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RESEARCH PAPER

Comparison of liposomal formulations incorporating BMP-2 peptide to induce bone tissue engineering

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ABSTRACT

Objective(s): Fabricating a biomimetic scaffold platform combined with controlled release of bioactive agents is a practical approach for bone tissue engineering. Controlled delivery of peptides and growth factors which play a significant role in osteogenesis is an important issue reducing the associated adverse effects and leading to cost-effectiveness.

Materials and Methods: We developed two liposomal formulations of bone morphogenetic protein-2 (BMP-2) peptide designated as F1 and F2 with controlled release properties. Due to high negative zeta potential of F1 formulation, the surface of the liposomes was decorated with positively charged BMP-2 peptide while the peptide was encapsulated in F2 formulation. Then, we evaluated the hypothesis that whether the electrostatically loaded peptide could act as a ligand and improve the cellular uptake and osteogenic differentiation of mesenchymal stem cells.

Results: Both formulations were less than 100 nm in size. The release study revealed that both formulations showed a sustained release pattern for 21 days. However, the cumulative releases were 60% and 40% in F1 and F2 formulations, respectively. Flow cytometry analysis indicated that cell internalization of F1 liposomes was more than the other formulation. In the next step, F1 and F2 formulations were attached covalently to our previously developed nanofibrous electrospun scaffold and biocompatibility and osteogenic differentiation of each formulation were studied. The results indicated that the proliferation of the cells seeded on F1 liposcaffold was significantly more than F2 liposcaffold at days 1 and 3. Furthermore, F1 liposcaffold showed superior osteogenic differentiation through measurement of alkaline phosphatase activity which could be due to the higher release pattern of F1 liposomes and their improved cellular uptake.

Conclusion: Our findings revealed that controlled release BMP-2 decorated liposomal formulations immobilized on nanofibrous electrospun scaffold platform could be a promising candidate for bone regeneration therapeutics and merits further investigation.

Keywords: *BMP-2 peptide, Bone regeneration, MSCs, Liposome, Osteogenic differentiation, Scaffold*

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INTRODUCTION

Bone tissue engineering has faced a big challenge while trying to mimic the complex extracellular structure of the bone matrix in terms of structure, composition and the controlled release of bioactive agents. Developing a 3D, porous nanofibrilar scaffold platform mimicking bone collagen fibrils is a fundamental requirement for bone regeneration. Among various platforms, electrospun nanofibers have gained dramatic interest as ECM-mimicking structures due to their nanofibrous framework and the capability to

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deposit inorganic materials such as hydroxyapatite (HAP). Besides, their porous 3D structure and high surface area improve cell adhesion and proliferation [1, 2].

Rather than designing the template and extracellular environment to support the cells, controlled local delivery of growth factors and bioactive agents play a pivotal role [3, 4]. Bone morphogenetic protein-2 (BMP-2) is a potent growth factor in inducing bone regeneration [5, 6]. Also its use in spinal fusion was clinically approved. However, due to its short half-life, large clinical doses are used which off-site release cause adverse effects such as bone resorption, inflammatory reactions and tumorigenesis [7]. Rather than adverse reactions, the use of high doses of the growth factor leads to high costs and studies claimed that clinical efficacy of BMP strongly depends on its carrier [8, 9]. So that there is a growing need for developing stable, controlled release formulations with easy scale up properties. Another important issue which should be considered is the cost-effectiveness of such formulations. Due to potent biological effects of BMP-2, studies are moving towards taking advantage of BMP-2 peptide [8, 10, 11]. Synthetic 20-mer BMP-2 peptide KIPKASSVPTELSAISTLYL corresponding to residues 73-92 of the knuckle epitope of BMP-2 showed highest alkaline phosphatase activity and osteogenic differentiation of mesenchymal stem cells (MSCs) [12, 13]. Besides, studies have shown that the outstanding effect of BMP-2 protein is largely due to the interaction of the knuckle epitope with type 2 BMP receptor [14]. Lee et al. fabricated 3D scaffolds using osteopromotive domains of BMP-2 (which selectively bind to BMP receptors) to promote osteoblastic differentiation. They developed peptide-hydrophobic alkyl chain amphiphiles which were self-assembled in the presence of calcium ions to produce osteopromotive scaffolds [15]. Zhou et al. produced mesoporous silica nanoparticles (MSNs) which were functionalized with BMP-2 peptide. They showed that rather than improving biocompatibility of the MSNs, intracellular uptake of the nanoparticles were also enhanced as the peptide induced ligand- receptor internalization [16].

To design an ECM-mimicking scaffold platform, we previously developed BMP-2 peptide encapsulated liposomes which were covalently attached to the nanofibrous scaffold. The scaffold was made of HA coated electrospun PLLA nanofibers [1]. Herein, to enhance the osteogenic differentiation of the MSCs, we tried to improve the uptake efficiency of the liposomes by decorating the surface of the liposomal formulation with BMP-2 peptide. We hypothesize the BMP-2 peptide can act as the targeting ligand to improve the entrance of the nanoparticles into the MSCs. So that two liposomal formulations (BMP-2 peptide encapsulated or surface decoration of BMP-2 peptide) were prepared, covalently conjugated to previously designed nanofibrous scaffold to compare osteogenic differentiation of each platform.

MATERIALS AND METHODS

Materials

Poly-L-lactic acid (Mn 10000, PDI< 1.1) was purchased from sigma Aldrich, Germany. The lipids; dipalmitoyl phosphatidylcholine (DPPC), hydrogenated soy phosphatidylcholine (HSPC), distearoyl phosphatidylglycerol (DSPG), distearoyl phosphatidylcholine (DSPC), cholesterol [3] and methoxy-polyethylene glycol (M_w 2000)-distearoylphosphatidylcholine ($mPEG_{2000}$–DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL). BMP-2 peptide, with the sequence of KIPKASSVPTELSAISTLYL and FAM labeled peptide were purchased from China peptides (China) with purity of 95.42%. The purification percent was evaluated using reverse-phase high-performance liquid chromatography on a Kromasil 100-5C18 column with a 30-44% acetonitrile gradient in 0.1% trifluoroacetic acid–water, at flow rate of 1 mL/min (9 min) and detection at wavelength of 220 nm.

Preparation and characterization of liposomes

Liposomal formulations were prepared using film hydration and extrusion method as previously described [1]. Briefly, two liposomal formulations (Formulation 1: DPPC/DSPG/Cholestrol/DSPE-mPEGmaleimide:55/10/35/1 %molar ratios and Formulation 2: DPPC/Cholestrol/DSPE-mPEGmaleimide:64/35/1 %molar ratios) were produced. The thin lipid layer was prepared and hydrated with BMP-2 solution in phosphate buffer saline (10 mM, pH 7.4) alone and PBS containing the peptide, respectively. After incubation at 4°C for 24 h, the formulations were extruded using 200, 100, 50 nm polycarbonate membranes and dialyzed against PBS (pH 7.4, 10 mM).

To electrostatically load the BMP-2, F2 liposomal formulation was incubated with BMP-2 solution for 30 min at room temperature. Then, it was centrifuged at 4000 rpm for 10 min to remove the residual BMP-2 peptide. The prepared liposomes were used for further studies.

The particle size and surface charge and poly dispersity index (PDI) were determined through dynamic light scattering using Malvern zeta sizer (Nano-ZS; Malvern, UK).

To determine the release properties of the formulations, liposomes were loaded with FAM-labeled BMP-2 peptide. One milliliter of each formulation was put into the dialysis cassettes (6-8 kDa, Spectrum Laboratories) which was dipped in a glass beaker containing PBS (10 mM, pH 7.4 supplemented with FBS 5% and penicillin–streptomycin 1%). The sealed sterile glass beaker was kept in shaker incubator (60 rpm, 37 °C). At determined time points, 1 ml of the dialysate was withdrawn and replaced with fresh medium. The study was performed for 21 days. Fluorescence intensity of the collected samples was determined at multiplate fluorescence reader (Synergy HT; λ ex 520 nm, λ em 490 nm).

Uptake study

Adipose-derived MSCs were isolated from human lipoaspirates and characterized by flow cytometry and differentiation to adipogenic, osteogenic and chondrogenic cells (data is available at doi: 10.1016/j.nano. 2018.06.001). MSCs were cultured in 6-well culture plates at density of 10^4 cells/well and cultured for 24 h at 37 °C, 5% CO_2. Thereafter, the cells were incubated with either the F1/F2 liposomal formulation (loaded with FAM-labeled BMP-2) for 6 h. After wards, the cells were detached, and washed with cold PBS for 3 times (centrifugation at 1300 rpm, 7 min). Then, cells were suspended in PBS containing FBS (2%, v/v) and the fluorescence intensity of the internalized nanoparticles were determined using flow cytometry. The results were analyzed by Flow Jo software.

Preparation and characterization of electrospun nanofibers

PLLA electrospun nanofibers were produced and functionalized as described previously [1]. Surface morphology of the nanofibers was studied using Field emission scanning electron microscope (FESEM, Tescan Mira3). The sample was coated with gold and the fiber diameter was analyzed using image J software. Water contact angle was measured through placing a water droplet on the surface of the mat using the automated contact angle measuring system (OCA15 plus; Dataphysics Instruments, Filderstadt, Germany).

Attachment of liposomes onto the surface of the scaffold

Thiol-functionalized fibers were conjugated to liposomes using thiol-maleimide chemistry. Liposomal formulations (1 ml) were incubated with fibers for 4 hours at room temperature. Thereafter, the scaffolds were washed 3 times with PBS solution. The conjugation efficiency was determined using maleimide quantification assay kit (Abcam, USA) using the following formula:

Proliferation study

To evaluate the effect of BMP-2 peptide released from liposcaffolds on viability and proliferation rate of the mesenchymal stem cells (MSCs), the amount of double-stranded DNA content of samples was quantified using Quant-it PicoGreen assay (Invitrogen, Molecular Probes, Oregon, USA) at determined time points (days 1 and 3). To this aim, MSCs were seeded on F1 liposomal formulation conjugated to scaffold (F1 liposcaffold), F2 liposcaffold, scaffold containing BMP-2 peptide. DNA content was extracted using cell lysis buffer and thermal shock followed by centrifugation (2500×g, 15 min, 4 °C). The fluorescence intensity of the supernatant was quantified at Ex/Em 485/ 520 nm using microplate reader (Synergy HT, BioTek; USA).

Alkaline phosphatase assay

The effect of sustained release pattern of the peptide on osteogenic differentiation was studied using alkaline phosphatase assay. The enzymatic activity of ALP (as an early indicator of osteoblast differentiation) was quantified using ALP assay kit (Abcam, USA) at days 14 and 21. After washing the cells for 3 times using PBS, cells were lysed through osmotic and thermal shock, centrifuged (13,000×g, 10 min) and the supernatant was analyzed for ALP activity. The absorbance was evaluated at 405 nm using microplate reader (Tecan infinite M200, Switzerland) and normalized against total DNA concentration.

Statistical analysis

One-way ANOVA was used for statistical analysis using Prism software. P value ≤ 0.05 was

Table 1. Particle size and surface charge of liposomal formulations

	F1 (DPPC/DSPG/Cholestrol/DSPE-mPEGmaleimide:55/10/35/1 %molar ratios)	F2 (DPPC/Cholestrol/DSPE-mPEGmaleimide:64/35/1 %molar ratios)
Z- Average (nm)	74.1	92.3
PDI	0.198	0.183
Zeta potential (mV)	-19.9	-6.4
Loading Efficiency	99%	29%

considered significant. The results are reported as mean ± SD (Standard Deviation).

RESULTS AND DISCUSSION

Characterization of the liposomal formulations

Two different liposomal formulations (F1:DPPC/DSPG/Cholestrol/DSPE-mPEGmaleimide:55/10/35/1 %molar ratios and F2: DPPC/Cholestrol/DSPE-mPEGmaleimide:64/35/1 %molar ratios) were prepared using film rehydration method followed by membrane extrusion. BMP-2 peptide was encapsulated in F2 while it was electrostatically loaded on the surface of F1. DSPG was used in the formulation of F1 to enhance the negative surface charge of the nanoparticles. Isoelectric point of BMP-2 peptide is about 9.2 so at physiologic pH (7.4), the peptide is positively charged. Due to the opposite charges of the peptide and F1 liposomes, about 99% of the peptide was loaded onto the liposome whereas 29% of BMP-2 was encapsulated in the core of F2 liposomes. Particle size, surface charge, poly dispersity index and loading efficiency of each formulation is shown in Table 1.

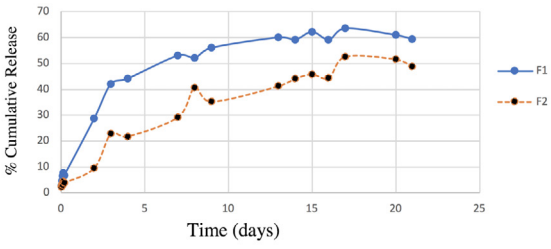

Fig 1. Cumulative release profile of FAM-labeled BMP-2 release from liposomal formulations F1 and F2 in PBS containing FBS 10% at 37 oC

To fabricate ECM-mimicking scaffolds, one of the main goals is to develop carriers with sustained delivery of bioactive agents. Previous studies showed that long term release of BMP-2 improved the bone formation *in vivo* [17-19]. Thus FAM-labeled BMP-2 peptide was loaded and release behavior of the formulations was studied. As shown in Fig 1, the cumulative release of F1 and F2 formulations was about 60% and 50% in 21 days, respectively.

Cellular uptake study using flow cytometry

Previous studies demonstrated that BMP-2 bound the domains at the cell surface which are related to signaling pathways to induce osteogenic differentiation [20]. Besides, it was shown that decoration of mesoporous silica nanoparticles with BMP-2 peptide improved cellular internalization of the nanoparticles [16]. To investigate whether the presence of BMP-2 could enhance the cellular uptake, MSCs were incubated with F1 and F2 liposomal formulations. While BMP-2 was electrostatically immobilized on the surface of F1 liposomes, it was encapsulated into the F2 liposomes. After 6 h of incubation, the cells were detached, washed and the green florescence intensity was evaluated using flow cytometry.

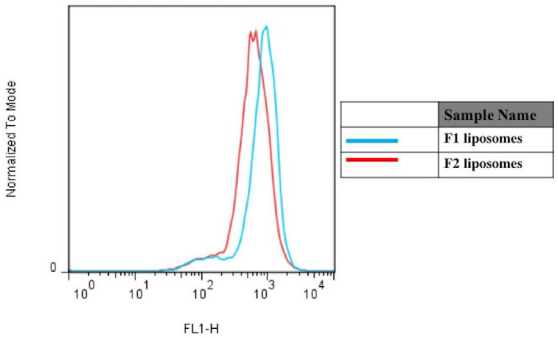

Fig 2. Flow cytometry analysis of MSCs in FL1 after treatment with F1 liposomal formulation (electrostatically bound to FAM-labeled BMP-2 peptide) and F2 liposomes (encapsulating FAM-labeled BMP-2)

Fig 2 showed that there was a right shift in the geometric mean (G mean) fluorescence intensity of F1 (G mean: 714) compared to F2 (G mean: 548)

indicating that immobilization of BMP-2 on the surface of liposomes might enhance the delivery efficiency of the nanoparticles into the MSCs cytoplasm.

Characterization of liposome-attached scaffolds (Liposcaffolds)

Liposomes were immobilized onto the surface of thiol-functionalized electrospun nanofibrous scaffolds, which were previously described [1]. Electrospun, porous, interconnected PLLA scaffold was fabricated with fiber average diameter of about 654 nm (Fig 3). The mat was completely hydrophilic with water contact angle of 0° (physicochemical characterization of the scaffold was completely discussed in our previous study [1]). Maleimide-functionalized liposomes were attached to the scaffold using thiol-maleimide chemistry. We analyzed the conjugation efficiency with maleimide quantification assay kit (Abcam, USA). The attachment efficiency was about 79% for both formulations.

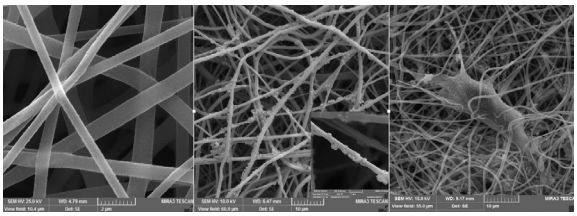

Fig 3. SEM image of electrospun nanofibrous scaffolds (a); The mean diameter of the fibers were 654 nm using image J software. Electrospun nanofibers were coated with nHA (nano hydroxyapatite) (b) and MSCs seeded on to the final scaffold (c)

Evaluating cytocompatibility and proliferation

To evaluate the biocompatibility of the liposcaffold and the effect of the peptide release on the MSCs attachment and proliferation, the cells were seeded on liposcaffolds and double stranded DNA of each sample was evaluated.

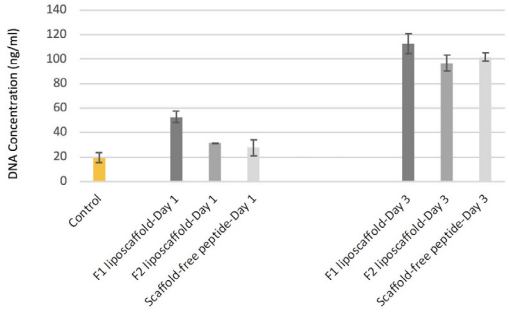

Fig 4. DNA concentration of samples following 1 and 3 days of seeding of MSCs onto the electrospun scaffold indicating biocompatibility of the liposcaffolds and the effect of BMP-2 peptide release on the cell proliferation. There was a significant increase in DNA concentration of F1 liposcaffold compared to F2 liposcaffold at days 1 and day 3 (Pvalue <0.05)

Fig 3c is the SEM image showing the MSCs integrated into the scaffold after 3 days.

Fig 4 shows that not only the fabricated scaffold was completely biocompatible but also due to the presence of BMP-2 peptide and its release, the proliferation of the attached cells was enhanced. It is obvious that at the days 1 and 3, the DNA content of the F1 liposcaffold was significantly more than F2 liposcaffold (P value < 0.05). This could be due to the faster release profile of the F1 liposomal formulation. Also, the immobilized BMP-2 peptide on the surface of the liposomes could act as a ligand and enhanced the entrance of the liposomes into the cells through receptor-mediated endocytosis as proved by cellular uptake study (Fig 2).

Quantification of alkaline phosphatase activity

To evaluate the effect of release of BMP-2 peptide, the activity of alkaline phosphatase (ALP) which is an early marker of osteoblast differentiation, was evaluated. Consistent with the results of DNA concentration, Fig 5 indicated that ALP activity of F1-liposcaffold was significantly more than F2-liposcaffold (P value < 0.05). This result could be due to the decoration of the F1 liposomes with BMP-2 peptide which could act as a ligand for the receptor-mediated endocytosis of the liposomes by the MSCs.

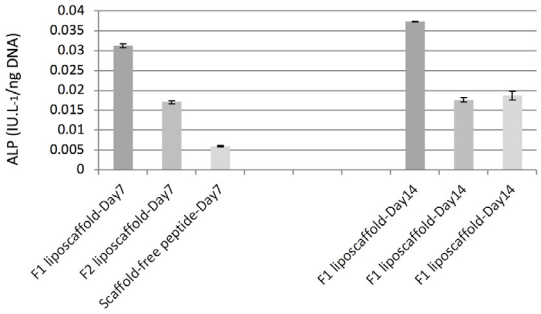

Fig 5. Alkaline phosphatase activity of the MSCs seeded on to the liposcaffold after 7 and 14 days. There was a significant increase of ALP activity in F1 liposcaffold compared to F2 liposcaffold at day 7 and day 14 (Pvalue <0.0001)

CONCLUSION

The main purpose of the current study was to design an ECM mimicking scaffold platform through modification of BMP-2 peptide releasing system which was covalently attached to electrospun PLA/nHA scaffold. To this aim, we prepared two liposomal formulations. BMP-2 peptide was electrostatically immobilized

on the surface of F1 liposomes whereas it was encapsulated into the F2 liposomes. We found that electrostatically-immobilized BMP-2 peptide could act as a ligand to improve the cellular internalization of the liposomes and promote the osteogenic differentiation of MSCs through increasing the ALP activity compared to the encapsulated peptide. Thus we believe that not only BMP-2 peptide is released in a sustained pattern from F1 formulation, but also it enhanced the particle internalization leading to more osteogenic differentiation.

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