeutic needs. Substantial efforts on the development of engineered three-dimensional (3D) scaffolds have been conducted in combination with bioactive molecules such as growth factors (3-6), genetic materials (3, 7), and drugs (8, 9), targeting bone precursor cells through composite designs (1, 10, 11) and physical/chemical modifications to create better bone regeneration therapies. The application of bioactive molecules without proper delivery systems suffers from several limitations including poor physiological stability, rapid clearance (or burst release), nonspecific targeting, and low cell membrane permeability (12). In this regard, the bioactive molecules often require supraphysiological dosages to counter the poor bioavailability, thereby increasing the potentials of adverse effects (13). Delivery systems can overcome the drawbacks by protecting the bioactive molecules through encapsulation (14), promoting entry into cells (13), and providing controlled drug release in a spatially targeted manner (12, 15). Several delivery systems using nanotechnology have reached the market. Most of these delivery systems have been approved for parenteral applications in the category of liposomal or lipid-based formulations with bioactive molecules. Liposomes have been intensively explored as drug delivery vehicles using naturally derived lipids as phospholipids (e.g., phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine) with cholesterol. However, the use of conventional phospholipid liposomes is limited by their poor stability.

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Sterosome is a recently developed liposomal nanocarrier system formed by nonphospholipids and high content of sterols. Mixtures of single-chain amphiphiles [e.g., stearylamine (SA) or palmitic acid] with high cholesterol content can produce stable unilamellar vesicles with high specific surface charges and very low permeability corresponding to their colloidal stability (*16*). Several reports have demonstrated a great potential of sterosome as a delivery vector for therapeutic and genetic molecules (*17*, *18*). However, most liposomal formulations including sterosome have low drug loading capacity (<10%), requiring the use of a large amount of nanocarriers that may cause undesirable side effects and cost considerations. Therefore, there is a need to develop advanced delivery systems reducing the use of carrier materials or increasing drug loading content.

Here, we first develop a smoothened agonist sterosome-immobilized 3D hybrid scaffold, which can promote osteogenesis and bone healing by activating the hedgehog signaling (Fig. 1). This new class of sterosome is created using small-molecule agonists [20S-hydroxycholesterol (OHC) and purmorphamine (PUR)] of the smoothened protein in the hedgehog pathway as liposomal carrier and drug cargo (Fig. 1A). Sterosome presents an inherent osteoinductive property occurring from OHC even without drug loading (19). OHC is a naturally occurring oxysterol and dictates pro-osteogenic and anti-adipogenic lineage commitment in mesenchymal stem cells (MSCs) by binding smoothened and activating hedgehog signaling (20, 21). Activation of hedgehog signaling enhances osteogenesis by binding of OHC to the extracellular domain of smoothened in hedgehog signaling (20). Furthermore, sterosome with PUR is more effective to promote osteogenesis by combinative up-regulation of hedgehog signaling. PUR is a trisubstituted purine derivative and activates the hedgehog signaling by directly binding and activating smoothened, as does OHC, whereas smoothened is regulated by two separate binding sites for PUR and OHC (21, 22). These compounds can exert synergistic effects on the up-regulation of hedgehog signaling. The proposed hybrid scaffold is fabricated by sequential procedures by introducing a bioinspired polymerized dopamine (PDA) coating on the surface of the 3D poly(lactic-co-glycolic acid) (PLGA) scaffold without complex and harsh chemical reactions as a biocompatible intermediate for further functionalization (Fig. 1B). Upon creation of the PDA layer

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Fig. 1. Schematic illustration of smoothened agonist sterosome and its hybrid scaffold for bone regeneration. (A) Smoothened agonist sterosome consisted of SA, OHC (osteogenic cholesterol), and PUR (smoothened agonist). (B) Fabrication process for PLGA 3D hybrid scaffold with sterosome by layer-by-layer modification via bioinspired dopamine chemistry, and the stepwise bone repair strategy through hedgehog signaling pathway. Smo, smoothened; PTCH, patched.

on the scaffold surface, the osteoinductive sterosome is immobilized on the PDA layer by Schiff base formation and Michael-type addition between the PDA and primary amine of the sterosome surface (23, 24). The PDA layer not only acts as an intermediate for sterosome immobilization but also provides a powerful route for enhancing cell-scaffold interactions and controlling drug release (25). First, sterosome immobilized on the hybrid scaffold with sequential modifications is verified by surface characterization of the scaffold. Next, an in vitro study is conducted to investigate the ability of the hybrid scaffold to support cytocompatibility and osteogenic differentiation of bone marrow stromal cells (BMSCs). Last, we use a critical-sized mouse calvarial defect model to confirm the efficacy of this system to promote bone regeneration. This study will systematically integrate small-molecule drugs (PUR), innately osteoinductive nanovector (smoothened agonist sterosome) with synergistic osteoinductive potential, and biocompatible scaffolds into a bone graft device that will promote better bone growth.

RESULTS AND DISCUSSION

Characterization of sterosomes

Specific properties of nanoparticles such as hydrodynamic size, surface charge, and functionality play pivotal roles in the application of bioactive payload as delivery platform. The physicochemical characterization of smoothened agonist sterosome was conducted by using dynamic light scattering (table S1 and fig. S1). The diameter of

sterosome was determined to be 157 ± 8 nm with a narrow distribution [polydispersity index (PDI), 0.15 ± 0.03]. Sterosome after encapsulation of PUR shows 214 ± 11 nm (PDI, 0.17 ± 0.03) in diameter with a PUR loading efficiency of $60.7 \pm 1.5\%$. The ζ -potentials of sterosomes without and with PUR loading were 35.7 ± 2.0 and 35.5 ± 0.7 mV, respectively, due to the protonation of the single-chain amphiphile (SA) at physiological pH. The effect of sterosomes on osteogenic differentiation of BMSCs was evaluated by monitoring the alkaline phosphatase (ALP) activity and the production of mineralized matrix (Fig. 2). ALP plays a pivotal role during the early stage of osteogenesis (26). The expression of ALP was increased dose dependently by sterosomes with or without PUR, as observed in direct staining and colorimetric quantification at day 4 (Fig. 2, A and B). In addition, PUR loading into sterosome further enhanced ALP activity, which was significantly higher than that detected in sterosome without PUR loading. These results indicate that the sterosome itself can induce osteogenic differentiation of BMSCs without drug loading and synergistically enhance osteogenesis with PUR. BMSC maturation was determined by using the Alizarin red S staining for extracellular calcium deposits as a late marker of osteogenesis at day 14 (Fig. 2, C and D). Sterosome treatment dose-dependently enhanced mineralization without PUR loading, which was significantly increased with PUR delivery compared to sterosome alone. We have performed additional study of demonstrating superior efficacy of our nanoparticulate system in comparison to PUR delivery from a similar vesicle but prepared using nonosteogenic cholesterol.

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Fig. 2. Bioactivity of smoothened agonist sterosomes in BMSCs. (A) ALP staining and (**B**) colorimetric quantification of ALP activity at day 4. (**C**) Mineralization stained with Alizarin red S and (**D**) colorimetric quantification of mineralization activity at day 14. Scale bars, 200 μm. Data were presented as means ± SD. Statistical analysis was determined by one-way ANOVA with Tukey's post hoc test; ***P* < 0.01, ****P* < 0.001. CM, culture medium; OM, osteogenic medium.

The treatment with either steorosomes (blank) or PUR delivery alone failed to achieve the expression level of osteogenic and hedgehog genes induced by our smoothened agonist sterosomes (fig. S2). These results show the inherent bioactivity of sterosome formed from OHC and synergistic osteoinductive potential with PUR, indicating a great potential of sterosome with PUR delivery for promoting bone regeneration.

Characterization of sterosome-immobilized hybrid scaffolds

The immobilization of sterosome on the surface of porous 3D PLGA scaffold was achieved through a PDA intermediate under weakly alkaline condition (pH 8.0) and observed using sterosome encapsulated with nile red as a fluorescence model drug (Fig. 3A). The presence of PDA intermediate resulted in the enhanced immobilization of sterosome. When the scaffolds were treated with nile red-loaded sterosome (1 mg ml⁻¹) for 1 hour, the amount (13.0 \pm 0.3 mg per scaffold) of sterosome detected on the surface of PDA-coated scaffolds was fourfold higher than that $(3.3 \pm 0.1 \text{ mg per scaffold})$ of bare PLGA scaffolds with no PDA coating. Furthermore, the amount of immobilized sterosome could be modulated by changing immobilization time and concentration of sterosome (fig. S3). Sterosome immobilization was further confirmed by using nile red-loaded, fluorescein isothiocyanate (FITC)-labeled sterosome. The fluorescence images showed substantial overlap between nile red-loaded and FITClabeled sterosome on the scaffold (Fig. 3B). The fluorescence dots of

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100 to 200 nm, which is the size range of sterosome, were observed on the scaffold surface, indicating that sterosome is able to immobilize and remain stable on the PDA intermediate. The outer shell of sterosome has primary amine originated from single-chain amphiphile, SA, which reacts covalently with PDA via Schiff base and Michael addition reactions under oxidizing condition (*27*).

The engineered surfaces of the scaffolds were analyzed by x-ray photoelectron spectroscopy (XPS) to confirm PDA coating and immobilization of sterosome. The XPS spectra confirmed that the chemical composition of the surfaces was altered by the PDA coating and sterosome immobilization. All the scaffolds had peaks at 530 and 285 eV in XPS spectra, which corresponded to oxygen (O 1s) and carbon (C 1s) of PLGA substrate, respectively (Fig. 3C). There was no difference in O 1s spectra among all scaffolds (fig. S4). However, there was an obvious distinction between C 1s peaks in high-resolution spectra. Although the C-C, C-O, and O-C=O peaks corresponding to PLGA were observed at 281.2, 283.2, and 285.3 eV on the surface of bare PLGA scaffold, respectively, both C-O and O-C=O peaks were extremely decreased through the sequential modification with PDA and sterosome (Fig. 3C). Elemental composition of the scaffolds was markedly changed, and the carbon atomic content was gradually increased (table S2). The atomic content of oxygen with the O/C ratio was steadily decreased over the modification process due to the scaffold surface covered with carbon-rich compound such as PDA, OHC, and SA. Moreover, a nitrogen (N 1s) peak at 397 eV

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Fig. 3. Characterization of surface-modified PLGA 3D scaffold with smoothened agonist sterosomes via bioinspired dopamine chemistry. (A) Quantification of immobilized sterosome on surface of PLGA and PDA-coated scaffold via PDA for 1 hour. Nile red–loaded sterosome was used as a model sterosome. The concentration of nile red–loaded sterosome is 1.0 g liter⁻¹. Data were presented as means \pm SD. ****P* < 0.001, two-tailed *t* tests. (B) Confocal laser scanning microscopy images of sterosome-immobilized scaffold (scale bar, 100 µm). Sterosome is labeled by FITC. Nile red is used as model cargo. (C) XPS spectra of (i) PLGA scaffold, (ii) PDA-coated scaffold, and sterosome-immobilized scaffold (iii) without second PDA layer or (iv) with second PDA layer. (D) XPS C 1s spectra of (i) PLGA scaffold, (ii) PDA-coated scaffold, and sterosome-immobilized scaffold (iii) without second PDA layer or (iv) with second PDA layer.

was detected in modified scaffolds, verifying the presence of a PDA and/or sterosome (fig. S5). These findings support the idea that sterosome is immobilized successfully on the surface of the scaffolds via a bioinspired PDA intermediate without any other chemical modification. Scanning electron microscopy (SEM) images revealed the porous structure of the modified scaffolds with rough surface morphology compared to that of bare PLGA scaffold, which may facilitate cell adhesion, proliferation, and differentiation (fig. S6) (28). This also implies successful grafting of sterosome. Liposomes can also be physically grafted onto scaffolds using a polymer hydrogel, but liposome-polymer association can lead to increase in viscosity and formation of aggregates, while too strong electrostatic interaction was found to lead to the perturbation of vesicle permeability or even disruption (29). PUR can be incorporated physically onto a scaffold surface, but this nonspecific adsorption-based delivery is often easily

affected by the surrounding environment with premature burst release and increased toxicity. Our previous calvarial defect healing study used similar PLGA scaffold treated with small molecular smoothened agonist (SAG), which has even higher agonist activity [effective concentration (EC) = 1 nM] than PUR (EC = 5 μ M), but the bone-forming effect was prominent only with high SAG doses over 1 mM (30).

Liposomal formulations (i.e., sterosome) provide a distinct advantage in controlling the release kinetics of encapsulated bioactive molecules. In particular, the sustained release of bioactive molecules (PUR in this study) can prolong its bioactivity and promote osteogenic differentiation of MSCs (*31*, *32*). Sterosome immobilized on the scaffold demonstrated the sustained release of loaded PUR, and the coating of the scaffolds with secondary PDA further reduced the initial burst release of PUR (fig. S7). Biocompatibility of sterosome-immobilized hybrid scaffolds

The biocompatibility of the scaffolds with various surfaces was determined by seeding BMSCs on the scaffolds and analyzing using Alamar Blue assay and live/dead fluorescence staining for 7 days. The metabolic activity of cells was normalized to the PLGA scaffold without any modification (Fig. 4A). The engineered surfaces with PDA did not significantly influence the metabolic activity (P > 0.05) of BMSCs, whereas sterosome-immobilized scaffold without second

PDA layer showed extremely low cell metabolic activity of ~10%. A possible description to the low metabolic activity in the sterosomeimmobilized scaffold without second PDA layer is that too high density of sterosome immobilized on the surface may restrict the cell attachment and growth. The PDA surface was shown to support cell adhesion and proliferation without the induction of toxic and abnormal responses (33). Furthermore, the number of cells in the scaffolds with PDA increased over time, demonstrating that this



Fig. 4. Cell adherence, proliferation, and bioactivity evaluation of scaffolds. (**A**) In vitro cell proliferation after 1, 4, and 7 days. The value was normalized by PLGA scaffold at day 1. (**B**) Representative confocal fluorescence images of BMSCs stained with calcein AM (live cells, green fluorescence) and ethidium homodimer (dead cells, red fluorescence) for day 7. Scale bar, 200 μ m. (**C**) ALP staining and (**D**) colorimetric quantification of BMSCs on scaffolds at day 4. (**E**) Alizarin red S staining and (**F**) quantified mineralized extracellular matrix of BMSCs on scaffolds at day 14. Data were presented as means \pm SD. ***P* < 0.01, ****P* < 0.001, one-way ANOVA with Tukey's post hoc test. ns, not significant in (D) and (F). (**G**) Representative confocal laser scanning microscopy images of BMSCs incubated on FITC-labeled, nile red–loaded sterosome-immobilized hybrid scaffold for 4 and 24 hours. Nucleus is stained with Hoechst 33342 (blue). Scale bar, 100 μ m. Sterosomes are labeled by FITC.

surface is capable of promoting the proliferation of cells. Consistent with the Alamar Blue assay, all of the scaffolds except for sterosome without second PDA layer had no significant difference on the appearance of BMSCs after incubation for 7 days, as evidenced by live (green) and dead (red) cells in the images (Fig. 4B). Most of the cells on the scaffolds with PDA intermediate were stained green and appeared to spread well with spindle shape. These results present that PDA intermediate can serve as excellent substrates to promote cell attachment, survival, and proliferation.

Osteogenic ability of sterosome-immobilized hybrid scaffolds

On the basis of the excellent biocompatibility of sterosome-immobilized hybrid scaffold, the osteogenic potential of the scaffolds was investigated by monitoring ALP activity (day 4) and mineralization (day 14) in BMSCs. The hybrid scaffold groups with sterosome or PUR-encapsulated sterosome showed highly intense ALP staining compared with the bare PLGA scaffold and PDA-coated scaffold, indicating that sterosome promotes osteogenic differentiation of BMSCs on the scaffold (Fig. 4C). ALP activity was significantly higher in the hybrid scaffold groups compared with PLGA scaffold and PDA-coated scaffold (Fig. 4D). ALP expression was much higher in the sterosome with PUR loading in comparison with the sterosome without PUR, indicating that PUR in sterosome. Furthermore, a similar tendency was observed in Alizarin red S staining, verifying the production of mineralized extracellular matrix (Fig. 4E). Alizarin red S staining was more in-

tense on the hybrid scaffolds compared with the control scaffolds, indicating extracellular mineral deposition. In addition, the marked increment of mineralized matrix was observed in the PUR-containing scaffold, as evaluated by quantification inferred from the stained scaffolds (Fig. 4F).

To investigate the interaction of sterosome on the scaffold with cells, we seeded BMSCs on nile red–encapsulated and FITC-labeled sterosome-immobilized scaffolds for 24 hours and directly monitored using confocal laser scanning microscopy (Fig. 4G). Fluorescence of cells that internalized FITC (sterosome) and nile red increased over time and was intensively observed along the porous strut of the scaffold, indicating successfully interaction of sterosome with the seeded cells on the scaffold surface.

Osteogenic gene expressions of BMSCs in these scaffolds were examined using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 5, A to E). Runt-related transcription factor 2 (Runx2) is a major regulator of osteoblast differentiation, enhancing ALP activity and expression of osteogenic gene such as osteopontin (OPN), osteocalcin (OCN), and collagen type I α 1 (Col1 α 1) (34). Osteogenic gene expression for PDA-coated scaffold was not significantly different from the PLGA scaffold control group, indicating that the PDA intermediate does not affect osteogenic differentiation of MSCs. In contrast, the expression levels of *ALP*, *Runx2*, and *Col1\alpha1* were found to be significantly up-regulated at day 4 in the hybrid scaffolds, most notably in sterosome with PUR (Fig. 5, A to C). Similarly, the expression of *OPN* and *OCN*, key osteogenic markers regulating the production of noncollagenous



Fig. 5. Gene expression related to osteogenesis and hedgehog signaling pathway in scaffolds. (**A**) *ALP*, (**B**) *Runx2*, and (**C**) *Col1a1* were evaluated to assess the osteogenic differentiation after 4 days of incubation. (**D**) *OCN* and (**E**) *OPN* were evaluated to assess the osteogenic differentiation after 14 days of incubation. (**F**) *Schematic illustration of hedgehog signaling pathway in the presence or absence of sterosomes.* (**G**) *PTCH* and (**H**) *Gli1* were evaluated after 24 hours of incubation. The results are normalized by expression level of *GAPDH* and then presented as relative ratios to the PLGA scaffold. *ALP, Runx2, Col1a1, OCN,* and *OPN* are osteogenic markers. *Gli1* and *PTCH* are hedgehog signaling pathway markers. Data were presented as means \pm SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, one-way ANOVA with Tukey's post hoc test.

bone matrix proteins (Fig. 5, D and E), was significantly increased for the sterosome with PUR compared to the sterosome alone and negative control groups on day 14. Collectively, these results suggest that our hybrid scaffolds (sterosome alone or with PUR) exert a very positive effect on the osteogenic differentiation of MSCs.

Hedgehog signaling pathway is known to play important roles in vertebrate embryonic development and tumorigenesis (35, 36). In the absence of hedgehog ligand, patched (PTCH) suppresses high expression and activity of smoothened (37). The binding of smoothened with its agonist such as OHC and PUR enables the activation of downstream Gli1 and PTCH transcription factors (Fig. 5F) (38, 39). We assessed the ability of sterosomes to promote smoothened activity by analyzing the transcription of hedgehog target genes, including PTCH and Gli1. qRT-PCR results showed that the expression levels of PTCH and Gli1 were significantly up-regulated in the hybrid scaffolds (Fig. 5, G and H). Sterosome with PUR had 1.4- and 2.1-fold greater level than sterosome alone in promoting gene expression of PTCH and Gli1, respectively. In addition to the inherent ability of sterosome to regulate hedgehog signaling, these findings highlight synergistically enhanced hedgehog signaling with PUR loading. PUR or OHC is reported to act at the smoothened transmembrane domains or in its N-terminal tail, respectively; thus, the two smoothened agonists are not competing for the same binding site (21, 22).

Bone regeneration capacity of sterosome-immobilized hybrid scaffolds in vivo

We demonstrated the bone regeneration capacity of the hybrid scaffolds in a critical-sized mouse calvarial defect model. We have evaluated four types of scaffolds in parallel for comparison, including the sterosome-immobilized scaffolds, and control scaffolds (PDAcoated scaffold and PLGA scaffold). All of the mice were found to remain in good health and did not show any defect complications throughout the 6 weeks of experiment. On Fig. 6, the micro-computed tomography (microCT) scanning images and their corresponding analysis were performed after 6 weeks of implantation to evaluate the quantity of the regenerated bone. The results showed that a substantial regeneration of new bone was observed after implantation of sterosome-immobilized scaffold with or without PUR loading compared to the control groups. As shown in Fig. 6A, the PURcontaining sterosome-immobilized scaffold led to the most effective bone healing. When the normalization was carried out on the original 3-mm defect area, the bone fractional areas of the PUR-containing sterosome scaffold (44.7%) and sterosome scaffold (31.1%) were remarkably larger than the control groups (11.1% for PLGA scaffold and 11.0% for PDA-coated scaffold) after 6 weeks (Fig. 6B).

Quantitative 3D analyses of microCT images were performed by selecting a 3-mm-diameter cylindrical volume of interest to determine bone volume density [bone volume (BV)/tissue volume (TV)], bone mineral density, and trabecular number. In Fig. 6C, the hybrid scaffolds resulted in a significant increase (P < 0.01 for the sterosome alone group and P < 0.001 for the PUR-containing sterosome group) in bone volume density compared to the controls. Moreover, even higher bone volume density was observed in the PUR-containing sterosome group (P < 0.05 compared to the sterosome alone group). Consistent with previous measurement, bone mineral density of the PUR-containing group was significantly higher than all the other groups, although the sterosome alone group showed remarkable increase in bone mineral density (Fig. 6D). In addition, trabecular number analysis (Fig. 6E) showed that the regenerated bone in the

PUR-containing sterosome scaffold was noticeably dense with the highest trabecular number compared to PLGA (P < 0.001), PDA-coated (P < 0.001), and sterosome-immobilized scaffold (P < 0.05). The results mentioned above indicate that the hybrid scaffold with PUR-containing sterosome exhibits best performance for ossification among all groups, and the sterosome-immobilized scaffold combined with osteoinductive compound, PUR, provides a promising strategy for bone regeneration.

Histological observation was also performed to verify the bone regeneration using hematoxylin and eosin (H&E) and Masson's trichrome staining (Fig. 6F). In sterosome-immobilized hybrid scaffolds, new bone was clearly visible and corresponded to a lamellar bone formation, whereas a precipitous cutoff was observed in the defects of control groups (PLGA- and PDA-coated scaffold) between the edge of the parietal bone and adjacent fibrous tissue with minimal bone regeneration. The implantation of the PUR-containing sterosome scaffold resulted in the most prominent new bone formation throughout the implantation site.

We finally conducted immunohistochemical staining of osteogenic markers, including Runx2 and OCN (fig. S8). In the control scaffolds, low levels of Runx2 and OCN were observed within the fibrous tissue associated with nonhealing defect sites. On the other hand, strong expression of Runx2 and OCN was observed for both sterosomeimmobilized scaffolds. In addition, the sterosome-immobilized scaffold with PUR loading demonstrated highly intense staining of Runx2 and OCN in the osteoblasts and osteocytes compared to the scaffold with sterosome alone, supporting the idea that sterosome and encapsulated PUR exhibits combinatory performance for enhanced bone regeneration.

Our previous reports demonstrated the successful use of similar PLGA scaffolds in various defective bone models, including femoral, cranial, and maxillofacial defects, and their mechanical property allowed easy handling and placing into the created defect (5, 8, 18, 19, 30). Moreover, we created custom scaffolds that can match patient's anatomic shapes by using medical images of patients in additive manufacturing, indicating its potential applications in clinical treatment (40). Further study will be performed to improve bone regeneration efficacy by delivering other small-molecule agonists of smoothened receptor with enhanced potency. Since the discovery of Hh-Ag1.1, the first smoothened agonist, several derivatives have been identified with enhanced activity and reduced toxicity, including Hh-Ag1.3 (also known as SAG) and Hh-Ag1.5 (EC = 1 nM). It is also possible to introduce osteoconductive materials (e.g., hydroxyapatite) onto scaffolds to enhance the recruitment of host cells into the implanted scaffold and better direct activity of progenitor cells for bone repair.

CONCLUSIONS

Together, the 3D hybrid scaffold immobilized with a liposomepackaged hedgehog small molecule (smoothened agonist sterosome) is successfully developed for bone repair. Sterosome not only shows its inherent osteogenic properties but also induces synergistic osteogenic differentiation of MSCs with PUR loaded in sterosome. Sterosome is successfully immobilized on the surface of porous 3D PLGA scaffold via a bioinspired PDA intermediate, which provides a biocompatible substrate for cell adhesion, survival, and proliferation. The created hybrid scaffold with the PUR-containing sterosome exhibits a favorable microenvironment for bone regeneration with



Fig. 6. In vivo bone regeneration after 6 weeks of implantation of smoothened agonist sterosome-immobilized hybrid scaffolds on mouse calvarial defect. (A) Representative 3D reconstructed images of calvarial defect at 6 weeks after implantation taken a superficial view. Quantified parameters of the regenerated bone including (B) bone fractional area, (C) bone volume density (BV/TV), (D) bone mineral density, and (E) trabecular number taken at the 3-mm-diameter cylindrical defect. All data were presented as means \pm SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, one-way ANOVA with Tukey's post hoc test. (F) H&E-stained (top) and Masson's trichrome-stained (bottom) histologic sections of calvarial decalcified sections after 6 weeks of implantation. S, remained scaffold; CT, connective tissue; NB, new bone. Scale bars, 100 µm.

the following properties: controlled bioactive molecule delivery, enhanced nanocarrier-cell interactions, and synergistic up-regulation of hedgehog signaling. These results demonstrate the potential of next-generation liposome-packaged small molecule and hybrid scaffold as a bone graft substitute for faster and more efficacious bone repair.

MATERIALS AND METHODS

Preparation of smoothened agonist sterosomes

Sterosome was prepared using the modified thin-film hydration technique. SA (2.2 mg, 0.008 mM; Sigma-Aldrich, St. Louis, MO) and OHC (5.0 mg, 0.012 mM; Tocris Bioscience, Bristol, UK) powders in the presence or absence of PUR (0.36 mg, 0.7μ M; Tocris Bioscience,

Bristol, UK) or nile red (0.36 mg, 1.1 µM; Sigma-Aldrich, St. Louis, MO) were mixed and dissolved in benzene/methanol (9:1 volume ratio). Lipid solution was kept under vacuum at 60°C until a thin lipid layer was observed, and all organic solvents were evaporated. The dried thin lipid film was hydrated in a tris buffer (2.4 ml), containing 50 mM tris and 140 mM NaCl at pH 7.4. The suspensions underwent five freeze/thaw cycles (liquid nitrogen for 2 min to a 70°C water bath for 10 min) with vortexing between successive cycles for good hydration. Afterward, the pH was readjusted, if necessary, with diluted HCl or NaOH solution. Sterosome was prepared by sonication using a high-power 500-W sonic dismembrator (20 s on, 5 s off, 20% amplitude, 25 W cm^{-2} of power intensity) for 20 min. The size and the ζ -potential of the prepared sterosomes were measured on a Malvern Zetasizer Nano ZS instrument. The 9:1 molar ratio of SA to FITC (Alfa Aesar, Haverhill, MA)-labeled SA (FITC-SA) was used to formulate FITC-labeled sterosome using a similar sterosome preparation procedure with tris buffer.

Fabrication of sterosome-immobilized hybrid scaffolds

The PLGA scaffold was immersed in a dopamine hydrochloride solution (5 g liter⁻¹ in 10 mM tris-HCl, pH 8.5) and incubated on a shaker for 1 hour at room temperature. Because the polydopamine (PDA) not only is deposited onto surfaces but also forms PDA aggregates in solution, the solution was replaced every 30 min with fresh solution to minimize the precipitation and adsorption of PDA aggregates formed in solution and to ensure continuous growth. The PDA-coated scaffold was then rinsed three times with 10 mM phosphate-buffered saline (PBS) (pH 7.4) to remove unattached dopamine. For sterosome immobilization, the PDA-coated scaffold was immersed in tris buffer (10 mM, pH 8.0), which contained sterosome. After 1 hour of incubation at room temperature with gentle shaking, the hybrid scaffold was then rinsed three times with 10 mM tris-HCl (pH 8.5) to remove unattached sterosome. Last, the hybrid scaffold was immersed in a dopamine hydrochloride solution (5 g liter⁻¹ in 10 mM tris-HCl, pH 8.5) and incubated on a shaker for 1 hour at room temperature and washed with 10 mM PBS (pH 7.4) three times. XPS spectra were obtained with AXIS Ultra DLD (Kratos Analytical Ltd.) with a monochromatic Al (K α) radiation source. Data analysis was performed using the CasaXPS software (version 2.3.19). The morphology of scaffolds was investigated by scanning electron microscopy (Nova NanoSEM 230; FEI, Hillsboro, OR). The scaffolds were mounted on aluminum stubs and captured under lowvacuum mode.

Quantification of sterosome immobilization

The nile red–encapsulated smoothened agonist sterosome (0.1 or 1.0 g liter^{-1}) was prepared in tris buffer (10 mM, pH 8.0) and incubated with the PDA-coated scaffold for predetermined time at room temperature with gentle shaking. The resulting scaffold was then rinsed three times with 10 mM tris-HCl (pH 8.5). Afterward, nile red–loaded sterosome from the hybrid scaffolds was extracted in a 10 mM PBS (pH 7.4) buffer containing 1.0% Tween 20 for 3 hours. The fluorescence of nile red was measured at 530 nm (excitation) and 590 nm (emission).

Release profile of PUR from hybrid scaffold

The scaffolds were immersed in 0.5 ml of 10 mM PBS (pH 7.4). The scaffolds were then incubated at 70 rpm at 37°C. At predetermined time, the medium was exchanged with fresh buffer. PUR was measured at an absorbance of 315 nm using ultraviolet spectrophotometer.

Cell adherence and proliferation on hybrid scaffold

The mouse BMSC (D1 cell) was supplied from the American Type Culture Collection (Manassas, VA). To quantify the cell proliferation on the scaffold, the BMSC/scaffold constructs were analyzed with the Alamar Blue Assay Kit (Life Technologies) at days 1, 4, and 7. Briefly, the BMSC/scaffold constructs were rinsed with PBS and incubated with sterilized 10% Alamar Blue solution for 3 hours. Alamar Blue fluorescence was measured at 535 nm (excitation) and 585 nm (emission). To visualize the cell proliferation on the scaffold, BMSCs were seeded onto the scaffold at the final concentration of 2×10^6 cells/ml, stained with a Live/Dead staining kit (Life Technologies), and observed under a confocal laser scanning microscope (Leica TCS-SP5 AOBS, Buffalo, IL) at day 7.

Interaction between cell and sterosome on hybrid scaffold

BMSCs were seeded onto the scaffold with FITC-labeled, nile redencapsulated sterosome at the final concentration of 2×10^6 cells/ml. After 4 or 24 hours, the cells were treated with Hoechst 33342 to stain the nuclei for 1 hour. After incubation, the medium was removed, and the cells were rinsed twice with Dulbecco's PBS. The cells were observed on a confocal laser scanning microscope (LSM 880; Zeiss, Germany).

Staining and quantification of ALP

For 2D assay, BMSCs were cultured in 24-well plates with growth medium [Dulbecco's modified Eagle's medium, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin]. After 100% confluence, the medium was replaced with an osteogenic medium (OM) containing 10% FBS, 1%, penicillin-streptomycin, 10 mM β -glycerophosphate, L-ascorbic acid (50 g liter⁻¹), 100 nM dexamethasone, and sterosomes at various concentrations (0, 1, 5, or 10 μ M OHC). After 4 days of culture, cells were fixed in 10% formalin, rinsed with 10 mM PBS (pH 7.4), and incubated in a solution containing nitro blue tetrazolium (Sigma-Aldrich, St. Louis, MO) and 5-bromo-4-chloro-3-indoxylphosphate (Sigma-Aldrich, St. Louis, MO) stock solutions in ALP buffer [100 mM tris (pH 8.5), 50 mM MgCl₂, and 100 mM NaCl] for 1 hour. The stained samples were visualized with an Olympus IX 71 microscope (Center Valley, PA).

For 3D assay, BMSCs were seeded onto the scaffold at the final concentration of 2×10^6 cells/ml and cultured for 1 day. The medium was replaced with an OM. After 4 days of culture, cells were fixed in 10% formalin, rinsed with PBS, and incubated in a solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indoxylphosphate stock solutions in ALP buffer for 1 hour. The stained scaffolds were visualized with an Olympus SZX 16 stereomicroscope. For the colorimetric measurement of ALP activity, cells were lysed in lysis buffer (0.1% Tween 20 in 10 mM PBS, pH 7.4) and measured at the absorbance of 405 nm by using ρ -nitrophenol phosphate substrate. Measurements were normalized to total protein content evaluated by the bicinchoninic assay (Thermo Fisher Scientific, Rockford. IL).

Staining and quantification of mineralization

Mineralized extracellular matrix was evaluated by using Alizarin red S staining. At 14 days after incubation, the BMSCs were immersed in 10% formalin for 15 min and rinsed three times with 10 mM PBS (pH 7.4). The fixed cells were incubated in 1% Alizarin red S solution for 5 min. The stained samples were observed with an Olympus

SZX 16 stereomicroscope. The quantification of mineralized extracellular matrix was performed with acetic acid extraction and neutralization with ammonium hydroxide. The colorimetric measurement was carried out at 405 nm. The statistical analyses were performed on the measured data. The data are presented as fold change normalized to the nontreated (OM or PLGA scaffold) group.

RNA extraction and qRT-PCR

Total RNA was extracted from cells cultured with the scaffold using TRIzol reagent (Life Technologies) and RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The concentration of purified total RNA was determined spectrophotometrically using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The ratio of absorbance at 260 and 280 nm, which represents the purity of RNA, was 1.5 to 1.8. Total RNA (0.5 µg) was reverse-transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA), and then 1 µl of complementary DNA (cDNA) was used for each reaction in the presence of 20 µl of LightCycler 480 SYBR Green Master Mix (Roche, Indianapolis, IN). RT-PCR was performed using LightCycler 480 PCR (Roche, Indianapolis, IN) under the following PCR conditions: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 58°C for 45 s. Gene expression was calculated using the C_T value, and fold changes in expression were determined with the $2^{-\Delta\Delta CT}$ method. The relative gene levels were calculated with reference to the housekeeping gene (GAPDH). The values were normalized to the average of PLGA scaffold values ($\Delta C_{\rm T}$). The specific forward and reverse primer sequences were as follows: ALP, 5'-GTTGCCAAGCTGGGAAGAACAC-3' (forward) and 5'-CCC-ACCCCGCTATTCCAAAC-3' (reverse); Runx2, 5'-CGGTCTC-CTTCAGGATGGT-3'(forward) and 5'-GCTTCCGTCAGCGT-CAACA-3' (reverse); Col1a1, 5'-AACCCGAGGTATGCTTGATCT-3' (forward) and 5'-CCAGTTCTTCATTGCATTGC-3' (reverse); OCN, 5'-GGGAGACAACAGGGAGGAAAC-3' (forward) and 5'-CAG-GCTTCCTGCCAGTACCT-3' (reverse); OPN, 5'-CTCCTGGCT-GAATTCTGAGG-3' (forward) and 5'-TGCCAGAATCAGTCAC-TTTCA-3' (reverse); PTCH, 5'-AAACGGCTACCCTTTCCTGTTC-3' (forward) and 5'-GACAATGATTCCAGCAGTCCAAG-3' (reverse); Gli1, 5'-ACACATTACCAAGAAGCACCG-3' (forward) and 5'-CAG-CTGGTTTTCCCCTTTAAC-3' (reverse); GAPDH, 5'-AGGTCG-GTGTGAACGGATTTG-3' (forward) and 5'-TGTAGACCATG-TAGTTGAGGTCA-3' (reverse).

Calvarial defect model

All surgical experiments strictly abided by the guidelines of the Chancellor's Animal Research Committee (ARC) at the University of California, Los Angeles. All full-thickness craniotomy defects (3 mm) were drilled on 8- to 10-week-old male C57BL/6 mice on the parietal bone. Each defect was cleaned and then implanted with the scaffold. Following the surgery, all animals were monitored until they regained sternal recumbency and transported to the vivarium for postoperative care. Buprenorphine was injected subcutaneously in all animals with a concentration of 0.1 mg kg⁻¹ for 3 days for pain management. All animals had free access to water including trimethoprim/sulfamethoxazole to avoid infection for 7 days.

MicroCT scanning and analysis

The harvested calvarial tissues from all experimental animals 6 weeks after implantation were immersed in 4% paraformaldehyde solution

at 25°C for 48 hours under gentle shaking. High-resolution microCT images were acquired for all the samples on SkyScan 1172 (Kontich, Belgium) with 0.5-mm Al filtration [184 μ A, 57 kilovolt peak (kVp)]. The exposure time was 190 ms, and 475 projections were acquired at the angle of 190°. The resolution was 10 μ m for both of the pixel size of projections and the voxel size of the reconstructed dataset. Visualization and reconstruction of the data were carried out with the OsiriX MD imaging software. The new bone surface area of all samples was determined using ImageJ [National Institutes of Health (NIH), Bethesda, MD] and normalized to the original defect surface area (3 mm in diameter). Bone volume density (BV/TV, %), trabecular number (mm⁻¹), and bone mineral density (%) were measured with the SkyScan CT-Analyzer program (Bruker microCT).

Histological evaluation

After microCT scanning, all fixed tissues were incubated in 10% EDTA solution for 7 days with gentle shaking, with one time refresh of the EDTA solution on day 3. After decalcification, samples were embedded into paraffin and cut at a thickness of 5 µm. H&E and Masson's trichrome staining were performed to determine new bone formation. The blue color, indicative of regenerated or mature bone, was observed with an Olympus IX71 microscope (Tokyo, Japan). The size of the remaining defect was determined with ImageJ, compared to the blank control group. Additional sections underwent immunohistochemical analysis. The deparaffinized sections were processed with citric acid for antigen retrieval and thereafter incubated with the primary antibody of Runx2 (M-70, sc-10758; Santa Cruz Biotechnology) and OCN (FL-95, sc-30045; Santa Cruz Biotechnology) and were detected by the HRP/DAB Detection IHC Kit (ab64261, Abcam). The sections were further counterstained with Mayer's hematoxylin.

Statistical analysis

Statistical analysis was performed using SigmaPlot 12.5 software. Data were presented as mean \pm SD for all results. Statistical significance was determined by two-tailed *t* test or one-way analysis of variance (ANOVA) with Tukey. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 were considered significant.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/ content/full/6/17/eaaz7822/DC1

View/request a protocol for this paper from *Bio-protocol*.

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Acknowledgments: We would like to thank the University of California Los Angeles Broad Stem Cell Research Center (UCLA BSCRC) Microscopy Core. Funding: This work was supported by grants from the NIH (R01 DE027332), the Department of Defense (W81XWH-18-1-0337), and the MTF Biologics. Author contributions: C.-S.L. and M.L. conceived the ideas for experimental designs, analyzed the data, and wrote the paper. C.-S.L., S.K., J.F., and H.S.H. conducted all the experiments and helped with paper preparation. T.A. performed the animal surgery. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 9 October 2019 Accepted 28 January 2020 Published 22 April 2020 10.1126/sciadv.aaz7822

Citation: C.-S. Lee, S. Kim, J. Fan, H. S. Hwang, T. Aghaloo, M. Lee, Smoothened agonist sterosome immobilized hybrid scaffold for bone regeneration. *Sci. Adv.* **6**, eaaz7822 (2020).