Liposomal delivery of demineralised dentine matrix for dental tissue regeneration

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Abstract

Current dental restorations have short longevity, consequently there is a need for novel tissue engineering strategies that aim to regenerate the dentine-pulp complex. Dentine matrix contains a myriad of bioactive growth factors and extracellular matrix proteins associated with the recruitment, proliferation and differentiation of dental pulp progenitor cells. Here, we show that demineralised dentine matrix (DDM), from non-carious dentine, can be encapsulated into liposomes for delivery to dental tissue to promote regeneration. Liposomes were formulated to encapsulate 0 - 100 μg/mL DDM, lysed with Triton X and used in VEGF and TGF-β1 ELISAs to quantify release. The encapsulation efficiencies were calculated to be 25.9% and 28.8% (VEGF/TGF-β1) for 50 μg/mL DDM liposomes and 39% and 146.7% (VEGF/TGF-β1) for 100 μg/mL DDM liposomes. All liposome formulations had no cytotoxic effects on a dental pulp stem cell (DPSC) clone, as shown by MTT, Caspase 3/7 assays and cell counts. The ability of the liposomes to stimulate DPSC chemotactic recruitment was tested by Boyden chamber chemotaxis assays. Unloaded liposomes alone stimulated significant progenitor cell recruitment, while DDM loaded liposomes further promoted chemotactic recruitment in a dose dependent manner. DDM liposomes promoted the upregulation of ‘osteodentine’ markers osteocalcin and RUNX2 in DPSCs after 9 days of treatment, determined by Real Time quantitative PCR. Furthermore, Alizarin Red S staining showed that unloaded liposomes alone induced biomineralisation of DPSCs and DDM liposomes further increased the amount of mineralisation observed. DDM liposomes were more effective than free DDM (10 μg/mL) at activating recruitment and osteogenic differentiation of DPSC, which are key events in the endogenous repair of
the dentine-pulp complex. The study has highlighted the therapeutic potential of bioactive DDM liposomes in activating dental tissue repair \textit{in vitro}, suggesting that liposomal delivery from biomaterials could be a valuable tool for reparative dentistry and hard tissue engineering applications.

Keywords: Dental Tissue Engineering, Liposomes, Demineralised Dentine Matrix, Odontogenesis, Dental Pulp Stem Cells, Reparative Dentinogenesis, Restorative Materials
Introduction

In response to demineralisation from trauma or caries induced acid injury, the dentine-pulp complex can undergo reparative dentinogenesis. This involves dental pulp stem cells (DPSCs) being recruited to below the site of injury and differentiating into odontoblast-like cells, which produce tertiary/reparative dentine\(^1,2\) to protect the underlying dentine. The mechanisms behind this endogenous response are not fully delineated, however the non-collagenous bioactive protein component of dentine is thought to play a key role.

Dentine contains a plethora of growth factors and matrix proteoglycans which are involved with the differentiation, survival, migration and proliferation of DPSCs and mineralisation of predentine\(^3\). Studies have highlighted that demineralised dentine matrix (DDM) and soluble whole tooth extract\(^4\) can induce matrix mineralisation, in addition to dental papilla cell\(^5\) and DPSC differentiation\(^6\). Our previous work has highlighted the bioactivity of DDM in dentine and bone repair, including its ability to promote survival, cell expansion, osteogenesis and mineralisation in DPSCs\(^6\) and bone marrow mesenchymal stem cells\(^7\) (BMMSCs). Additionally, \textit{in vivo} studies have shown its ability to promote bone and dentine regeneration\(^8-10\).

Despite the capacity of dentine to endogenously regenerate, the process is overwhelmed in the case of large lesions. Failure to regenerate dentine results in further exposure of the vital pulp which, if left untreated, can lead to irreversible pulpitis, the treatment for which is endodontic therapy or whole tooth extraction. In addition to these, there is an increased risk of abscess formation and systemic infections. Currently, the most common treatment is to remove the carious dentine and fill with
an inert amalgam or composite material. The current success rates of restorative dental treatments are variable, with around 50% of restorations failing within 10 years\textsuperscript{11} and around 25% of endodontic treatments failing\textsuperscript{12}. Recent research has aimed to identify small molecules that could be used in restorative materials to regenerate the dentine-pulp complex, for example glycogen synthase kinase (GSK3) antagonists\textsuperscript{13} and dentine sialoprotein\textsuperscript{14}. Previous work from our group and others\textsuperscript{15-17}, has investigated harnessing the bioactivity of DDM in dental restorations, by testing the use of etching/conditioning agents to liberate sequestered growth factors within dentine.

Liposomes are amphiphilic lipid vesicles which self-assemble in an aqueous environment. They are capable of protecting their cargo from proteolytic degradation by encapsulating drugs both in the lipid bilayer and the aqueous core, depending on the agent’s hydrophobicity. Liposomes are excellent tools for controlled, sustained drug delivery\textsuperscript{18} and have been used to deliver a range of molecules to engineer tissue and stimulate endogenous repair\textsuperscript{19}. The aim of this study was to investigate the ability of bioactive liposomes containing DDM to induce a mineralising phenotype to promote reparative dentinogenesis.

In this study, phosphatidylserine containing liposomes, a lipid which has been shown to promote bone mineralisation\textsuperscript{20,21}, were used to encapsulate and deliver DDM to a DPSC clone to promote mineralised tissue repair \textit{in vitro}.

\textbf{Materials and methods}
Materials

Phosphatidylcholine from egg yolk (≥ 99.0%), phosphatidylserine from Glycine max (soybean; ≥ 97%), cholesterol (≥ 99.0%). Reagents were, unless otherwise stated, purchased from Sigma Aldrich.

Routine cell culture

The primary human dental pulp stem (hDPSCs) cell colony A3 was isolated as previously described²², and cultured in alpha-MEM (containing ribonucleosides and deoxyribonucleosides) supplemented with 10 % (v/v) heat-inactivated foetal bovine serum (FBS; ThermoFisher), 4 mM L-glutamine (ThermoFisher), 100 μM L-ascorbate 2-phosphate; 100 units/mL penicillin G sodium, 0.1 μg/mL streptomycin sulphate and 0.25 μg/mL amphotericin.

Preparation of Demineralised Dentine Matrix (DDM)

Demineralised Dentine Matrix (DDM) was prepared using extracted human teeth from informed, consenting patients at the Cardiff University School of Dentistry, University Hospital Wales. Ethical approval was granted by the Research Ethics Committee under South East Wales Tissue Bank approval (12/WA/028). The DDM was prepared as previously described⁷. The lyophilised DDM was reconstituted in Tris buffered saline (TBS). Total protein was measured using a BCA assay (ThermoFisher) and diluted in TBS (0 μg/mL - 100 μg/mL).

Preparation of DDM Liposomes

Lipids were dissolved in chloroform (ThermoFisher) at 1 mg/ml (w/v) at a ratio of 7:2:1 (w/w) Phosphatidylcholine: Phosphatidylserine: Cholesterol. Chloroform was removed by rotary evaporation (BUCHI Rotavapor R-300) at 332 mbar for 15 mins (water bath
at 50°C) or until a dry lipid film was produced. Liposomes were formed by resuspending the lipid bilayer film in DDM/TBS to a final concentration of 1 mg/ml (w/v). Liposome batches were then extruded, using a Lipex extruder (Northern Lipids), through a 100 nm polycarbonate filter 10 times to produce small unilamellar liposome vesicles of ~ 100 nm ± 20 nm. The liposomes were sized by dynamic light scattering using a Zetasizer Nano ZS (Malvern). Unentrapped material was removed by Sephadex G50 column separation or ultracentrifugation (Sorvall Discovery 100SE ultracentrifuge) at 100,000 g for 1 h at 4°C.  

Assessment of DDM Encapsulation into Liposomes

Liposomes were separated from unentrapped material by Sephadex G50 column filtration, as described in Torchilin & Weissig (2003)23, and were subsequently lysed with 1% (v/v) Triton X and the amount of VEGF and TGF-β1 release was determined using a commercial ELISA (VEGF, R & D systems; TGF-β1, ThermoFisher) following manufacturer's instructions.

Cell Treatments

A3 DPSCs (2.2×10⁴/cm²) were seeded in 6 well plates and treated with DDM liposomes at concentrations of 0 μg/mL - 100 μg/mL (lipid concentration), free DDM (10 μg/mL) or osteogenic media (supplemented alpha-MEM plus 10 nM dexamethasone and 100 μM β-glycerophosphate) for 3, 9, 21 and 35 days. DDM liposomes (DDM concentrations of 0 μg/ml, 1 μg/mL, 10 μg/mL and 100 μg/mL) were ultracentrifuged, resuspended in supplemented alpha-MEM and sterile filtered. After treatments, cells were scraped in RLT RNA lysis buffer (Qiagen) for molecular analysis.

Cytotoxicity Assays
A3 DPSCs (1x10^4/cm^2) were seeded in 96 well plates (white walled for the Caspase-Glo 3/7 assay; Greiner Bio One). DPSCs were treated with DDM liposomes (0 μg/mL - 100 μg/mL DDM) at liposome concentration of 10 ng/mL, for 3, 9 and 21 days. After 3 days, a Caspase-Glo 3/7 assay (Promega) was performed by adding 1 volume of Caspase-Glo 3/7 reagent and incubating for 1 h at room temperature before measuring luminescence. Cell viability was assessed by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterrazolium bromide) assay. 25 μL MTT solution (5 mg/mL) was added to DPSCs and incubated for 3 h at 37°C, media aspirated and the MTT precipitate solubilised by the addition of 100 μL dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm, with a reference at 630 nm. Cells in additional wells were also counted after 3 days.

**Boyden Transwell Recruitment Assay**

DPSCs (1x10^5) were seeded into transwell inserts and placed on top of media containing liposomes (100 μg/mL). Recruited cells attached to the transwell inserts were washed with PBS, fixed with 10 % (v/v) formaldehyde, stained with 0.1% (w/v) crystal violet and imaged in PBS at 20x magnification on a Nikon Eclipse TS100 light microscope. Representative images were taken and cells were counted using ImageJ image analysis software (NIH).

**Quantitative Real Time PCR (qRT-PCR)**

RNA was isolated using the RNeasy Kit (Qiagen), following manufacturer’s instructions. RNA yield was quantified using a NanoVue spectrophotometer (GE Healthcare). Total RNA (1 μg) was used to generate total cDNA using MMLV Reverse transcriptase (Promega). Osteogenic marker expression was analysed in a SYBR green (Primer Design) qPCR reaction performed on a QuantStudio 6 Flex Real-Time
PCR System (ThermoFisher), using gene specific primers for Runx-related transcription factor 2 (RUNX2), Osteocalcin, and GAPDH, as an endogenous control. The results were analysed using the ΔΔCT method according to MIQE guidelines.24,25

The primer sequences were as follows: GAPDH F 5’ TTCTTTTGCCTGTCGGCAGCGGA 3’, GAPDH R 5’ GTGACCAGGCGCCCAATACGA 3’, RUNX2 F 5’ CCCGTGGCCTTCAAGGT 3’, RUNX2 R 5’ CGTTACCCGCCCATGACGA 3’, Osteocalcin F 5’ GGCAGCGAGGTAGTGAAGAG 3’, Osteocalcin R 5’ CTCACACACCTCCCTCCT 3’. All primers were validated using total human cDNA standard curves, and have 90-110 % amplification efficiency.

Alizarin Red S staining

DPSCs were treated with DDM liposomes (100 μg/mL) for 35 days, fixed with 10 % (v/v) formaldehyde and incubated with 2 % (w/v) Alizarin Red S (pH 4.2) for 20 mins. After staining, the cells were washed 4 times with deionised distilled water and images taken at 20x magnification.

Statistical methods

Statistical analysis, where appropriate, was conducted using a two-tailed paired student’s t-test comparing two groups. Data is expressed as a mean ±SEM.

Results

Demineralised dentine matrix was encapsulated into liposomes for delivery to cells
Liposomes encapsulating 0-100 μg/mL DDM were formulated with an average size of 126.5 ± 40 nm (Table 1) and an average polydispersity index (PDI) of 0.168. There was no significant (p>0.05) variation in size or PDI between liposomes encapsulating different DDM concentrations. The efficacy of the DDM encapsulation was calculated by measuring VEGF and TGF-β1 release from lysed liposomes, a growth factors known to be highly expressed in DDM26,27. 100 μg/mL DDM liposomes encapsulated the greatest amount of VEGF and TGF-β1, encapsulating 3.1 and 330 pg/mL respectively (Tables 2A and B). 50 μg/mL DDM liposomes encapsulated 1.06 pg/mL VEGF and 96 pg/mL TGF-β1 and 10 μg/mL DDM liposomes encapsulated 0.9 pg/mL VEGF and 126 pg/mL TGF-β1. The calculated encapsulation efficiency for each growth factor was similar for 50 μg/mL DDM liposomes (25.9% and 28.8% for VEGF and TGF-β1 respectively), however the encapsulation efficiency varied for 100 μg/mL DDM liposomes, with greater efficiency for TGF-β1 (146.7%) than VEGF (39.0%). Due to the limit of detection of the ELISA kits, encapsulation efficiencies for 1 and 10 μg/mL DDM liposomes were unable to be determined.

All DDM liposomes tested (DDM concentrations of 0, 1, 10, 100 μg/mL; liposome concentrations of 10 ng/mL) caused no cytotoxic effects on a hDPSCs colony (p>0.05), after 3, 9 or 21 days treatment (Figure 1A).

*DDM liposomes promoted the chemotactic recruitment of hDPSCs*

Next the effects of DDM liposomes on proliferation, survival and recruitment of DPSCs, key events in dentine repair process, were tested. Liposomal treatments for 72 h had no significant (p>0.05) change in cell proliferation, assessed by cell counts (Figure 1B). In line with our previous findings, free DDM (10 µg/mL) treatment for 72 h promoted the survival of hDPSCs6 as shown by the reduction in apoptotic marker
Caspase 3/7 (Figure 1C; p<0.05) using a Glo-Caspase 3/7 luciferase assay. However, DPSCs treated with different concentrations of DDM liposomes had no significant reduction in Caspase 3 activity (p>0.05).

A Boyden transwell chemotaxis assay showed that TBS and DDM liposomes significantly (p<0.0001) promote chemotactic recruitment of hDPSC (Figures 2A and B). There was a dose dependent increase in DPSC chemotaxis with increasing doses of DDM liposomes over 48 h. TBS liposomes alone stimulated the chemotaxis of DPSC compared to the control (p<0.0001), but there was further significant increase in chemotaxis when DPSCs were treated with 100 µg/mL DDM liposomes (p<0.01).

**DDM liposomes promoted the expression of osteogenic markers in hDPSCs**

The effect of DDM liposomes on promoting osteogenic differentiation in hDPSCs was investigated via qRT-PCR for ‘osteodentine’ markers, RUNX2 and osteocalcin, which are early and late markers of odontogenesis process. After 3 days, there was an increased trend (p>0.05) in RUNX2 expression in DPSCs treated with free DDM (~2 fold increased) and the higher concentrations of DDM liposomes treatments (10 and 100 µg/mL; ~ 4 fold and 1.5 fold increase respectively; Figure 3A). This peak of expression was significantly lost in 10 (p<0.001) and 100 µg/mL (p<0.0001) DDM liposome treated hDPSCs at day 9. The peak of expression was later in cells treated with free DDM and 1 µg/mL DDM liposomes as at day 9 there was a ~4 fold increase in RUNX2 expression compared to day 3 control (p>0.05). Of note, TBS liposomes induced significant (p<0.05), ~1.2 fold increase in RUNX2 expression at day 3, which was lost at day 9.

Free DDM and 1 µg/mL DDM liposomes caused a ~12 fold (p<0.05) and ~26 fold (p>0.05) increase in osteocalcin expression, respectively, after 9 days (Figure 3B). In
these conditions, the relative expression of osteocalcin decreased after 21 days of treatment. For the higher DDM liposome concentrations (10 and 100 µg/mL) the levels of osteocalcin transcript significantly increased after 21 days of treatment (p<0.05), compared to 9 days. Again, TBS liposomes caused a significant (p<0.001) increase in osteocalcin mRNA levels after 21 days of culture, compared to day 9.

**DDM liposomes induced mineralisation of hDPSCs**

Mineralisation, a marker of late osteogenesis, was examined in hDPSCs treated with DDM liposomes. After 35 days, there was increased Alizarin Red S staining in hDPSC treated with DDM liposomes (10 and 100 µg/mL DDM) compared to untreated, osteogenic media and free DDM (10 µg/mL) controls (Figure 3C). Interestingly, TBS liposomes also increased DPSC mineralisation compared to the untreated controls.

**Discussion**

Dentine contains a ‘cocktail’ of factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), matrix metalloproteinases (MMPs), members transforming growth factor- Beta (TGF-β) superfamily (TGF-β 1, 2, 3, bone morphogenetic protein (BMP) 2, 4 and 7) which synergistically coordinate dentine repair, recruitment and osteo-/odontoinduction of stem cells. In addition, small leucine rich proteoglycans, biglycan and decorin, and dentine specific molecules such as dentine sialoprotein (DSP) and dentine phosphoprotein (DMP-1) have been shown to contribute towards DDM bioactivity. In line with this, we found, when liposomes were used to deliver DDM to a human DPSC
clone, they stimulated chemotactic recruitment, odontogenic differentiation and biomineralisation.

*In vivo*, for dentine regeneration to occur, DPSCs are first required to migrate towards tertiary dentine to the site of injury, where they subsequently proliferate and provide a source of progenitors for odontogenic differentiation to produce new dentine\(^1\). Here, biocompatible TBS phosphatidylserine liposomes were sufficient to stimulate the chemotactic recruitment of hDPSCs, and DDM liposomes further promoted DPSCs chemotaxis in a dose dependent manner, suggesting that DDM liposomes are releasing their cargo to allow paracrine signalling. In this way, DDM liposomes are initiating the first crucial events in dentine repair. A previous study from our group\(^7\) showed that only low DDM doses (0.1 µg/mL) were able to promote migration of BMMSC and higher doses (1-10 µg/mL) were inhibitory to migration. Similarly, several studies have demonstrated that low doses of DDM promote cellular wound healing through stimulating migration, differentiation\(^{31,32}\) and angiogenesis\(^{33}\), however higher doses were found to have an inhibitory effect. Here, 10 µg/mL DDM promoted hDPSC migration and, despite being a smaller dose according to encapsulation efficacies, DDM liposomes promoted more migration than free DDM, we postulate this is due to the slow, consistent release of multiple chemokines having a greater combined effect.

Liposomal delivery of growth factors have been employed to promote wound healing, and cardiac, cartilage, and bone regeneration\(^{34-37}\). Many studies have profiled drug retention in liposomes over time and demonstrated that liposomes have a slower release rate of the encapsulated drug/growth factor compared to free drug\(^{38-40}\). Giannoni et al.,\(^{36}\) observed that the rate of TGF-β1 release from liposomes in fibrin clots was lower compared to free drug release over an initial 5-day period. A body of research\(^{41,42}\) has demonstrated that a sustained release of growth factors from
biomaterials is more effective at producing a cellular response and driving mineralised tissue repair than an initial burst. Likewise, the sustained release of DDM from liposomes within a material is proposed to be more successful than the delivery of individual growth factors due to the synergistic multi-osteoinductive growth factors and matricellular components, which can sequester bioactivity and orchestrate endogenous dentine-pulp complex repair.

Encapsulation of DDM into liposomes allows for a sustained, controlled, local delivery of DDM over time, reducing the need for high doses of free DDM, which has a lengthy production time and uses a limited resource of non-carious human teeth. Research has recently shown that whole DDM is more effective at driving bone repair than individual fractions separated by heparin affinity chromatography, thus a proportion of the entire DDM may be necessary to be encapsulated to drive full dental repair. To establish the amount of DDM encapsulation and therefore the dose of DDM administered to cells, single growth factor ELISAs were used for VEGF and TGF-β1. This highlighted that the liposomes are likely to differentially encapsulate individual constituents of DDM, as TGF-β1 had a much greater encapsulation efficiency at 100 µg/mL than VEGF (146.7% compared to 39.0%). Therefore, it is difficult to assess the exact delivery of DDM constituents to cells using liposomal carriers. Further work is needed to assess the exact encapsulation in DDM liposomes, and to delineate how DDM liposomes elicit their response, e.g. through cargo release or uptake into DPSCs. The migration data suggests DDM release from liposomes, however we cannot rule out that liposomes may enter cells by endocytosis or membrane fusion, as many have previously shown, to deliver DDM components to produce the observed mineralising phenotype.
We observed an increase in mineralisation with control (TBS) liposomes, which was further increased when liposomes were loaded with bioactive DDM- suggesting that DDM liposomes are inducing differentiation. The biomineralisation observed with control liposomes was likely to be the result of phosphatidylserine in the liposomal formulation. Phosphatidylserine is known to bind calcium ions to promote mineralisation\(^{46}\), and has previously been used to coat prosthetic implants, which has promoted osteoblast attachment, mineralisation and osseointegration in both \textit{in vitro} and \textit{in vivo} experiments\(^{47,48}\). Control liposomes also promoted the expression of osteodentine markers, and chemotaxis, suggesting that the phosphatidylserine containing liposomal carrier is beneficial for delivering DDM for promoting mineralised tissue.

Data presented in this study suggests that liposomes are highly efficient at delivering low, sustained doses of DDM that are required for stimulating dental tissue repair. Data presented in this study suggests that liposomes are highly efficient at delivering low, sustained doses of DDM that are required for stimulating dental tissue repair. The dentine regeneration seen \textit{in vivo}, referred to as tertiary dentine, has been reported to be histologically and biochemically similar to bone\(^{49}\). In addition, some proteins that are classically considered as dentine specific are downregulated in tertiary dentine (DSP and DMP-1)\(^{50}\). This study assessed therefore osteodentine markers RUNX2 and osteocalcin in response to DDM liposomes. RUNX2 is a key transcription factor involved in early osteogenesis and odontogenesis, but it is downregulated in terminal differentiation\(^{51,52}\). This trend was observed in DPSCs treated with high concentrations of DDM liposomes (10 and 100 µg/mL DDM), where RUNX2 expression was upregulated at day 3 but significantly downregulated at day 9. Osteocalcin is an extracellular dentine and bone matrix protein recognised as a late stage marker of
odontoblast and osteoblast differentiation\textsuperscript{53,54}. Higher concentrations of DDM liposomes and free DDM induced osteocalcin expression after 21 days. Taken together, the analysis of markers suggests that DPSCs treated with DDM liposomes are undergoing differentiation into odontoblast-like cells, highlighting the potential therapeutic use of these liposomes in regenerative dentinogenesis.

Bioactive DDM liposomes could be applied in various biomaterials for hard tissue engineering purposes. Members of our group have previously optimised a highly efficient liposomal drug delivery system in bone cements\textsuperscript{55}, integrating liposomes in polymethyl methacrylate (PMMA) bone cements with minimal integral artefacts. This study has particularly highlighted the potential use of these liposomes in restorative dental materials, for example, liposomes could be released from glass ionomer cements or modified hydrogels (reviewed in Mourtas et al.,\textsuperscript{56}), to harness the tooth’s natural repair mechanisms. Alternatively, the liposomes could even be directly applied to the pulp during a pulpotomy to enhance pulp dentine regeneration.

This work has shown that bioactive dentine growth factors and matrices can be incorporated and released from liposomes to recruit and differentiate DPSC, promoting dentinogenesis \textit{in vitro}. The enhancement of dentine remineralisation and activation of local DPSCs would, in turn, delay the progression of dental disease, thereby improve the longevity of dental restorations.

\textbf{Acknowledgements}

This work was supported by Medical Research Council (MRC) UK Regenerative Medicine Platform, Acellular Approaches for therapeutic delivery Hub Partnership award and a MRC research grant (G0900954).
Author Disclosure Statement

No competing financial interests exist

References


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Figure legends

Table 1) Size and polydispersity index of DDM liposomes. A Zetasizer Nano ZS was used to measure the average size and polydispersity index of each batch of liposomes (0-100 μg/mL) by dynamic light scattering. N=8 ±SD.

Table 2) DDM was encapsulated into liposomes for delivery. 0, 10, 50, 100 μg/mL DDM liposomes, were separated from unentrapped material by Sephadex G50 column filtration, then lysed using 1% (v/v) Triton –X to release encapsulated cargo and subsequently used in a VEGF (A) and TGF-β1 (B) ELISA, with TBS and free DDM (10, 50 and 100 μg/mL) as controls. From the measured release of growth factor in free DDM and liposomal encapsulated DDM, encapsulation efficiencies for 10, 50 and 100 μg/mL DDM liposomes were calculated. N=3 ±SEM.

Figure 1) DDM liposomes had no cytotoxic effects in hDPSCs. DPSCs were treated with different concentrations of DDM liposomes (0-100 μg/mL; liposome concentration of 10 μg/mL) for 3, 9 and 21 days and then used in a MTT assay (A) to assess cell viability. The cells were then counted with a hemocytometer (B) or used in a Caspase-Glo 3/7 assay (C), which measures apoptotic caspase 3 activity using a bioluminescence substrate. N=3 ±SEM significance is indicated by *p<0.05, and ***p<0.001 (unpaired student’s t test compared to control).

Figure 2) DDM liposomes promoted the chemotaxis of hDPSCs in a dose dependent manner. DPSCs were seeded into Boyden transwell inserts, placed on
liposome treatments (100 μg/mL), serum free media (SFM), free DDM (10 μg/mL) or control (basal alpha-MEM) and allowed to pass through a 0.8 μm membrane for 48 h. Membranes were then fixed and stained with 0.1% (w/v) crystal violet, respective photos were taken (B) and recruited cells counted (A). N=3 ±SEM significance indicated by *p<0.05, **p<0.01, ****p<0.0001 (unpaired student’s t test compared to control).

Figure 3) DDM liposomes promoted the osteogenic differentiation of hDPSC progenitor cells. DPSCs were treated with DDM liposomes (0-100 μg/mL DDM, 10 μg/mL liposome), free 10 μg/mL, or control for DDM for 3, 9 and 21 days. qRT-PCR was performed using primers to amplify RUNX2 (Runt-related transcription factor 2), an early osteodentine marker, at day 3 and day 9 (A) and osteocalcin (OC), a late osteodentine marker, at day 9 and day 21 (B), their expression compared to endogenous control GAPDH. The data is shown relative to day 3 control (A) and day 9 control (B). After 35 days of treatment the amount of calcium deposition was assessed by Alizarin Red S staining using 2% (w/v) Alizarin Red S, cultures treated with 100 μg/mL DDM liposomes and representative pictures taken at 20x magnification. N=3 ±SEM significance indicated by *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (unpaired student’s t test compared to control).
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<td>96±31.3</td>
<td>330±18.2</td>
</tr>
<tr>
<td>% Encapsulation efficiency (TGF-β1 from lysed liposomes/TGF-β1 in free x 100)</td>
<td>0</td>
<td>undetermined</td>
<td>28.82%</td>
<td>146.66%</td>
</tr>
</tbody>
</table>
Figure 2:

A

![Graph showing relative chemotaxis with different treatments.](image)

B

![Micrographs of different treatments.](image)
Figure 3: