A Novel Nanoparticle-Enhanced Photoacoustic Stimulus for Bone Tissue Engineering

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In this study, we introduce a novel nanoparticle-enhanced biophysical stimulus based on the photoacoustic (PA) effect. We demonstrate that the PA effect differentiates bone marrow-derived marrow stromal cells (MSCs) grown on poly(lactic-co-glycolic acid) (PLGA) polymer films toward osteoblasts. We further show that the osteodifferentiation of the MSCs due to PA stimulation is significantly enhanced by the presence of single-walled carbon nanotubes (SWCNTs) in the polymer. MSCs, without the osteogenic culture supplements (0.01 M β -glycerophosphate, 50 mg/L ascorbic acid, 10^{-8} M dexamethasone), were seeded onto plain glass slides, glass slides coated with PLGA, or glass slides coated with SWCNT-PLGA films and photoacoustically stimulated by a 527 nm Nd:YLF pulse laser, with a 200 ns pulse duration, and 10 Hz pulse frequency for 10 min a day for 15 consecutive days. The study had four control groups; three baseline controls similar to the three experimental groups but without PA stimulation, and one positive control where MSCs were grown on glass slides without PA stimulation but with osteogenic culture supplements. The osteogenic differentiation of all the groups was evaluated using quantitative assays (alkaline phosphatase, calcium, osteopontin) and qualitative staining (alizarin red). After 15 days, the PA stimulated groups showed up to a 350% increase in calcium content when compared with the non-PA stimulated positive control. Further, within the PA stimulated group, the PLGA-SWCNT group had 130% higher calcium values than the PLGA film without SWCNTs. These results were further corroborated by the analysis of osteopontin secretion, alkaline phosphatase expression, and qualitative alizarin red staining of extracellular matrix calcification. The results indicate that PA stimulation holds promise for bone tissue engineering and that the nanomaterials which enhance the PA effect should allow the development of biophysical rather than biochemical strategies to induce osteoinductive properties into tissue engineering scaffolds.

Introduction

THE NEED FOR ADVANCES in bone regeneration ensues from the serious conditions of bone loss due to disuse, disease or trauma, and age.¹⁻⁴ Bone tissue engineering seeks to develop strategies to reverse bone loss without the limitations and drawbacks of current autografting and allografting approaches. A key component of bone tissue engineering is the scaffold, which provides structural support and functions as a carrier of cells and bioactive molecules necessary for the formation of new bone tissue. Ideally, the scaffold should provide sufficient support for growing bone tissue, degrade as bone tissue grows, be biocompatible, and be highly porous, with high pore interconnectivity. Most current scaffolds including ceramics, metals, polymers, and nano-reinforced materials⁵⁻⁸ are osteoconductive, in that they only support *de novo* bone formation. To incorporate

osteoinductive (stimulate progenitor cell differentiation into mature bone) properties into scaffolds, biochemical strategies such as scaffold surface treatments and incorporation of covalent or noncovalent peptides, nano-sized hydroxyapatite particles, growth factors, and cytokines have been explored.⁹⁻¹²

The photoacoustic (PA) effect occurs when a surface absorbs an intense electromagnetic pulse—usually light, which results in rapid heating and a corresponding rapid expansion near the surface. This expansion results in an intense acoustic wave that propagates away from the initial illuminated surface area at the speed of sound in the material.¹³ Since its first demonstration in 1881, the PA effect has been harnessed for imaging and spectroscopy in material sciences, engineering, and medicine.^{14,15} More recently, nanomaterials such as gold nanoparticles and single-walled carbon nanotubes (SWCNTs) that absorb strongly in the visible, near

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infrared, or the radiofrequency regions have been employed as contrast agents for PA imaging.^{16–19} To the authors' knowledge, the efficacy of PA stimulation for bone tissue regeneration, which, in theory, combines the benefits of electromagnetic and acoustic effects, has not yet been extensively explored.

The aim of this study was to investigate the effect of pulse-laser-induced PA stimulation on marrow stromal cells (MSCs) seeded on poly(lactic-co-glycolic acid) (PLGA)- and SWCNT-incorporated PLGA films in static culture. In the present study, the widely accepted biocompatible, biodegradable, and FDA approved PLGA was used as polymer matrix to evaluate carbon nanotube-enhanced PA stimulus (as a means of biophysical rather than as a biochemical strategy) for bone tissue engineering. Recently, the effect of laser-induced PA stimulation on the differentiation of MSCs was investigated in static culture media without osteogenic supplements (0.01 M β -glycerophosphate, 50 mg/L ascorbic acid, and 10^{-8} M dexamethasone [Dex]).²⁰ It was found that a brief (10 min) daily exposure of multipotent MSCs to pulse-laser-induced PA stimulation promotes their differentiation toward osteoblasts and that this osteodifferentiation of the MSCs is further enhanced by the presence of nanoparticles (SWCNTs or gold nanoparticles).¹⁶ Experiments were designed to provide answers to the following questions: (1) Does PA stimulation of MSCs differentiate the MSCs toward osteoblasts when incubated without osteogenic supplements on a biodegradable, biocompatible PLGA polymer widely used as a scaffold in tissue engineering? (2) Do small concentrations of SWCNTs dispersed in this polymer enhance the osteodifferentiation of the MSCs?

Materials and Methods

Experimental design

Mouse MSCs obtained from ATCC (CRL12424) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin under standard culture conditions. For the experimental groups, the cells were cultured in the following three ways and photoacoustically stimulated: (1) cells cultured on bare glass cover slips, (2) on PLGA polymer film, and (3) on a PLGA-SWCNT composite film. The three baseline controls were MSCs cultured on glass, PLGA, and PLGA-SWCNT, respectively, but not exposed to PA stimulation. The positive control in this study, Dex, consisted of MSCs grown on glass cover slips in osteogenic supple-

mented media (0.01 M β -glycerophosphate, 50 mg/L ascorbic acid, and 10^{-8} M Dex). All the above groups had a sample size of $n=4$ and are described in Table 1.

Each osteodifferentiation of the MSCs was evaluated at 4, 9, and 15 days using cell growth, alkaline phosphatase (ALP), and calcium assays; also, an osteopontin (OPN) assay was performed every 2–3 days. At day 15, alizarin red staining was also performed to visually detect the presence of calcium deposition.

PLGA and PLGA-SWCNT film fabrication

A modified version of the protocol used by Karp *et al.*²¹ for creating polymer-coated glass slips was employed. PLGA films were created both with and without SWCNTs. In both cases, PLGA (50:50) pellets (Sigma) were weighed and dissolved at a concentration of 73 mg/mL in chloroform by heating the solution in a sealed glass vial at 60°C for 1 h. For the PLGA-SWCNT films, purified SWCNTs (HiPco SWCNTs; Unidym) were added at 0.5% (w/w) to the films. The liquefied PLGA and PLGA-SWCNT solutions were applied in 100 μ L aliquots to 15 mm round glass cover slips. The cover slips were maintained on a hot plate at 60°C until the chloroform evaporated and the films were firm. The films were then stored at 4°C until ready for use.

Cell culture

MSCs were cultured onto 10 cm tissue culture plates in standard media containing DMEM (Gibco) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin until the cells were at least 90% confluent. All MSCs were cultured in a 37°C incubator with 95% humidity and 5% CO₂ and handled with standard tissue culture techniques. The cells were passaged, plated onto 15 mm round glass cover slips placed in 18 mm $\times$ 18 mm square glass bottom wells (Nunc), and maintained with 1.2 mL standard media. The cells for the positive control were grown on plain glass cover slips and were given 10^{-8} M dexamethasone (Sigma), 10 mM β -glycerophosphate (Sigma), and 50 mg/L L-ascorbic acid (Sigma) osteogenic supplements, which have been shown to induce differentiation.^{22,23} The media in all the wells were changed every 2–3 days, and the collected media were stored at 4°C for OPN quantification. At each time point (4, 9, and 15 days) after stimulation, round cover slips from each experimental group were washed with phosphate-buffered saline (PBS) and moved to a fresh 18 mm $\times$ 18 mm square well containing 2 mL of double distilled water per sample.

TABLE 1. SUMMARY OF EXPERIMENTAL GROUPS SHOWING EITHER THE PRESENCE (YES) OR ABSENCE (NO) OF THE RESPECTIVE COMPONENTS

S. No.	Experimental group	PLGA film	SWCNTs	Osteogenic media	PA stimulation performed
1	Light	No	No	No	Yes
2	PLGA	Yes	No	No	Yes
3	PLGA-SWCNT	Yes	Yes	No	Yes
4	Light Control	No	No	No	No
5	PLGA Control	Yes	No	No	No
6	PLGA-SWCNT Control	Yes	Yes	No	No
7	Dex	No	No	Yes	No

PLGA, poly(lactic-co-glycolic acid); SWCNTs, single-walled carbon nanotubes; Dex, dexamethasone; PA, photoacoustic.

The samples were stored at -20°C until the assays were performed.

PA protocol

MSCs were stimulated using a 527 nm Nd:YLF short pulse laser (Photonics Industries GM-30). The laser pulses have nominal 200 ns pulse duration, 10 mJ pulse energy, and are delivered at a rate of 10 Hz to the media. The stimulation was carried out for 10 min per day (~ 6000 pulses/day) for 4, 9, or 15, consecutive days. The 10 min stimulation time per day was chosen based on other optical and ultrasound stimulation work reported in the literature.^{24–28} The cells are held in a fixture approximately 20 cm above the optical table containing the laser. A 45° reflecting mirror below the fixture redirects the horizontal laser beam vertically upward, where it enters the bottom of the well. The total beam travel distance from the laser is ~ 2 m, and the beam diameter is approximately 15 mm at the well bottom (Fig. 1). Baseline control cells were removed from the incubator and allowed to reach room temperature, without stimulation to maintain similar conditions as the experimental group.

Cell growth analysis

DNA was quantified using a *Picogreen Elisa* kit (Cat no. P7589; Invitrogen), which fluorescently quantifies DNA present within a sample.⁴ Quantification of cell number is possible by comparing the experimental sample DNA with the DNA in a known number of MSCs. The previously frozen cover slips containing cells were thawed and sonicated for 5 min to lyse the cells. A 96-well plate was then prepared with 100 μL /well of Tris-EDTA buffer provided with the kit. One hundred microliters of standards or samples were

added in triplicate to the buffer, followed by 100 μL of Picogreen reagent. The plate was incubated at room temperature in the dark for 10 min. The fluorescent signal was read at 480 nm excitation and 520 nm emission wavelengths using a microplate reader (Biotek).

ALP assay

In a 96-well plate, 100 μL of p-Nitrophenyl Phosphate (pNPP) Liquid Substrate System (Cat. no. N7653; Sigma) was added to 100 μL of the sample or standard (4 nitrophenol; Sigma) in triplicate and incubated for 1 h at 37°C . The ALP produced by the cells hydrolyzes pNPP, the reagent forming p-nitrophenol. The reaction was stopped using 100 μL of 0.2 M NaOH, and absorbance was read at 405 nm on a 96-well microplate reader (Biotek) to detect the p-nitrophenol.⁴

Calcium assay

The samples for this assay were prepared by adding 1 M acetic acid to an equal volume of the solution in each well and left on a shaker overnight to digest the biological components and dissolve the calcium into solution. Using calcium chloride as a standard, 20 μL of either standard or sample was added in triplicate to a 96-well plate. Two hundred eighty microliters of Arsenazo III Calcium Assay Reagent (Cat no. 140-20; Diagnostic Chemicals) was then added to each of the wells.⁴ The reagent is a calcium binding chelate that changes color when the dissolved calcium in the sample is chelated. Absorbance was measured at 650 nm on a 96-well Biotek microplate reader.

OPN assay

The aspirated media changed out every 2–3 days was collected and used for this assay. The Mouse OPN *Elisa* kit (Cat no. MOST00; R&D Systems) was used to quantify OPN. The sample media was diluted 10,000-fold in DMEM, and the assay was performed in duplicate. Fifty microliters of the samples or standards along with 50 μL of the reagent provided with the kit were added to the OPN polyclonal antibody coated wells. The plate was incubated for 2 h at room temperature to allow the OPN to bind to the antibodies. The samples were then aspirated, and an enzyme-linked polyclonal antibody reagent was added for 2 h at room temperature. The samples were aspirated again, and 100 μL of substrate reagent was added to each well and kept for 30 min in the dark, during which time the enzymatic reaction occurs. Addition of hydrochloric acid (100 μL /well) stopped the reaction. OPN levels were quantified by measuring absorbance at 450 nm on a 96-well Biotek microplate reader.

Alizarin Red staining

To prepare for staining, the 15 mm cover slips of the various groups were washed with PBS and fixed with 70% ethanol on ice for 1 h. The samples were washed with deionized water and stained with 500 μL of 40 mM alizarin red (Sigma-Aldrich) solution (pH 4.2) for 10 min at room temperature. The alizarin red solution was aspirated, and the wells were washed with deionized water. The samples were incubated with PBS (with no Mg or Ca) for 15 min at room temperature, and optical images were taken.

FIG. 1. Experimental setup of laser photoacoustically stimulating the cells perpendicular to the cellular surface. Key components include the Nd:YLF laser and beam, the 45° mirror, and the cell well (A). Detail of cell well, showing incident laser pulse, carbon nanotube layer, and cell media (B). MSCs, marrow stromal cells. Color images available online at www.liebertonline.com/tea

Statistical analysis

The quantitative data gathered from the ALP, calcium, and DNA assays were analyzed using a one-way analysis of variance. The data are presented as the mean $\pm$ standard deviation for $n=4$ samples. Tukey's "Honestly Significantly Different" multiple comparison test was used to determine the significance of the differences seen between the experimental groups in the different assays. The comparisons were carried out with a 95% confidence interval ($p < 0.05$).

Results

Cell growth analysis in response to nanoparticle-enhanced PA stimulation

The plot of cell growth at days 4, 9, and 15 is presented in Figure 2. The number of cells in each group was found to increase between day 4 and day 9 and plateau between day 9 and day 16. There was no statistically significant difference in the cell numbers between the different groups at days 4, 9, and 15.

Nanoparticle-enhanced PA stimulation on ALP activity

The ALP assay (Fig. 3) shows a difference between the PA stimulated experimental groups and their nonstimulated controls at all three time points. At days 9 and 15, the PLGA-SWCNT group showed significantly greater ($p < 0.05$) ALP expression than any of the other groups. Among PA stimulated groups, at day 9, PLGA-SWCNT showed 34% and 16% greater expression than PLGA and Light, respectively, and at day 15, PLGA-SWCNT showed 10% and 65% greater expression than PLGA and Light, respectively. The increase in ALP production from days 9 to 15 is less substantial than days 4 to 9 results. For instance, between days 4 and 9, ALP activity for PLGA-SWCNT increased by 290%; whereas between days 9 and 15, ALP expression only increased an

FIG. 2. The cell growth was quantified for each group after 4, 9, and 15 days in culture. The nonstimulated samples include cells cultured on a glass slide (Light Control), a PLGA film (PLGA Control), a PLGA film incorporated with SWCNTs (PLGA-SWCNT Control), and a glass slide containing osteogenic supplemented media in the cell culture well (Dex). The stimulated samples were exposed to the laser for 10 min a day, and they include cells cultured on a glass slide (Light), a PLGA film (PLGA), and a PLGA film incorporated with SWCNTs (PLGA-SWCNT). Note at 15 days in culture, there was a significant difference between stimulated samples and their non-PA stimulated counterparts ($*p < 0.05$). PLGA, poly(lactic-co-glycolic acid); SWCNTs, single-walled carbon nanotubes; Dex, dexamethasone.

FIG. 3. Quantitative analysis for ALP expression for non-stimulated and stimulated cells with and without PA stimulation after 4, 9, and 15 days in culture. The nonstimulated samples include cells cultured on a glass slide (Light Control), a PLGA film (PLGA Control), a PLGA film incorporated with SWCNTs (PLGA-SWCNT Control), and a glass slide containing osteogenic supplemented media in the cell culture well (Dex). The stimulated samples were exposed to the laser for 10 min a day, and they include cells cultured on a glass slide (Light), a PLGA film (PLGA), and a PLGA film incorporated with SWCNTs (PLGA-SWCNT). Note that $*p < 0.05$ is significantly different between the stimulated samples and their nonstimulated counterparts. ALP, alkaline phosphatase.

additional 30%. The positive control Dex maintained higher ALP expression than the nonstimulated control groups at days 9 and 15, but was less than the PA stimulated groups.

Nanoparticle-enhanced PA stimulates calcium release

The results of the calcium assay, indicative of calcium matrix deposition, are shown in Figure 4. All the stimulated samples showed a temporal increase in calcium content, whereas the nonstimulated controls, with the exception of Dex, had negligible levels of calcium throughout the experiment. After 4 days, PLGA-SWCNT displayed a 13%, 12%, and 34% greater amount of calcium than PLGA, Light, and Dex, respectively. This result was increased to 64% and 75.22% at day 9, and through day 15, where PLGA-SWCNT samples had a 131%, 146%, and 347% greater calcium expression than PLGA, Light, and Dex, respectively. By day 9, the calcium matrix deposition began and matured by day 15. The positive control, Dex, followed the same trend as the PA stimulated samples but had lower levels of calcium than the PA stimulated samples at all time points.

Nanoparticle-enhanced PA stimulates OPN secretion

The results of the OPN assay are presented in Figure 5. Over the 15 day sequence test, it is clear that the baseline control groups had low levels of OPN secretion. The positive control and PA stimulated samples continuously increased in OPN secretion over time. The PA groups were always higher than the positive control. When the PA groups were compared with the positive control after 15 days in culture, there was a six- to sevenfold increase in the PA groups for Light, PLGA, and PLGA-SWCNT groups, respectively. Further, within the PA groups at day 15, the PLGA-SWCNT

FIG. 4. Quantitative analysis of calcium matrix deposition for stimulated and nonstimulated cells with and without PA stimulation after 4, 9, and 15 days in culture. The nonstimulated samples include cells cultured on a glass slide (Light Control), a PLGA film (PLGA Control), a PLGA film incorporated with SWCNTs (PLGA-SWCNT Control), and a glass slide containing osteogenic supplemented media in the cell culture well (Dex). The stimulated samples were exposed to the laser for 10 min a day, and they include cells cultured on a glass slide (Light), a PLGA film (PLGA), and a PLGA film incorporated with SWCNTs (PLGA-SWCNT). Note that at all time points, there was a significantly higher level of calcium for the stimulated samples compared with their nonstimulated counterparts ($*p < 0.05$). At days 9 and 15 in culture, there was also a significantly greater amount of calcium for stimulated samples cultured on the PLGA-SWCNT film in comparison to all other groups ($**p < 0.05$).

group was 11% and 22% higher than Light and PLGA groups, respectively. The increase in OPN secretion in PA-stimulated samples was evident after the first media change, occurring on the third day of PA stimulation, and continued to have high levels through the remainder of the study.

Nanoparticle-enhanced PA stimulates matrix mineralization (alizarin red staining)

Figure 6 shows representative optical images of PLGA-SWCNT, PLGA, Dex, and Light. The deep red color for the PA stimulated samples indicates the formation of a calcified matrix, which is less intense for the positive control group containing dexamethasone. The purple color in the Dex sample represents the underlying cells. These results are consistent with the quantitative calcium data, which show Dex deposits an extracellular matrix, but the level of deposition for the PA stimulated groups surpasses that of the nonstimulated Dex group. Although there appears to be a purple color present for the Light group, this occurs because the matrix for these samples was delicate and started to break off during the ddH₂O washing process. The matrices present on the PLGA and PLGA-SWCNT samples were less sensitive, so they did not experience this problem.

Discussion

The aim of this study was to investigate the effect of pulse-laser-induced PA stimulation on MSCs seeded on PLGA- and SWCNT-incorporated PLGA films in static culture. This study was performed toward our goal of evaluating the efficacy of this nanoparticle-enhanced biophysical stimulus for bone tissue engineering. PLGA was chosen as the polymer matrix, because it is biocompatible, biodegradable, FDA approved for clinical use and represents a widely used

FIG. 5. After 15 days of PA stimulation for 10 min per day, old media captured from regular media changes was used to determine OPN concentrations, a later stage osteogenic marker. The nonstimulated samples include cells cultured on a glass slide (Light Control), a PLGA film (PLGA Control), a PLGA film incorporated with SWCNTs (PLGA-SWCNT Control), and a glass slide containing osteogenic supplemented media in the cell culture well (Dex). The stimulated samples were exposed to the laser for 10 min a day, and they include cells cultured on a glass slide (Light), a PLGA film (PLGA), and a PLGA film incorporated with SWCNTs (PLGA-SWCNT). It can be seen that OPN had consistently higher expression in the PA stimulated groups, with the PLGA-SWCNT group having the greatest expression. Note that at all time points, there was a significantly higher level of OPN for the stimulated samples compared with their nonstimulated counterparts ($*p < 0.05$). At all time points, there was no significant difference in the OPN levels across the stimulated groups except for day 11. At day 11, the PLGA stimulated group showed a significant decrease in OPN levels compared with other stimulated groups. OPN, osteopontin.

polymer in the fabrication of tissue engineering scaffolds.²⁰ The extent of differentiation of the MSCs toward osteoblastic lineages for the stimulated and the nonstimulated groups was quantitatively determined by analysis of known indicators for cell proliferation (cellular DNA analysis), osteogenesis (production of ALP, deposition of a calcified matrix (Ca content analysis), and OPN expression). The alizarin red staining was used as qualitative technique to visually confirm the calcium deposits present in the extracellular matrix and to corroborate the trends of the calcium content analysis (Fig. 6).

The cell growth of all the PA and control groups was not statistically different on days 4, 9, and 15. All groups showed an increase in cell growth and a growth plateau between day 9 and day 15. This general trend of cell growth for all groups over the 15-day period is consistent with what is normally observed for this cell type, increase in matrix deposition and mineralization may be the reason for the plateau in cell growth between day 9 and day 15 (Fig. 5). The matrix encasement of the DNA may make the Picogreen diagnostic kit underestimate the DNA content in each sample.⁴ Alternatively, between days 9 and 15, all the groups become visibly confluent. Once they reach this state of confluency, they are incapable of further proliferation, because there is no available surface area to allow for cellular adhesion. The

FIG. 6. Alizarin red optical images from left to right, PA stimulated PLGA-SWCNT (PLGA-SWCNT), PA stimulated PLGA (PLGA), osteogenic supplemented control (Dex), PA stimulated direct light (Light). Circle diameters correspond to 15 mm. PA, photoacoustic. Color images available online at www.liebertonline.com/tea

plateau in cell growth between days 9 and 15 may also be an indication of poor cell proliferation or cell death. However, the extensive mineralization and changes in expression of osteoblastic markers (Figs. 2–6) observed in all of the groups suggest that the cells remain viable.

The ALP assay provides a quantitative marker of early stage osteogenic activity.²⁹ ALP activity for the PA stimulated groups was always statistically greater ($p < 0.05$) than their nonstimulated controls, and the Dex group also surpassed the nonstimulated controls. These results are consistent with our previous studies showing that PA stimulation causes increased ALP expression before matrix maturation.³⁰ The addition of SWCNTs into the PLGA matrix significantly increased ALP expression by day 9 of stimulation. ALP is secreted by osteoblasts during the matrix maturation stage, making it an early stage marker for osteogenesis, and this causes ALP expression typically to decrease before complete matrix deposition.³¹ This explains why ALP activity only displays a slight increase from days 9 through 15, because the matrix maturation likely occurred between days 9 and 15.

Calcium, on the other hand, is a late stage marker for osteogenic differentiation and is assessed based on the deposition of a calcified matrix.⁴ The PA groups and Dex all displayed their highest calcium expression after 15 days in culture (Fig. 4), and the mineralization can further be visualized in Figure 6 using Alizarin Red staining. Alizarin red binds to the calcium deposited in the extracellular matrix and is a marker for matrix mineralization, a precursor to the calcified matrix associated with bone.³² The bright red color present in the PA stimulated samples indicates the formation of the matrix. The Light group also appeared to form a mineralized matrix, which was confirmed by the calcium assay. The Light samples, though, were more delicate, and the matrix started to chip off the glass coverslip during the stain washing process, making the underlying cellular layer more visible in Figure 6 than the other PA stimulated samples. The Dex group displays a matured extracellular matrix, but it is less substantial than the other stimulated groups, thus indicating that the rate and level of mineralization is greater for PA stimulated samples than for the positive control. Based on the calcium assay results, the addition of SWCNTs to the PLGA scaffold (PLGA-SWCNT Control) does not cause an increase in calcium matrix deposition, suggesting that SWCNTs may not be independently osteoinductive. When the PLGA-SWCNT samples undergo stimulation, the presence of SWCNTs enhances the osteoinductive properties of PLGA as shown by the significant difference ($p < 0.05$, displayed in Figure 4 by the asterisks [*] between the PA stimulated groups and their controls), as well as the significant difference ($p < 0.05$, displayed by the double asterisks [**] between the PLGA-SWCNT group and

the two other stimulated groups). The results further indicate that the mechanism of the osteoinduction is a combination of the photochemical and biophysical (acoustic) effects caused by the laser. The photochemical effect is thought to cause mitochondrial changes, whereas the biophysical effect (generation of acoustic waves) has been shown to effect conformational and biochemical changes to the cell membrane, thus leading to downstream alterations in bone-specific genes leading to enhanced cellular proliferation.^{27,28}

OPN is an early stage marker of osteogenesis and is secreted into the extracellular media.⁴ In the present study, the OPN assay measures the OPN that has been secreted into the media. It is an early stage marker and is secreted by osteoblasts during development.³³ After as little as three days of stimulation, the PA stimulated samples had already started to secrete OPN into their extracellular fluid, which continued to increase until it peaked around day 13 for the PA stimulated samples (Fig. 5). The OPN secretion for PLGA-SWCNT always surpassed all other groups at all time points. The PA stimulated Light and PLGA also showed high levels of OPN expression, much greater than their respective controls. The PLGA-SWCNT Control group had low levels of OPN secretion. Taken together, it can be inferred that the PA stimulation with or without the presence of SWCNTs improves the osteoinductive properties of PLGA.

Recently, SWCNTs have been shown to improve the mechanical properties of the polymers nanocomposites and scaffolds as reinforcing agents used for load-bearing bone tissue engineering applications.^{34–36} Further, studies show that SWCNT-incorporated biodegradable polymer scaffolds are osteoconductive and allow noninvasive magnetic resonance imaging to evaluate nanotube release during the polymer degradation process and their biodistribution on release from the polymer matrix.^{37,38} Our results indicate that PA stimulation of SWCNT-incorporated bone tissue engineering polymer scaffolds should assist the process of osteoinduction. This approach is novel, as it introduces a nanoparticle-based biophysical rather than biochemical cue to affect the osteodifferentiation of MSCs with potential implications for other bone tissue engineering strategies. For instance, MSCs could be labeled *ex vivo* with nanoparticles that enhance the PA effect, seeded onto a carrier scaffold, implanted into a bone defect, and stimulated toward osteoblasts. Further, our nonpharmacological strategy based on the bone's sensitivity to mechanical/acoustic signals does not possess the traditional limitations of pharmacological growth factor-based approaches for bone regeneration, such as unstable biological activity, short half life, and minimal tissue penetration.³⁹

The limitation of the current set up for future *in vivo* applications is the use of a visible wavelength laser that would not be suitable for generation of acoustic waves from the

nanoparticles *in vivo* due to the laser's limited tissue penetration. Thus, for *in vivo* applications, a pulsed, near-infrared, or microwave electromagnetic source with deeper tissue penetration should be more suitable.

Conclusion

We have demonstrated that photoacoustically stimulated MSCs cultured on PLGA films without osteogenic supplemented media have increased levels of osteodifferentiation with up to a 350% increase in calcium content compared with nonstimulated samples. The osteodifferentiation of the MSCs were further enhanced (131% increase in calcium content) when the PLGA films were incorporated with small amounts of SWCNTs. Our results indicate that the SWCNT-enhanced PA effect improves the osteoinductive properties of PLGA, thereby introducing a novel biophysical rather than biochemical strategy to assist the process of osteoinduction using bone tissue engineering strategies.

Acknowledgments

This study was supported by the Office of the Vice President of Research at Stony Brook University and NIH Director's New Innovator Award (IDP2OD007394-01).

Disclosure Statement

No competing financial interests exist.

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Received: December 06, 2010

Accepted: March 10, 2011

Online Publication Date: April 27, 2011