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TITLE: Development of Smoothened Agonist Nonphospholipid Liposomal Nanoparticles for Bone Repair

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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14. ABSTRACT
Non-healing bone injuries represent a source of morbidity for combat casualties and military veterans, exacting both a devastating individual toll on the lives affected as well as an enormous socioeconomic burden. The manipulation of Hedgehog (Hh) signaling is a promising alternative for improved bone regeneration. In particular, the Hh activating small molecule SAG targets bone and vascular formation to induce bone healing. The present study seeks to develop a nanoparticle packaged Hh small molecule for use as a widely applicable bone graft substitute. To achieve this, we developed a novel class of liposomes formulated with single-chain amphiphiles and high content of sterols (sterosomes), resulting in significantly increased nanoparticle stability compared to conventional phospholipid. The validity of sterosome nanoparticles, along with the addition of SAG, was ensured by hydrodynamic characterizations before progression to scaffold loading and *in vivo* application. SAG-loaded sterosome was consistently bioactive and its osteoinductive potential was verified. We then developed a strategy to immobilize sterosome onto the surface of poly (lactic-co-glycolic acid) scaffolds using dopamine intermediates to achieve controlled drug delivery in the defect site. The sterosome-immobilized scaffolds significantly improved osteogenesis through activation of Hh signaling pathway. This study suggests a useful therapeutic biomaterial design for clinical bone repair.

15. SUBJECT TERMS
Non-phospholipid liposome, scaffold, smoothened agonist, hedgehog signaling, bone regeneration

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1. INTRODUCTION

Non-healing bone injuries represent a source of morbidity for combat casualties and military veterans, exacting both a devastating individual toll on the lives affected as well as an enormous socioeconomic burden. The manipulation of Hedgehog (Hh) signaling is a promising alternative for improved bone regeneration. Our research group has shown that Hh signaling diverts mesenchymal stem cells (MSC) toward a bone-forming fate and away from competing cell fates. Moreover, the Hh activating small molecule SAG targets bone and vascular formation to induce bone healing. In a coordinate research effort, we have shown that non-phospholipid liposomes composed of Stearylamine and Oxysterol (SA/Oxy) have intrinsic bone inducing capabilities, and are well designed to deliver the small molecule SAG to sites of bone injury. In aggregate, the present proposal seeks to develop a nanoparticle (NP) delivered small molecule for faster, safer, and more efficacious bone repair than currently available treatment strategies. Here, we will perform key preclinical safety and efficacy studies for clinical translation of a nanoparticle packaged Hh small molecule for use as a widely applicable bone graft substitute, to be accomplished in two specific aims.

2. KEYWORDS

Non-phospholipid liposome, scaffold, smoothened agonist, hedgehog signaling, bone regeneration

3. ACCOMPLISHMENTS

What were the major goals of the project?

The project contains two aims as stated in the SOW:

Aim 1: Optimize SAG-loaded liposomal nanoparticles for mouse calvarial defect repair.
Aim 2: Determine the safety of SAG-loaded liposomal nanoparticles for mouse calvarial defect repair.

These aims are composed of two subtasks:

Subtask 1: Osteoinductive scaffold fabrication and batch validation
Subtask 2: SA/Oxy NP production and batch validation

What was accomplished under these goals?

- Develop SAG-loaded Oxysterol (Oxy) liposomes and validate bioactivity

Oxy liposomes were prepared by self-assembly of SA and 20S-Oxy using thin film hydration technique. **Table 1** shows the hydrodynamic size, polydispersity index (PDI), ζ-potentials, and loading efficiency of the liposomes. The size distribution of Oxy liposomes was 142.5 ± 4.9 nm for Oxy liposomes and 145.4 ± 2.7 nm for SAG-loaded Oxy liposomes (SAG-Oxy liposome) with approximately 0.2 of PDI (**Figure 1A**). The ζ-potentials of both liposomes were highly positive, around 55-60 mV.

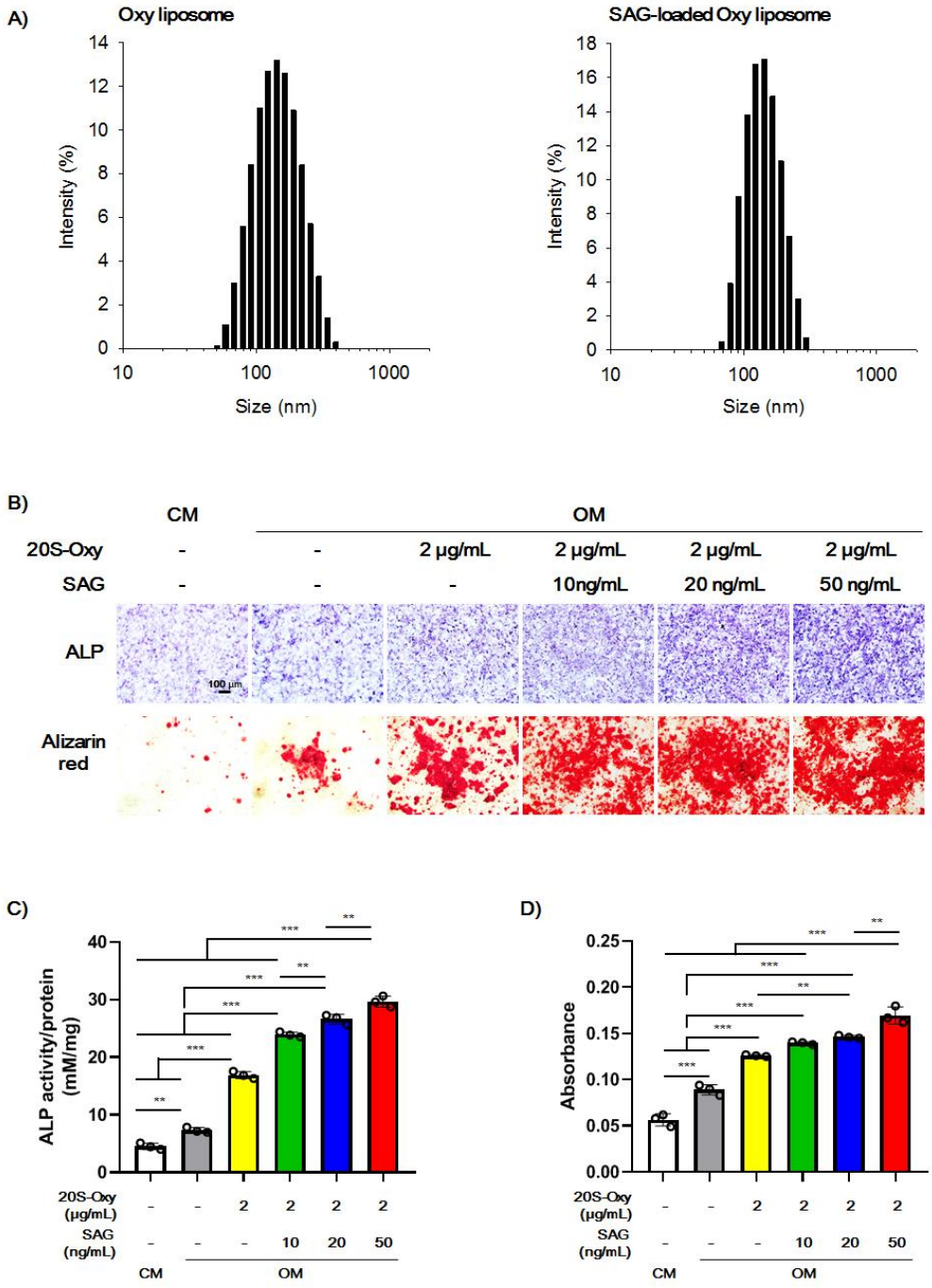


Figure 1. Characterization of Oxysterol (Oxy) liposomes. **A**) Size distributions of Oxy liposomes and Smoothened Agonist (SAG)-loaded Oxy liposomes. (**B**, upper line) Alkaline phosphatase (ALP) staining and **C**) colorimetric quantification of ALP activity at day 4 of osteogenic differentiation. (**B**, bottom line) Mineralization stained with Alizarin red S and **D**) colorimetric quantification of mineralization activity at day 14 of differentiation. Scale bars are 200 μm. All data were presented as means ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ as determined using a one-way ANOVA with Tukey's post hoc test. CM = Culture medium, OM = Osteogenic medium.

Table 1. Characterization of Oxy liposomes and SAG-loaded Oxy liposomes.

	Oxy liposome	**SAG-loaded Oxy liposome**
Appearance	Clear	Clear
Size (nm)	142.5 ± 4.9	145.4 ± 2.7
PDI	0.231 ± 0.042	0.154 ± 0.028
Zeta potential (mV)	60.3 ± 3.5	55.9 ± 1.2
Loading efficiency	-	52.6 ± 2.9 %

ALP, an early stage marker of osteogenic differentiation, was assessed at day 4 of differentiation. The Oxy liposomes without SAG-loading induced ALP expression compared to non-treated groups (**Figure 1B**, upper panels). The ALP staining synergistically intensified with SAG-loading and demonstrated a dose-dependent increase with ascending SAG content. A quantitative analysis of ALP expression confirmed this finding, with ascending SAG-loading concentrations leading to a dose-dependent 3.7- to 6.6-fold increase in comparison to culture medium (CM) (**Figure 1C**). Alizarin red S (ARS) staining was further carried out to determine matrix mineralization (**Figure 1B**, bottom panels). Similar to ALP expression, more intense mineral deposition was observed in Oxy liposome-treated groups. Furthermore, SAG-Oxy liposomes induced a dose dependent increase in mineralization. Photometric quantification of ARS confirmed these results (**Figure 1D**), with mineralization was increased up to 3.0-fold in SAG-Oxy liposome treatment groups.

Key outcomes and conclusions:

Hydrodynamic characterizations ensure the validity of Oxy liposomes, along with the addition of SAG as drug cargo, before progression to scaffold loading and *in vivo* application. SAG-loaded Oxy liposomes was consistently bioactive and its osteoinductive potential was verified by staining and colorimetric assay.

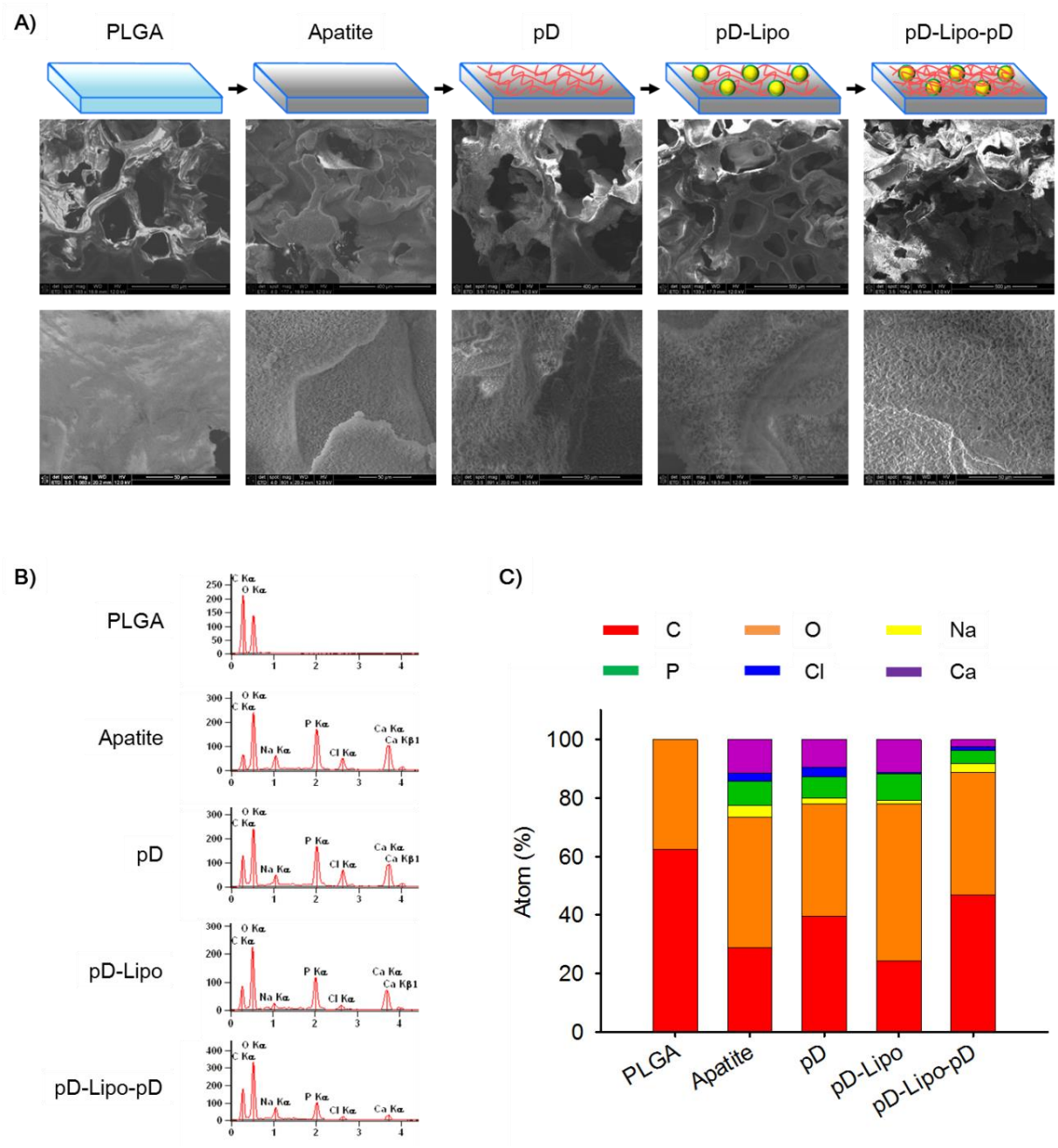
- Combine SAG-loaded Oxy liposome with 3D PLGA scaffolds

Oxy liposome-coated scaffolds were fabricated by a series of coating with polydopamine (pD) substrate and Oxy liposome as follows: Apatite-PLGA was coated by sequential incubation in pD solution, Oxy liposome solution, and pD solution. Scanning electron microscopy (SEM) was used to characterize the microstructure of scaffolds (**Figure 2A**). All the scaffolds showed the typical porous structure with highly interconnected about 200-300 μm pore size, whereas PLGA scaffold prior to any modification displayed a flat strut surface. Apatite-PLGA scaffolds upon immersion in stimulated body fluid (SBF) were fabricated with nubby textured structures on the surface along the strut. After the serial functionalization with pD, and Oxy liposome on the scaffold, there was no obvious change in microstructure morphology of PLGA and Apatite-PLGA.

The scaffolds were further analyzed by energy dispersive X-ray spectroscopy (EDX) to uncover the engineered surfaces (**Figure 2B**). The spectra showed that the chemical composition of the surfaces was dramatically altered by the apatite-coating, resulting in new peaks for calcium (Ca) and phosphorus (P), which was close to the theoretical Ca/P ratio of HA (**Figure 2C** and **Table 2)**. After

functionalization with pD and Oxy liposome, the atomic composition of both Ca and P was subsequently decreased due to the cover with the carbon-rich layers of pD and Oxy liposomes.

Next, we evaluated the pD-mediated functionalizing efficacy of Oxy liposomes onto the scaffold surface using nile red-loaded Oxy liposomes, of which nile red was used as a fluorescence model drug (**Figure 2D**). There were intense red fluorescence signals on pD-coated Apatite-PLGA scaffolds after coating with nile red-loaded Oxy liposomes. However, the signals were extremely low on Apatite-PLGA scaffolds without a pD layer. Furthermore, the coating amount of Oxy liposome could be managed by controlling reaction time and concentration of the liposome (**Figure 2E**).



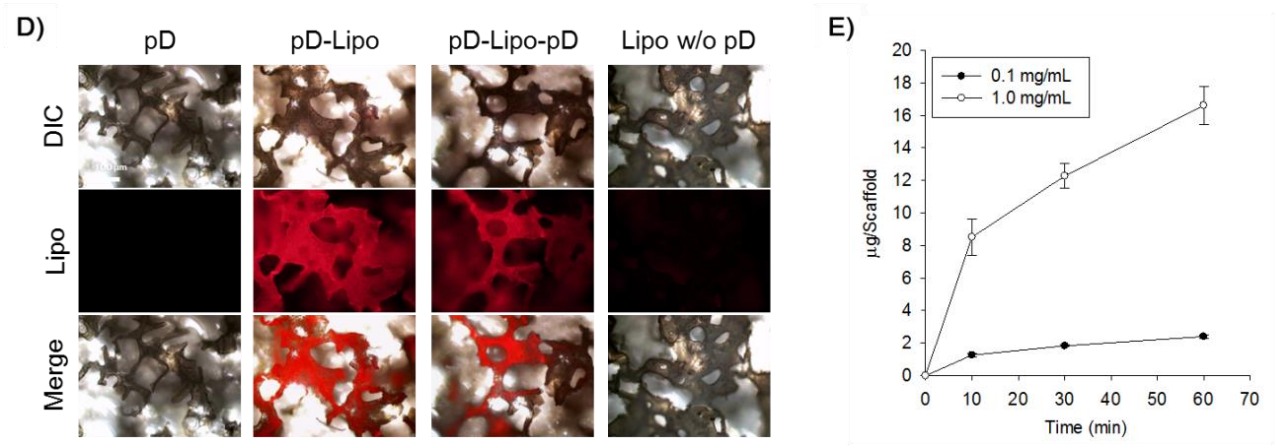


Figure 2. Characterization of PLGA scaffolds with Oxysterol (Oxy) liposomes. **A**) Scanning electron microscopy (SEM) images and **B**) EDX spectra of PLGA, Apatite-coated PLGA (Apatite-PLGA), pD-coated Apatite-PLGA, pD-Lipo-coated Apatite-PLGA, and pD-Lipo-pD-coated Apatite-PLGA scaffolds. **C**) Elemental composition of the scaffold surface, determined by EDX. **D**) Fluorescence microscopy images of Oxy liposome on Apatite-PLGA scaffolds in the presence or absence of pD layer. Nile red was used as a model cargo. **E**) Quantification of Oxy liposomes on surface of pD-coated scaffold for 1 h. Nile red-loaded Oxy liposome was used as a model liposome. Data were presented as mean ± SD. PLGA = Poly(lactic-co-glycolic acid), Lipo = Oxy liposome, pD = Polydopamine.

Table 2. Elemental composition of the scaffold surface determined by energy dispersive X-ray spectrometry. PLGA = Poly(lactic-co-glycolic acid), Apatite-PLGA = Apatite-coated PLGA, pD = polydopamine, Lipo = Oxy liposome.

Atom %	PLGA	Apatite-PLGA	pD-coated Apatite-PLGA	pD-Lipo-coated Apatite-PLGA	pD-Lipo-pD-coated Apatite-PLGA
C	62.54±1.60	28.81±1.15	39.51±1.59	34.12±1.69	46.80±1.70
O	37.46±1.58	44.76±1.21	38.37±1.12	46.59±1.37	41.95±1.17
Na	-	3.81±0.29	2.07±0.25	1.60±0.17	2.97±0.29
P	-	8.47±0.29	7.24±0.27	6.82±0.32	4.64±0.24
Cl	-	2.61±0.24	3.32±0.24	1.26±0.13	1.17±0.11
Ca	-	11.54±0.31	9.50±0.27	9.62±0.52	2.47±0.26

Key outcomes and conclusions:

We successfully immobilized Oxy liposomes on apatite-coated 3D PLGA scaffolds using bio-inspired polydopamine adhesives to ensure favorable microenvironments for cell growth and local therapeutic delivery. The loading capacity of Oxy liposome was controlled by modulating the reaction time and concentration of liposomes added.

- Evaluate bioactivity of liposome-immobilized scaffolds

To evaluate the biocompatibility of the Oxy liposome-engineered scaffolds, we seeded mouse BMSCs onto the 3D porous scaffolds with various surfaces and allowed them to adhere for 4 h, followed by further incubation in culture medium for 7 days. No toxic effect of the pD-functionalization was observed as evidenced by consistent metabolic activity in Alamar Blue assay (**Figure 3A**) and high viability with Live/Dead staining (**Figure 3B**). The metabolic activity also showed that the seeded cells proliferated with no significant differences over time in all groups except for Oxy liposome-coated scaffolds without additional pD-coating. Even though the toxicity of Oxy liposome-coated scaffold without additional pD-coating was detected in both assay and staining, the cytocompatibility was recovered and the cells demonstrated adherence with the additional pD layer. Live/dead staining after 7 day culture demonstrated that the seeded cells were viable and uniformly adherent to the functionalized scaffolds, with the exception of Oxy liposome-coated scaffolds without additional pD-coating.

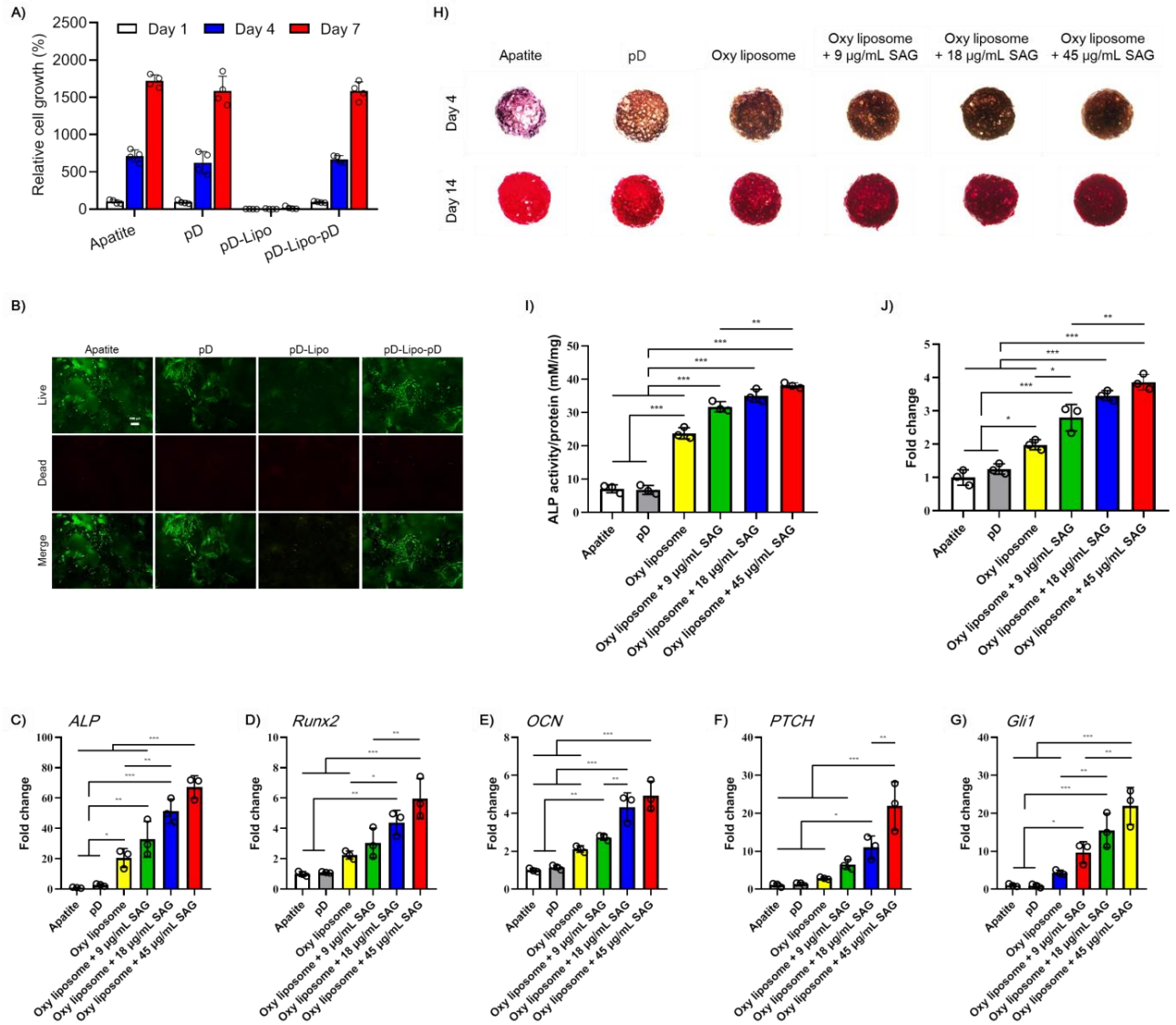


Figure 3. Cell adherence, proliferation, and bioactivity evaluations of SAG-loaded Oxysterol (Oxy) liposome-coated scaffolds. **A**) *In vitro* cell proliferation after 1, 4, and 7 days. The value was normalized by Apatite-PLGA scaffold of day 1. **B**) Representative fluorescence images of BMSCs stained with calcein AM (live cells, green fluorescence) and ethidium homodimer (dead cells, red fluorescence), day 7. Scale bar is 200 μm. **(C-G)** Gene expression related to osteogenesis and Hh signaling pathway. **C**) *ALP*, **D**) *Runx2*, **E**) *OCN*, **F**) *PTCH*, and **G**) *Gli1* were evaluated after 7 d incubation. **H**, top line) ALP staining and **I**) colorimetric quantification of ALP activity at day 4. **H**, bottom line) Mineralization stained with Alizarin red S and **J**) colorimetric quantification of mineralization activity at day 14. All data were presented as means ± SD. $^{**}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as determined using a one-way ANOVA with Tukey's post hoc test. ALP = Alkaline phosphatase, Runx2 = Runt-related transcription factor 2, OCN = Osteocalcin, PTCH = Protein patched homolog 1, Gli1 = GLI Family Zinc Finger 1.

Following the investigation of biocompatibility on the scaffolds, we turned to evaluate the osteogenic capacity and the regulation of Hh signaling of Oxy liposome-coated scaffolds toward BMSCs at the molecular level. The quantitative real-time polymerase chain reaction (qRT-PCR) result revealed that the expression levels of osteogenic markers including *ALP*, *Runx2*, and *OCN* in cells seeded on the Oxy liposome-coated scaffolds were upregulated by 19.8-, 2.2-, and 2.1-fold, respectively, compared with those in cells seeded in the Apatite-PLGA scaffolds without Oxy liposomes (**Figure 3C**, **D**, and **E**). In particular, the scaffolds with SAG-loading led to much greater influence on the expression levels of these osteogenic genes compared with corresponding levels in the other groups (Apatite-, pD, and Oxy liposome-coated scaffolds). The scaffolds with the highest SAG contents (Oxy liposome +45 ug/mL SAG) showed increased expressions of all osteogenic markers.

Next, we assessed Hh pathway signaling activation in cells seeded on the scaffolds by qRT-PCR (**Figure 3F** and **G**). The Hh pathway markers, *Ptch1* and *Gli1*, were upregulated by Oxy liposome coating on the scaffolds, although there are no significant differences. The levels of these genes expression were significantly increased among SAG-Oxy liposomes treated scaffolds, and showed a dose-dependent increase with SAG content.

We also examined the osteoinductive effect of Oxy liposome-coated 3D porous scaffolds, when seeded with BMSC and cultured in osteogenic medium. Consistent with the results of qRT-PCR, more intense ALP staining was observed in Oxy liposome-coated scaffolds compared to the control groups at day 4 (**Figure 3H**, top panels). Moreover, the level of ALP staining was gradually increased with ascending content of SAG-loading. Quantitative analysis of ALP activity confirmed this impression, demonstrating a significant increase not only between the Oxy liposome-coated scaffolds and the control groups, but also between Oxy liposome- and SAG-loaded Oxy liposome-coated scaffolds (**Figure 3I**). ARS staining (**Figure 3H**, bottom panels) further analyzed the accumulation of mineralized matrix during osteogenic differentiation of BMSCs. The results showed that the deposition of mineralized matrix was markedly enhanced in Oxy liposome-coated scaffolds, and further intensified with SAG-loading into Oxy liposomes (**Figure 3J**).

Key outcomes and conclusions:
SAG-loaded Oxy liposomes were successfully integrated with PLGA scaffolds and induced a significant and dose-dependent increase in Hh-mediated osteogenic differentiation.

What opportunities for training and professional development has the project provided?

This project provided a number of opportunities for Dr. Chung-Sung Lee and Dr. Xiao Zhang, postdoctoral researchers, to learn various drug delivery techniques. They also acquired in-depth knowledge of signaling molecules and mechanisms involved in osteogenic differentiation. Novel findings from the project have been presented at the following conferences:

1. UCLA School of Dentistry Research Day, Los Angeles, California, March 4, 2020.

How were the results disseminated to communities of interest?

We have disseminated our novel findings by presenting our work and interacting with other investigators and leaders in the field at the following conferences:

1. Lee CS, Kim S, Fan J, Hwang H, Aghaloo T, Lee M. Hedgehog agonist sterosome coated scaffold for bone repair UCLA School of Dentistry Research Day, Los Angeles, California, March 4, 2020.
2. Hsu C, Lee C, J, Sono T, Negiri S, Xu J, Lee M, James AW. Smoothened agonist-loaded non-phospholipid liposomes for bone regeneration. Military Health System Research Symposium (MHSRS), Kissimmee, FL (accepted for a presentation).

What do you plan to do during the next reporting period to accomplish the goals?

We demonstrated that Oxy liposomes, along with the addition of SAG as drug cargo, enhanced Hh signaling and osteogenic differentiation of MSCs. During the next year, we will continue to evaluate *in vivo* efficacy of SAG-loaded liposomal nanoparticles in a critical size calvarial defect in collaboration with JHU.

We successfully immobilized SAG-loaded Oxy liposomes onto the surface of PLGA scaffolds using a mussel-inspired polydopamine intermediate. Although SAG-loaded liposomes seem to improve osteogenesis, Oxy liposomes were found to be cytotoxic at higher concentration due to its strong cationic nature. During the next year, we will continue to develop a nanocarrier system to deliver SAG in a more efficient and safer manner. Recently, cell-derived exosomes gained considerable interest as alternative promising carriers due to its intrinsic cell targeting properties, intracellular transport and nearly non-immunogenic responses. Moreover, the surface of delivery vehicles could be modified with bone-binding moieties to target bone tissue and stay concentrated in the defective sites while minimizing off-target effects.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

This study developed a novel class of liposomes composed of non-phospholipid molecules formulated with single-chain amphiphiles and high content of sterols (sterosomes). The high sterol

content in our sterosomes induces well-ordered lipid bilayer chains with very limited permeability and significantly increased nanoparticle stability compared to conventional phospholipid. This system can be advantageous for delivery of small molecular drugs or other therapeutic genes. Liposomes are generally made from pharmacologically inactive substances. Oxysterols used in this study are interesting sterol molecules for applications targeting damaged bone treatment. Including oxysterol into our liposomal formulation not only increased nanoparticle stability but also stimulated cells to develop into bone-forming cells. We combined this breakthrough to develop a hybrid scaffold as a bone graft substitute product by covalently immobilizing drug-loaded liposomes onto three dimensional scaffolds via a bio-inspired polydopamine intermediate without complicated chemical modification. This is the first demonstration of a hedgehog agonist bone graft device for faster and more efficacious bone repair. The additional knowledge gained from this study may suggest nanocarrier design strategies loading bioactive agents into functional non-phospholipid bilayers to improve clinical efficacy of current therapeutic agents.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

6. PRODUCTS

- **Publications, conference papers, and presentations**

Journal publications.

1. Lee CS, Kim S, Fan J, Hwang HS, Aghaloo T, Lee M. Smoothened Agonist Sterosome Immobilized Hybrid Scaffold for Bone Regeneration. Science Advances 6(17):eaaz7822, 2020. Federal support acknowledged.

2. Lee CS, Hsu CY, Xu J, Sono T, Lee M, James A. Smoothened agonist-loaded non-phospholipid liposomes for bone regeneration. Under review. Federal support acknowledged

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

1. Lee CS, Kim S, Fan J, Hwang H, Aghaloo T, Lee M. Hedgehog agonist sterosome coated scaffold for bone repair UCLA School of Dentistry Research Day, Los Angeles, California, March 4, 2020.
2. Hsu C, Lee C, J, Sono T, Negiri S, Xu J, Lee M, James AW. Smoothened agonist-loaded non-phospholipid liposomes for bone regeneration. Military Health System Research Symposium (MHSRS), Kissimmee, FL (accepted for a presentation).

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Min Lee
Project Role:	PI
Researcher Identifier	orcid.org/0000-0003-2813-2091
Nearest person month worked:	2
Contribution to Project:	Dr. Lee is responsible for the overall design and conduct of the project, oversight of all research activities, budget management, publication writing and scientific report preparation.
Funding Support:	NIH/NIDCR

Name:	Chung-Sung Lee
Project Role:	Postdoctoral Researcher
Researcher Identifier	orcid.org/0000-0001-5813-6056
Nearest person month worked:	12
Contribution to Project:	Dr. Lee has fabricated SA/oxysterol drug delivery systems, and also performed material characterization and data analysis
Funding Support:	N/A

Name:	Xiao Zhang
Project Role:	Postdoctoral Researcher
Researcher Identifier	orcid.org/0000-0002-0242-1939
Nearest person month worked:	4
Contribution to Project:	Dr. Zhang has performed the daily research and participated in the bioactivity test of drug-loaded liposomes as well as molecular biology analyses.
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Johns Hopkins University

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All *in vivo* studies and analyses were performed in the partner organization. The partner's research group has expertise in orthopaedic injury and analysis in murine models, as well as the manipulation of Hedgehog signaling during bone repair.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Independent reports were submitted from the Initiating PI and Partnering PI.

QUAD CHARTS:

Not applicable

9. APPENDICES:

Award Chart

PR170115P1: Development of Smoothened Agonist Nonphospholipid Liposomal Nanoparticles for Bone Repair

PI: Min Lee, University of California, Los Angeles, CA
Topic Area: Nanomaterials for Bone Regeneration
Budget: $1,047,148
Mechanism: W81XWH-17-PRMRP-IIRA

0802, 0803, 0817, 0822

15 July 2018 – 14 July 2021

Study Goals:

To develop a nanoparticle delivered small molecule for faster, safer, and more efficacious bone repair than currently available treatment strategies

Specific Aims:

Aim 1: Optimize SAG-loaded liposomal nanoparticles for mouse calvarial defect repair
Aim 2: Determine the safety of SAG-loaded liposomal nanoparticles for mouse calvarial defect repair

Key Accomplishments and Outcomes:

Publications:

1. Lee CS, Kim S, Fan J, Hwang HS, Aghaloo T, Lee M. Smoothened Agonist Sterosome Immobilized Hybrid Scaffold for Bone Regeneration. *Science Advances* 6(17):eaaz7822, 2020.

Patents: N/A
Funding Obtained: N/A