RESEARCH ARTICLE

Controlled Release of Polyethyleneimine-Simvastatin Acid Nanoparticles from Conducting Matrices and Their Osteogenic Potential

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Abstract

Aim: The aim of the present study was to develop polyethyleneimine-simvastatin acid nanoparticles (PEI-SVA-NPs) loaded conducting scaffolds as drug delivery for osteogenesis in bone tissue regeneration. **Materials and Methods:** The PEI-SVA-NPs were prepared by nanoprecipitation by mixing PEI and SVA solutions. The freeze-dried NPs were loaded at 0.1, 0.5, and 1 mg onto the conducting scaffolds comprising chitosan grafted polyaniline/chitosan-gelatin matrices for drug release, biocompatibility, and alkaline phosphatase (ALP) activity studies. **Results and Discussion:** The PEI-SVA-NPs loaded conducting matrices exhibited slower drug release profiles compared with the free drug ones. Biocompatibility of the matrices was excellent on MC3T3-E1 cells. The PEI-SVA-NPs loading showed higher efficacy on promotion of osteogenesis than the free drug loading. Increasing ALP activity was found with increasing the PEI-SVA-NPs loading. **Conclusion:** The PEI-SVA-NPs loaded conducting matrices exhibited controlled release, biocompatibility, and osteogenic properties. The results strongly support the possibility of these matrices to be useful for bone tissue engineering application.

Key words: Drug release, nanoparticle, osteogenesis, polyethyleneimine, scaffold, simvastatin

INTRODUCTION

Cytokines and growth factors such as bone morphogenetic protein-2 (BMP-2), transforming growth factor-β, and vascular endothelial growth factor are known to enhance bone tissue engineering.[1] Compared with these macromolecules which are expensive and instable, the small molecules such as statins have been reported as bioactive substances to promote osteogenesis.[2] Statins are effective cholesterol-lowering drugs which are specific inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in cholesterol synthesis.[3] The drugs are known to promote bone formation by increasing BMP-2 mRNA expression, enhancing vascular endothelial growth factor expression[4] and reducing bone reabsorption by subsiding RANKL and cathepsin K expression.[5]

Simvastatin (SV) is one of the statins commonly used in lowering blood cholesterol levels. Local delivery of SV has gained importance in enhancing the anabolic effect in bone repair.[6] Several strategies have been attempted to control release and enhance osteogenic efficacy of SV for bone tissue regeneration. Promotion effects of SV loaded in methylcellulose gel under a polylactic acid dome membrane which was applied locally to introduce mandibular bone healing have been reported.[7] SV loaded poly-(dl-lactide-co-glycolide) acid microparticles were proven for localized controlled release of SV and increasing proliferation, differentiation, and mineralization of osteoblastic cell lines.[8] Poly(ethylene glycol)-poly(ε-caprolactone) micelles containing SV were efficient on enhancing osteoblast differentiation and mineralization by stimulation of the BMP-2 expression. These micelles showed

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prolonged release and higher osteogenic potential compared with the free drug.[9] Simvastatin acid (SVA) is an active metabolite of SV and it is known to stimulate osteogenesis by accelerating the expression of BMP-2 in osteoblasts.[10] SVA coated beta-tricalcium phosphate particles have been reported to accelerate bone formation by enhancing ALP activity, calcium deposition, and mRNA expressions of alkaline phosphatase (ALP) and osteopontin.[11]

Fabrication of polyethyleneimine (PEI)/SVA nanoparticles (PEI-SVA-NPs) by nanoprecipitation for osteogenesis application has been granted (Indian patent-201741008709).[12] PEI has been applied as non-viral vectors for gene delivery of ideal characteristics such as high transfection, less cytotoxicity, less immunogenicity, no carcinogenicity, and controlled expression.[13] High transfection efficiency of PEI is attributed due to a phenomenon known as the "proton sponge effect" which mimics the viral transfection.[14] Among different molecular weights of PEI, the branched 25 kDa polymer has been considered as an efficient gene delivering agent and gold standard.[15] PEI-DNA polyplexes incorporated onto collagen-based scaffolds were capable to promote bone regeneration by prolonged and elevated transfection efficiency.[16]

Conducting scaffolds comprising electroactive materials grafted onto biodegradable scaffolds are known as excellent templates for tissue formation.[17] Polyaniline (PANi), a conducting polymer, exhibiting high conductivity, biocompatibility, and thermal stability has been reported to be useful in biomedical application and controlled release drug delivery.[18] Grafting of PANi with the chitosan (chi-g-PANi) has been reported to provide the conducting properties to the final composite.[19] Osteogenic differentiation can be promoted by enhancing cell-cell communication, cell adhesion, and cell differentiation.[20]

The present study was aimed to control release of SVA from the PEI-SVA-NPs loaded conducting matrices comprising chi-g-PANi/chitosan-gelatin for bone tissue engineering [Figure 1]. Surface morphology, drug release, biocompatibility, and ALP activities of the matrices were determined. Controlled drug release of the NPs loaded matrices was expected to enhance ALP activities of the MC3T3-E1 cells conditioned with these matrices.

MATERIALS AND METHODS

Materials

Polyethylenimine, branched (average Mw 25kDa), chitosan (medium molecular weight), fish gelatin (gel strength 300 g bloom), and glutaraldehyde (25% in water) were purchased from Sigma-Aldrich, Germany. MC3T3-E1 (preosteoblast) cell line was purchased from the American Type Culture Collection (ATCC), Virginia, USA. Alpha-minimum essential medium (α-MEM), antibiotics, and enzymes were purchased from Gibco (Gibthai, Bangkok, Thailand). All other chemicals were obtained as reagent grade.

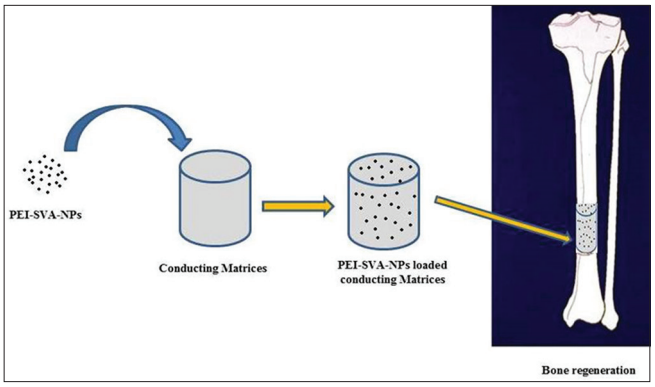

Figure 1: Scheme of loading polyethyleneimine-simvastatin acid nanoparticles onto conducting matrices for bone regeneration

Preparation of SVA solution

SVA solution was prepared by hydrolysis of SV in alkali solution.[21] Briefly, an accurate amount of 420 mg SV was dissolved in 10 ml of 95% ethanol at room temperature. Then, 1.5 ml of 0.1 M NaOH was added into the solution under warming at 50°C, and the reaction was allowed for 2 h. The resultant solution was adjusted to pH 7.2 by addition of 0.1 M HCl, and the volume was adjusted to 10 ml with milliQ water. The obtained SVA solution of 10 mM was filtered through a 0.45 μm membrane and kept at −20°C until used.

Preparation and characterization of PEI-SVA-NPs

PEI-SVA-NPs were prepared following a patented method (Indian patent-201741008709).[12] Nanoprecipitation was performed by mixing 14:25 (v/v) of 0.05% PEI and 10 mM SVA and solutions under constant stirring. The obtained NPs were freeze-dried (FTS systems, Tokyo, Japan). Morphology of the NPs dispersed in phosphate-buffered saline (PBS) was determined by transmission electron microscopy (TEM). Thin films of NPs were prepared by dropping 10 μl of the samples on the carbon-coated copper grids. The excess fluid was removed from grids using pieces of blotting paper. The films on the TEM grids were allowed to air dry. The image analysis of NPs was performed using TEM instrument (JEOL-2010, Tokyo, Japan) operated at accelerating voltage of 200 kV.

Preparation of chi-g-PANi conducting matrices

Chi-g-PANi was prepared by previously described method[18] and incorporated onto the chitosan-gelatin matrices by freeze-drying technique. Briefly, 6% gelatin solution was prepared by dissolving gelatin in water at 50°C under constant stirring

for 20 min until complete solution. A solution of 0.9% chitosan was prepared by dispersing chitosan in 0.1 M acetic acid under stirring at room temperature for 5 h. Amount of chi-g-PANi equivalent to 0.3% w/w of the chitosan solution was mixed and stirred overnight. Then, chi-g-PANi/chitosan and gelatin solutions were blended at 1:1 w/w ratio. The mixture was cross-linked with 5% glutaraldehyde (1:200 w/w glutaraldehyde:polymer mass) for 3 h under stirring at room temperature for 3 h. The mixture was poured into the polystyrene molds with the diameter of 15 mm and the height of 5 mm, kept at 4°C for 24 h, then −20°C for 24 h, followed by freeze-drying (FTS systems, Tokyo, Japan). The surface morphology of the matrices was observed by scanning electron microscopy (SEM, JEOL, JSM-5200, Tokyo, Japan) at an operating voltage of 25 kV.

Loading of PEI-SVA-NPs onto conducting matrices

The conducting matrices were cut into circular discs of a dimension of 1 cm diameter and 0.5 cm height. The freeze-dried NPs were dispersed in 500 µl PBS, pH 7.4, then, the dispersion equivalent to 0.1, 0.5, and 1 mg of NPs was loaded onto each of the matrices placed on the 12-well cell culture plates. Incubation was allowed overnight at 37°C for complete adsorption of PEI-SVA-NPs onto the matrices.[22] The matrices with free SVA loading were prepared following the same procedure.

In vitro drug release

The PEI-SVA-NPs and the free drug loaded matrices were carefully placed into the 12-well plates containing 2 ml of PBS, pH 7.4 at 37°C as the release medium under gentle shaking. At predetermined time intervals, 1 ml of the medium was collected from each well and replaced with the fresh PBS.[22] The experiment was carried out at least in triplicate. Concentrations of SVA in the collected samples were determined by high-performance liquid chromatography (HPLC) analysis (Perkin Elmer Series 200, Shelton, CT, USA) using a Thermo series chromatograph consisting of a C18, 5 µm, 150 mm × 4.60 mm column at 25°C ± 2°C and the UV/vis detection performed at a wavelength of 239 nm. 20 microliters of samples were introduced to the column, and the separation was performed by isocratic elution with a mobile phase of 70:30 v/v of acetonitrile: 0.03 M potassium phosphate buffer, pH 4.5 at a flow rate of 1.0 ml/min. Detection of each HPLC samples was carried out at least in triplicate. Various concentrations of SVA solutions (0–500 µg/ml) were determined by HPLC using the same conditions to create the standard calibration curve and calculate the amounts of SVA released from the samples.

Cell culture

MC3T3-E1 cells 1x10^7 cells/mL were cultured in 75-ml cell culture flasks containing 15 ml of α-MEM supplemented with 10% fetal bovine serum and 1% antibiotics (100 U/mL of penicillin G and 100 µg/mL streptomycin), and ascorbic acid (50 mg/ml) incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.[23] The culture media were changed on the other day. After the cell cultures reached 70–80% confluence, the cells were trypsinized using 5 ml of trypsin solution (0.05% trypsin, 0.53 mM EDTA). Subcultures not more than three passages were harvested for the subsequent studies. The cell suspensions were prepared at desired density by dilution with culture media.

Determination of biocompatibility

Biocompatibility of the PEI-SVA-NPs loaded matrices was determined according to ISO 10993-5.[24] Cell viability of MC3T3-E1 cells was determined by MTT assay to confirm non-toxicity of the samples compared with 100 ppm zinc acetate solution as cytotoxic agent and culture media as control. The matrices (1.5 cm diameter, 0.5 cm height) were sterilized by ethylene oxide gas and incubated in 1 ml culture media at for 48 h. A volume of 100 µl cell suspension (4×10^5 cells/ml) was seeded the 96-well cell culture plates and incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 24 h. Then, culture media was removed and substituted with the sample extracts. The cells were incubated with the samples for 24 h. The MTT assay was performed after removing the samples and washing the cells twice with PBS. MTT solution (0.5 mg/ml) 100 µl was added into the cells on each well. After incubation at 37°C for 4 h, the supernatant of each well was carefully discarded. After addition of 100 µl DMSO as a solvent, the absorbance at 570 nm of each sample was measured using microplate reader (Biotek PowerWave® X microplate spectrophotometer, USA). Cell viability was calculated as following Equation (1).

$$\text{Cell viabliity}[\%] = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (1)$$

Determination of ALP activities

The PEI-SVA-NPs and the free SVA loaded matrices were determined for their osteogenic potential by measurement of ALP activities of MC3T3-E1 cells conditioned with these samples. Briefly, 100 µl of MC3T3-E1 cells at a density of 4×10^5 cells/ml was seeded onto the matrices in 24-well plates. After cell seeding, the samples were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C at 37°C to allow cell attachment. After 4 h, 1 ml of culture media was added into each well and the cells were conditioned with these samples for 5 days. The ALP activities of the treated cells were determined on day 1, day 3, and day 5. The matrices were collected and the cells were lysed with triton-X and allowed to sonicate in ice bath. The cell lysates were obtained and centrifuged at 12,000 g at 4°C for 15 min. The supernatants of the samples were collected for the ALP determination using *p*-nitrophenyl phosphate method.[25]

Statistical analysis

The results are expressed as mean ± standard deviation. Student's *t*-test was utilized to assess statistical significance of the data. The sample size was at least $n = 3$ and $P < 0.05$ values were considered statistically significant.

RESULTS AND DISCUSSION

Preparation of PEI-SVA-NPs

The PEI-SVA-NPs were produced by nanoprecipitation method (Indian patent-201741008709).[12] Surface morphology of the NPs determined by TEM as shown in Figure 2. The NPs were in spherical shape with the particle size <200 nm. It can be assumed that this strong interaction between the positively charged PEI and the negatively charged SVA moieties may lead to the tight coiling and condensing behaviors, which is a specific character of PEI.[26]

SEM determination of Chi-g-PANi conducting matrices

Matrices characters such as porosity, pore size, inter-connecting pores, and surface-to-volume ratio are critical for an artificial tissue construct.[27] The SEM micrograph of the conducting matrices is shown in Figure 3. The well interconnected pores have been reported as one of the key factors which determine the matrices efficacy.[28] The matrix porosity can allow permeation and transportation of vital nutrients, as well as removal of metabolic wastes to facilitate ingrowths of cellular architectures.

Drug release study

Drug release from the PEI-SVA-NPs incorporated conducting matrices was investigated under sink condition in PBS, pH 7.4 at 37°C. As shown in Figure 4, release of SVA from the NPs loaded matrices was controlled over 24 h. The drug was released from the NPs loaded matrices in a sustained manner with a reduced initial burst release compared with the free drug loading. Burst release was attributed to the free drug weakly adsorbed on the porous matrices. Slower drug release was observed in the matrices with increasing the amount of NPs. After 24 h, the matrices with 1 mg NPs loading exhibited 30% drug release, followed by 55%, and 85% from the 0.5 mg, and 0.1 mg NPs loading, respectively. The sustained release profiles of the NPs loaded matrices could be attributed to strong interaction between the PEI and SVA.[26]

Biocompatibility of drug loaded matrices

The *in vitro* cytotoxicity of the PEI-SVA-NPs loaded conducting matrices was determined with MC3T3-E1 cells

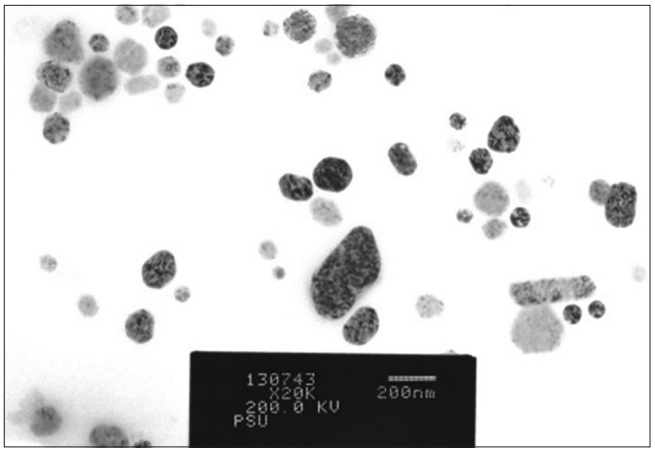

Figure 2: Transmission electron microscopy micrograph of the polyethyleneimine-simvastatin acid nanoparticles

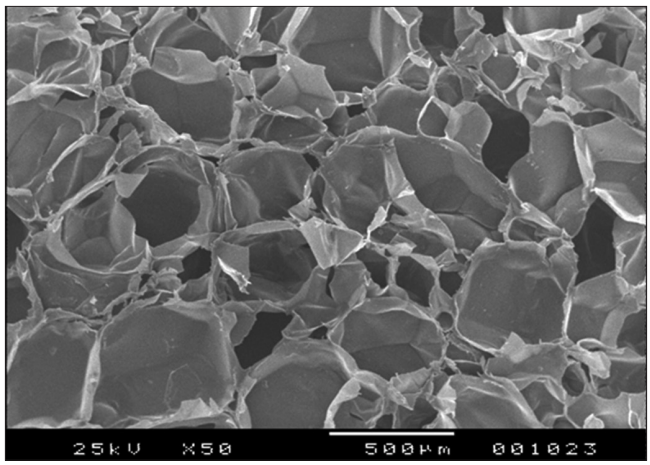

Figure 3: Scanning electron microscopy micrograph of chi-g-polyaniline chitosan/gelatin scaffold

by cell viability assay. The matrices with increasing NPs and free drug loading did not exhibit cytotoxic effect on the cells [Figure 5]. Higher cell viability is revealed in the samples of NPs loading compared with the free drug loading. The results implied that controlled release characteristics of the NPs loaded matrices could contribute to less toxicity of the samples.

ALP activity on PEI-SVA-NPs loaded matrices

SV has been reported to induce BMP-2 expression and enhance osteogenesis.[29,30] Miro/NPs have been studied as delivery systems of SV for bone tissue engineering. Controlled release of SVA from the three PEI-SVA-NPs loaded conducting matrices revealed as mentioned above. To investigate osteogenic ability of these matrices, ALP activity of MC3T3-E1cells was detected as an initial osteogenic marker. No enhancing effect was observed on the cells conditioned with 0.1 mg free drug loaded matrices. Even though free SVA was available, there was no marked increase in ALP activity due to transient osteogenic effect of

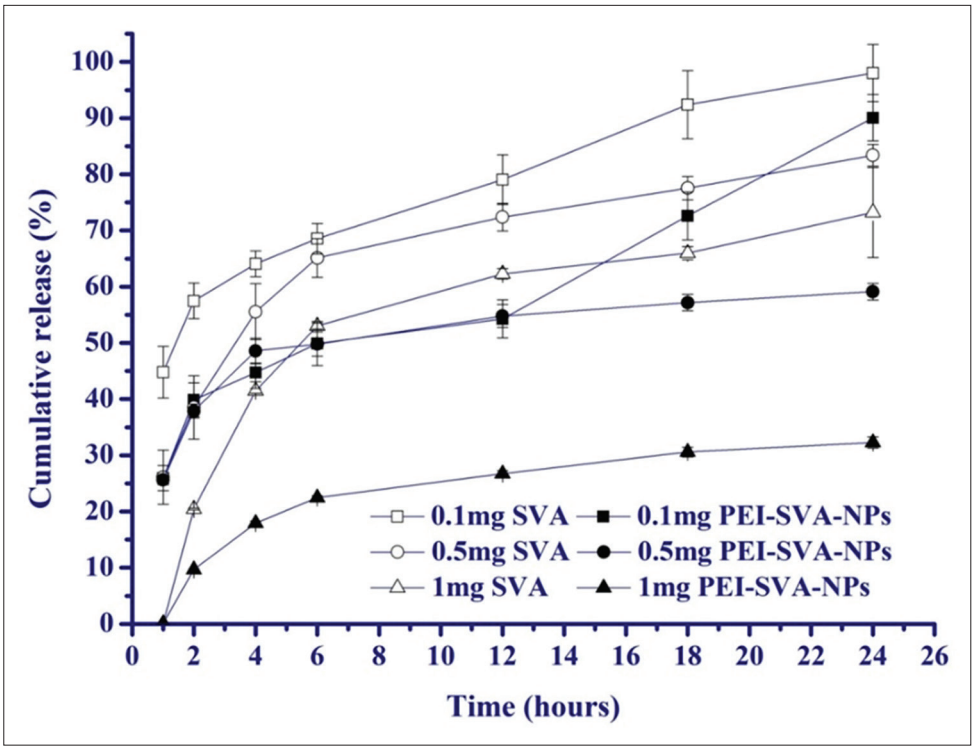

Figure 4: Release of simvastatin acid (SVA) from polyethyleneimine-SVA nanoparticles loaded conducting matrices (mean ± standard deviation, *n* = 4)

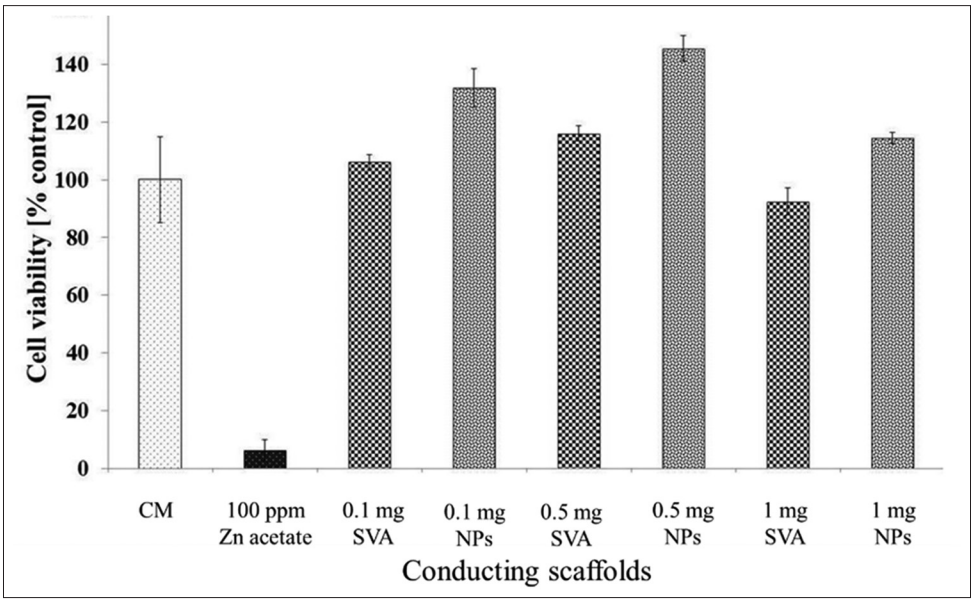

Figure 5: Biocompatibility of polyethyleneimine-simvastatin acid nanoparticles loaded conducting matrices on MC3T3-E1cells (mean ± standard deviation, *n* = 3)

free drug. Significant difference on ALP activity between the 0.1 mg PEI-SVA-NPs and the free drug loaded matrices was observed from 1-5 days ($P < 0.05$). The highest ALP activity on day 5 of cells conditioned with the matrices loaded with 1 mg PEI-SVA-NPs can be considered to be attributed to controlled drug release [Figure 6]. Results from the present study closely align with the above literatures indicating enhanced ALP activity in the presence of conducting polymer and PEI-SVA-NPs.[20,31]

CONCLUSIONS

In the present study, PEI-SVA-NPs were prepared and loaded onto the conducting matrices as a drug delivery system for SVA. The conducting matrices showed excellent architecture which can be essential for cellular activities. Controlled release properties and biocompatibility of the PEI-SA-NPs loaded matrices were revealed. These matrices were efficient

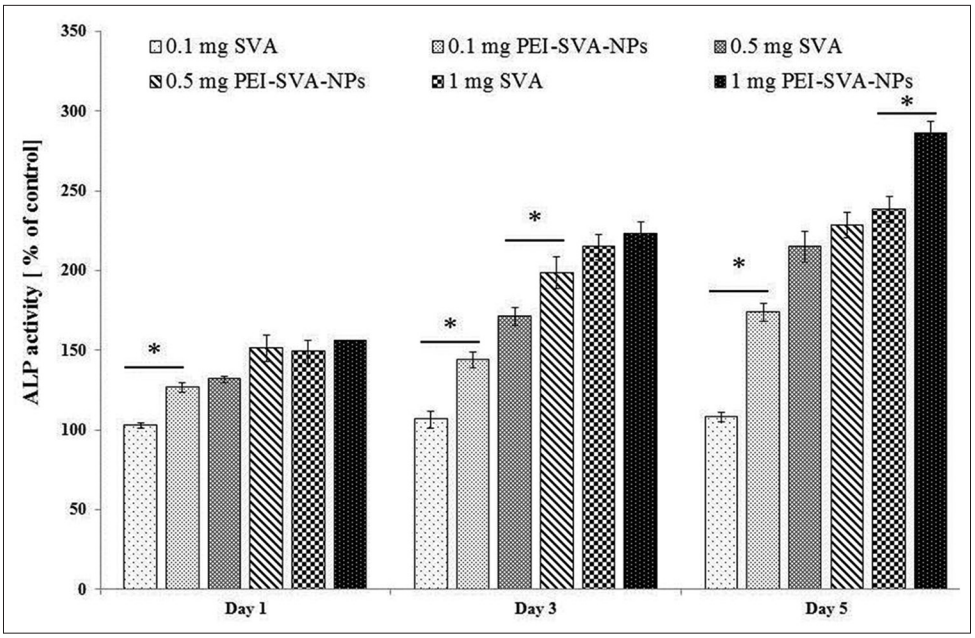

Figure 6: Alkaline phosphatase activity of polyethyleneimine-simvastatin acid nanoparticles loaded conducting matrices on MC3T3-E1cells (mean ± standard deviation, $n = 4$).Significant differences between the groups are shown as $^*P < 0.05$

to promote osteogenesis on MC3T3-E1cells and proven to be useful as drug delivery for bone tissue engineering.

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