

Innovative Collagen Nano-Hydroxyapatite Scaffolds Offer a Highly Efficient Non-Viral Gene Delivery Platform for Stem Cell-Mediated Bone Formation

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Treatments combining nanotechnology with gene and stem cell-based therapies are increasingly showing potential in the field of tissue engineering. In this study, we describe the development of a bioactive and biodegradable collagen nano-hydroxyapatite (coll-nHA) scaffold with the capacity to be used as a gene delivery platform to promote transfection of mesenchymal stem cells (MSCs). Firstly, a nano-hydroxyapatite (nHA) synthesis technique was optimized, and the ability of the nHA particles to act as non-viral vectors for delivery of plasmid-DNA (pDNA) encoding bone morphogenetic protein 2 (BMP2), a gene capable of stimulating MSCs along the osteogenic lineage,^[1] was determined. The nHA particles were then combined with collagen scaffolds, with an architecture previously optimized in our laboratory for bone repair, to yield highly porous, mechanically stable, osteoconductive and osteoinductive coll-nHA scaffolds. The ability of this scaffold to act as a gene-activated matrix (GAM) for BMP2 delivery was demonstrated with successful transfection of MSCs resulting in high levels of calcium production.

Commonly, viruses including adenovirus and lentivirus have been used as gene delivery vectors to efficiently transfer a gene of interest to the host genome.^[2,3] However, using viral vector-based technology possesses numerous disadvantages not least the known safety concerns associated with insertional mutagenesis following viral integration^[4] and the adverse immune responses observed in patients.^[5] Additional problems include the time consuming and labour intensive process involved in

the production of such viruses, thereby limiting their potential for tissue engineering.

Therefore, we sought to develop a safe and effective non-viral based gene delivery vector to overcome the associated viral vector safety problems while being amenable to cost-effective mass production. Typically however, non-viral transfection methods have demonstrated lower efficiencies than that of their viral counterparts^[6] but have an enhanced safety profile. Lipid and polymer-based gene delivery vectors are the most common and efficient non-viral delivery methods utilised but these are often associated with significant cytotoxicity thereby reducing their clinical relevance.^[7] To overcome this cytotoxic issue, we have developed non-aggregating nHA particles^[8] for gene delivery.

Calcium phosphate has long been used as a method of DNA transfection in various cell lines.^[9] As hydroxyapatite is a calcium phosphate mineral, it possesses many advantages for use as a gene delivery vector. nHA particles are biocompatible^[10] and bioresorbable, non-toxic and cost effective to produce, and they exhibit a high binding affinity for DNA most likely due to interactions between calcium ions in the apatite and the negatively charged phosphate groups in DNA.^[11] A nHA synthesis method has been developed within our laboratory to yield homogenous non-aggregating particles <200 nm in size.^[8] Following extensive testing to demonstrate their ability to act as gene delivery vectors when combined with MSCs, the nHA particles were investigated as BMP2 carriers in 2D osteogenic culture and additionally in the 3D environment of collagen-based scaffolds. BMP2 was assessed due to its known therapeutic capacity for bone repair.^[1,12,13]

In addition to using the nHA particles as gene delivery vectors, they were also used to develop an innovative coll-nHA scaffold. The nHA particles were combined with highly porous collagen-based scaffolds developed within our laboratory, with a structure optimized for bone repair,^[14–17] to yield highly biocompatible, mechanically superior, osteoconductive and osteoinductive coll-nHA scaffolds.^[18] Finally, we assessed the capacity of our 3D coll-nHA scaffolds to act as GAMs. We evaluated whether the subsequent culture of MSCs on the collagen and coll-nHA scaffolds containing nHA-BMP2 particles results in conditions sufficient to promote and enhance *in vitro* osteogenesis compared to non-transfected MSCs on these scaffolds.

Firstly, we performed optimization experiments to yield the most effective nHA particles for successful cellular transfection (Supporting Information). The transfection efficiency of nHA

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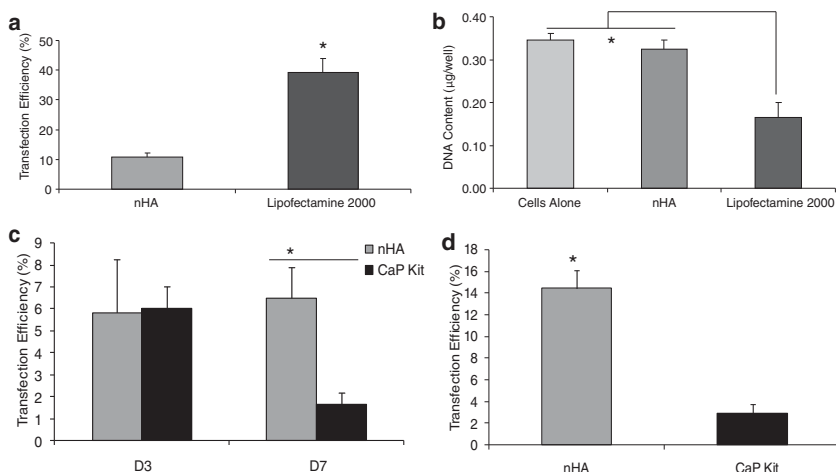


Figure 1. Nano-hydroxyapatite particle transfection efficiency and cell viability analysis. Quantification of GFP expression by FACS analysis revealed enhanced transfection efficiency with the positive control, Lipofectamine 2000, compared to nHA particles (a). Upon quantification of DNA content, there was a significant decrease in Lipofectamine 2000-pLuc transfected cells compared to nHA-pLuc transfected cells and cells alone (b). When nHA-GFP transfection efficiency was compared to a commercial CaP kit, quantification of GFP expression demonstrated a significant increase following nHA transfection by day 7 (c). Quantification of GFP expression in transfected hMSCs at day 3 also demonstrated a significant increase in transfection efficiency using 5 μg plasmid DNA compared to the commercial CaP kit (d). Error bars represent standard error of the mean ($n = 3$). * $P \leq 0.05$.

particle transfection was determined to be ~12% compared to ~40% for Lipofectamine 2000 transfection using green fluorescent protein (GFP) pDNA (Figure 1a). Following quantification of DNA content to assess proliferation, nHA-luciferase (nHA-pLuc) transfected rat MSCs (rMSCs) demonstrated similar DNA content levels to non-transfected cells while a significant decrease was observed in Lipofectamine 2000-pLuc transfected rMSCs indicating a potent cytotoxic effect using this transfection method (Figure 1b). The transfection efficiency obtained using these nHA particles was also compared to a commercially available calcium phosphate (CaP) transfection kit. MSCs transfected with nHA revealed a significant increase in GFP expression at day 7 compared to those transfected with the commercial CaP kit (Figure 1c). Furthermore, when transfection using the nHA particles was translated from rat to human MSCs (hMSCs) and compared to the CaP transfection kit, even greater levels of transfection were obtained than that following rMSC transfection at the same pDNA concentrations (Figure 1d). Interestingly, transfection using the nHA particles was also more efficient in hMSCs compared to rMSCs. This study thus demonstrates the superior capacity of these novel nHA particles to act as efficient non-viral delivery vectors for MSC transfection compared to other commercially available transfection delivery vectors and their potential for use in clinical applications with human cells.

Having demonstrated the ability of these nHA particles to act as competent gene delivery agents, the osteogenic ability of rMSCs following nHA-BMP2 transfection was assessed in monolayer using two different concentrations of BMP2: 1 μg and 3 μg. Alizarin red staining revealed enhanced calcium deposition within the nHA-BMP2 rMSCs compared to rMSCs alone and nHA-pDNA-free rMSC controls (Figure 2a). Similarly, von

Kossa staining displayed a comparable trend with nHA-BMP2 rMSCs demonstrating enhanced phosphate deposition compared to controls (Figure 2b). Quantification of calcium deposition corroborated the staining results, showing a significant increase in calcium deposition within the nHA-BMP2 transfected cells compared to the controls (Figure 2c). As both BMP2 concentrations resulted in equivalent calcium content, this indicates the nHA particles are providing an efficient osteoinductive effect negating the requirement for high levels of BMP2 to induce the enhanced osteogenic response.

The final stage of the study assessed the potential of coll-nHA and nHA-free collagen alone scaffolds to act as GAMs using the nHA particle transfection technique and compared to Lipofectamine 2000 (Supporting Information). The osteogenic potential of MSCs on coll-nHA scaffolds was assessed and compared to collagen alone scaffolds following nHA-BMP2 particle transfection. Results obtained were significant in two ways. Firstly, alizarin red (Figure 3a) and von Kossa staining (Supporting Information) revealed enhanced staining on the coll-nHA scaffolds

compared to the collagen scaffolds in all treatment groups indicating the improved osteogenic properties of these scaffolds due to the osteoinductive and osteoconductive nature of the nHA particles. Further enhanced staining was then observed within both scaffold types following nHA-BMP2 transfection compared to the rMSCs alone and nHA-pDNA-free rMSC groups demonstrating the potent ability of the nHA particles to act as efficient gene delivery vectors when combined with both scaffold types. In addition, osteocalcin expression, indicative of differentiation into an osteoblastic phenotype, was enhanced in the coll-nHA nHA-BMP2 group over all other groups evaluated, (Figure 3b), supporting alizarin red and Von Kossa staining. When calcium deposition was assessed, the highest levels were observed within the coll-nHA scaffolds demonstrating their superior ability to act as efficient gene delivery platforms (Figure 3c).

In conclusion, this study describes the development of a safe and effective non-viral gene delivery vector that overcomes the common viral-based gene delivery vector safety problems. We demonstrated the superior capacity of these novel non-aggregating nHA particles to act as efficient non-viral delivery vectors for rMSC and hMSC transfection compared to other commercially available sources. Incorporation of these nHA particles combined with pDNA in collagen-based scaffolds resulted in the development of a therapeutic GAM. Enhanced osteogenesis was then observed following nHA-BMP2 transfection in both 2D and 3D cultures when using low levels of pBMP2 demonstrating their innate capacity for promoting bone formation. When nHA-BMP2 particles were combined with the additional osteoinductive and osteoconductive nature of coll-nHA scaffolds, the inherent bone forming capacity was considerably enhanced. From a general tissue engineering perspective, the results demonstrate the promise of these coll-nHA scaffolds

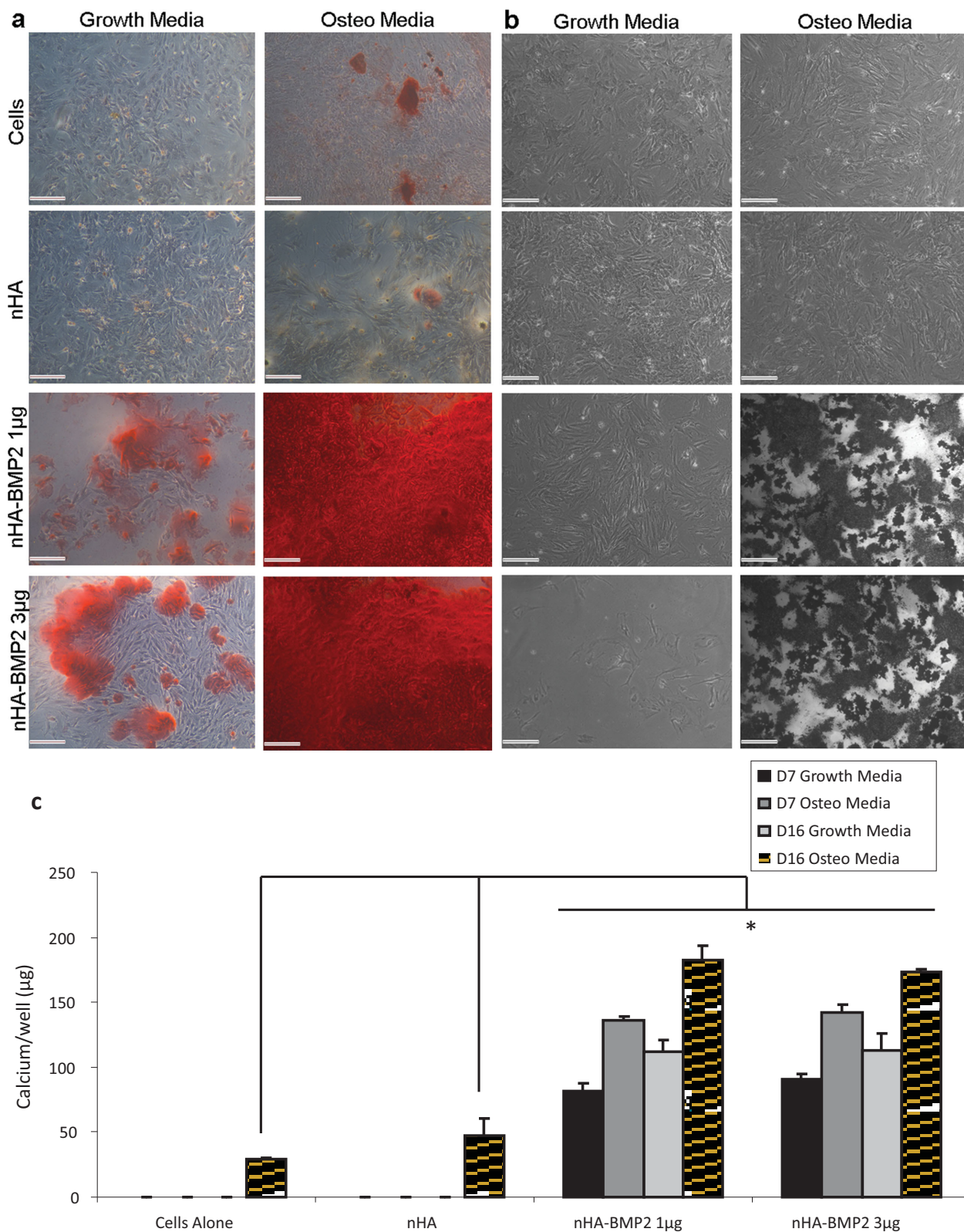


Figure 2. Enhanced 2D osteogenesis following nHA-BMP2 transfection. Brightfield images of alizarin red (a) and von Kossa (b) stained cells demonstrated enhanced staining in nHA-BMP2 treatment groups (growth and osteogenic media treated) compared to the cells alone treatment group. Magnification bar = 200 μ m. Similarly, calcium quantification demonstrated a dose dependent increase in calcium levels in both nHA-BMP2 transfected growth and osteogenic media treated cells at days 7 and 16 (c). Error bars represent standard error of the mean ($n = 3$). * $P \leq 0.05$ compared to cells alone.

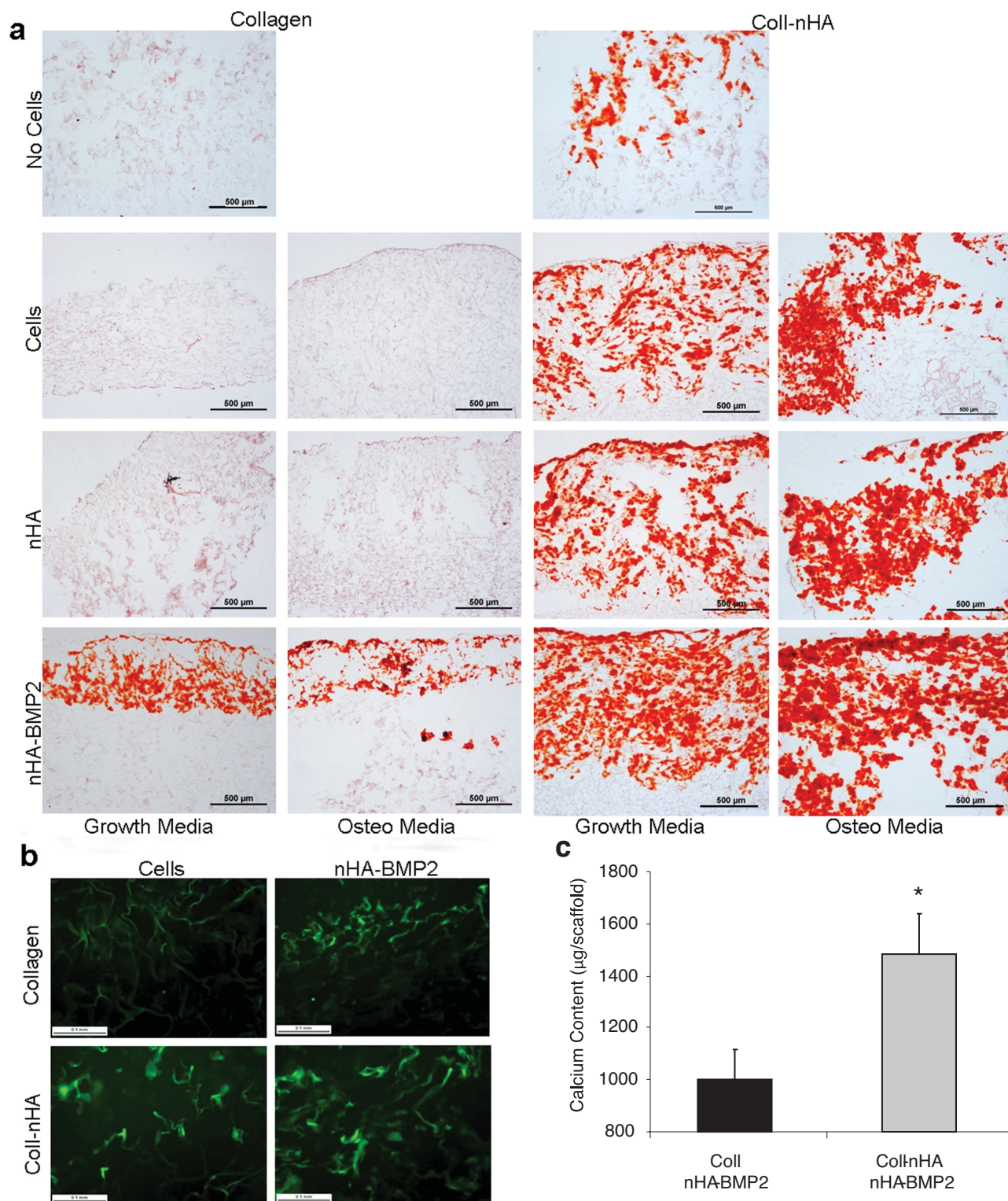


Figure 3. Enhanced 3D osteogenesis following nHA-BMP2 transfection. Alizarin red staining revealed enhanced calcium deposition on coll-nHA scaffolds under all treatments evaluated compared to collagen alone scaffolds (a). Most intense staining was observed on coll-nHA scaffolds with nHA-BMP2 transfection following osteogenic media treatment. Magnification bar = 500 μm . Osteocalcin immunofluorescence corroborates alizarin red staining demonstrating enhanced expression within the coll-nHA nHA-BMP2 scaffold compared to the cells alone and collagen nHA-BMP2 scaffold following osteogenic media treatment (b). Magnification bar = 100 μm . Calcium quantification of nHA-BMP2 transfected scaffolds demonstrated a significant increase in calcium content on coll-nHA scaffolds (c). Error bars represent standard error of the mean ($n = 3$). * $P \leq 0.05$ compared to the collagen nHA-BMP2 scaffold.

as effective gene delivery platforms and thus demonstrate their potential as superior alternatives to existing bone graft treatments.

Experimental Section

nHA particle synthesis optimization and nHA-pDNA complex preparation: nHA particles were precipitated as previously described.^[8] The phosphate solution was prepared in the presence or absence of Darvan dispersing agent—no Darvan (0% v/v Darvan), low Darvan (0.017% v/v Darvan), and high Darvan (0.1% v/v Darvan) to determine the effect of Darvan addition on transfection efficiency. Filtered (0.2 μ m filters) nHA solution (150 μ l) was then added to 3 μ g pDNA and 6.25 μ l 250 mM CaCl₂ for transfection with a final volume of 150 μ l nHA-pDNA suspension added per well in 12 well plates.

A Quant-iT PicoGreen dsDNA kit (Invitrogen) was used according to the manufacturer's protocol to determine the pDNA binding efficiency of nHA particles synthesized with and without Darvan. This kit was also used to assess DNA content following GFP transfection. Particle size distributions were measured using dynamic light scattering (DLS; ZetaSizer 3000 HS, Malvern Instruments). Zeta Potential (ZP) readings ($n = 6$) were conducted on freshly prepared suspensions using a ZetaSizer 3000 HS (Malvern instruments) to determine stability of nHA particles within the solution.

Plasmid propagation: pMaxGFP (Amara, Lonza), pGaussian-Luciferase (pLuc; New England Biolabs) and pBMP2,^[19] encoding the reporter genes, green fluorescent protein (GFP), luciferase and bone morphogenetic protein 2 (BMP2) respectively were used in this study. Plasmid amplification was carried out by transforming One Shot® TOP10 Chemically Competent *E. coli* bacterial cells according to the manufacturer's protocol and the pDNA was collected using Maxiprep kits (Qiagen).

Cell culture: rMSCs were isolated from the femora and tibiae of male Wistar rats with ethical approval (REC 237) from the Research Ethical Committee, Royal College of Surgeons in Ireland, Dublin. Cells were then expanded in culture by direct plating and cultured under normal growth conditions at 37 °C in 5% CO₂ in standard rMSC growth medium (Dulbecco's Minimum Essential Medium) supplemented with 2% penicillin/streptomycin, 1% L-glutamine, 1% GlutaMAX, 1% non-essential amino acids and 10% foetal bovine serum (FBS; BioSera). hMSCs were obtained as a kind gift from the Regenerative Medicine Institute (REMEDI), NUI Galway, Ireland. All procedures were performed with informed donor consent and ethically approved by the Clinical Research Ethical Committee at University College Hospital, Galway. Cells were cultured under normal growth conditions at 37 °C in 5% CO₂ in standard hMSC growth medium (Dulbecco's Minimum Essential Medium supplemented with 2% penicillin/streptomycin and 10% FBS).

2D transfection study: MSCs were plated in 12 well plates at a density of 5×10^4 cells/well. Media was changed 24 hrs after plating, and 1 hr before transfections were conducted, media was replaced. The commercial lipid transfection agent Lipofectamine 2000 (Invitrogen) was used as a positive control, according to the manufacturer's protocol. The prepared nHA/Lipofectamine:pDNA complex solutions (150 μ l) were added to the wells and incubated for 15 mins before growth media was added. OptiMEM reduced-serum media was required for the Lipofectamine 2000 transfected cells, and this solution was removed 6 hrs after transfection and replaced with regular growth media. Cells were assessed for transgene expression on day 3 post-transfection using fluorescence microscopy and days 3 and 7 using flow cytometry (BD FACSCanto II, BD Biosciences). A Live/Dead Viability/Cytotoxicity kit (Invitrogen) was used for calcein staining of the rMSCs to identify live cells according to the manufacturer's protocol. The nHA particles were also compared to a CaP transfection kit (Sigma) to determine transfection capabilities at day 3 (hMSCs and rMSCs) and day 7 (rMSCs). Equal pDNA levels (5 μ g) were assessed and transfection was performed according to the manufacturer's protocol.

Scaffold fabrication: Collagen and collagen nHA (coll-nHA) scaffolds investigated in this study were manufactured using a technique developed in our laboratory.^[14–18] For the coll-nHA scaffolds, nHA particles were synthesized as previously described and added to the collagen slurry during the blending process. A nHA suspension was added to the collagen slurry yielding a 1:1 scaffold of nHA:collagen. Slurries were freeze-dried and scaffolds were cross-linked and sterilized using a dehydrothermal (DHT) treatment.^[20] Cylindrical scaffolds (9.5 mm diameter, 4 mm height) were further cross-linked with 14 mM N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC) and 5.5 mM N-hydroxysuccinimide (NHS) in dH₂O.^[21]

3D transfection study: The pDNA-complexes were added to scaffolds by soak loading 75 μ l of the pDNA-complex solution drop-wise onto each side of the scaffold surface with a 15 min incubation step between additions. Scaffold samples were then seeded with 2.5×10^5 rMSCs on each side of the scaffold including a 15 min incubation step between additions. Constructs were cultured in growth media for 24 hrs before being replaced with either growth media for transgene expression profile experiments or osteogenic media (growth media with ascorbic acid (50 μ g/ml), β -glycerophosphate (10 mM) and dexamethasone (100 nM)) for osteogenesis experiments. Constructs were cultured under standard conditions (37 °C, 5% CO₂), and the media was replenished every 3–4 days up to 35 days. Constructs ($n = 3$) were analysed for calcium content or constructs ($n = 1$) were fixed in 10% formalin for processing prior to histological analysis.

Luciferase assay: A LumiFlex GLuc assay (New England Biolabs) was used to quantify the expression of luciferase in media samples from pLuc-transfected MSC seeded constructs according to the manufacturer's protocol.

Calcium quantification: Quantification of calcium deposition within 2D and 3D osteogenesis experiments was performed using the Calcium Liquicolor kit (Stanbio Laboratories) according to the manufacturer's protocol.

Mineralization: Wax embedded scaffold sections (10 μ m) were stained with either Von Kossa or alizarin red to evaluate cell-mediated matrix deposition and mineralization within the scaffolds according to standard protocols.

Immunofluorescence: Wax embedded scaffold sections (10 μ m) were permeabilized with 0.1% Triton-X100 for 10 mins and blocked with 0.1% Triton-X100/2% bovine serum albumin in phosphate buffered saline (PBS) for 2 hrs at room temperature. Cells were incubated with osteocalcin (1:50) at 4 °C overnight, washed three times with PBS, then incubated with secondary antibody (1:200) for 1 h at room temperature in the dark and washed as before, prior to coverslipping.

Statistical analysis: Results are expressed as mean \pm standard deviation. Two-way analysis of variance (ANOVA) followed by a pair wise multiple comparison procedure (Tukey test) was used. Statistical significance was declared at $p \leq 0.05$.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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