

Potent bioactive bone cements impregnated with polystyrene-g-soybean oil-AgNPs for advanced bone tissue applications

Elif Ilhan, Zeynep Karahaliloglu, Ebru Kilicay, Baki Hazer & Emir Baki Denkbaz

To cite this article: Elif Ilhan, Zeynep Karahaliloglu, Ebru Kilicay, Baki Hazer & Emir Baki Denkbaz (2019): Potent bioactive bone cements impregnated with polystyrene-g-soybean oil-AgNPs for advanced bone tissue applications, Materials Technology, DOI: [10.1080/10667857.2019.1661157](https://doi.org/10.1080/10667857.2019.1661157)

To link to this article: <https://doi.org/10.1080/10667857.2019.1661157>



Published online: 28 Sep 2019.



Submit your article to this journal [↗](#)



Article views: 4



View related articles [↗](#)



View Crossmark data [↗](#)



Potent bioactive bone cements impregnated with polystyrene-g-soybean oil-AgNPs for advanced bone tissue applications

Elif İlhan^a, Zeynep Karahaliloglu^b, Ebru Kilicay^c, Baki Hazer^{d,e,f} and Emir Baki Denkbaz^{g,h}

^aDepartment of Nanotechnology Engineering, Bülent Ecevit University, Institute of Science, Zonguldak, Turkey; ^bFaculty of Science, Department of Biology, Aksaray University, Aksaray, Turkey; ^cZonguldak Vocational High School, Bülent Ecevit University, Zonguldak, Turkey; ^dDepartment of Aircraft Airframe Engine Maintenance, Kapadokya University, Nevşehir, Turkey; ^eDepartment of Chemistry, Bülent Ecevit University, Zonguldak, Turkey; ^fDepartment of Nanotechnology Engineering, Bülent Ecevit University, Zonguldak, Turkey; ^gBioengineering Division, Hacettepe University, Institute of Pure and Applied Sciences, Ankara, Turkey; ^hFaculty of Engineering, Department of Biomedical Engineering, Başkent University, Ankara, Turkey

ABSTRACT

Postoperative infection in orthopaedic and trauma surgery is one of the most feared complications. Recently, the high prevalence of multidrug-resistant bacteria has made the antibiotic treatment ineffective; thus novel non-antibiotic alternative approaches to this problem are urgently needed. Based on these expectations, in this work, in order to enhance the cytocompatibility and antibacterial performances of poly (methyl methacrylate (PMMA) and beta-tricalcium phosphate (β -TCP) bone cements were impregnated with polystyrene (PS)-g-soybean oil graft copolymer containing AgNPs (PS-Agsbox), and we assessed the antimicrobial activity of the fabricated bone cements against *Staphylococcus aureus* and *Escherichia coli*. Nanoparticles at concentration of 1.25% (5 β -TCP) w/w in β -TCP bone cements were able to inhibit pathogens growth, while a concentration of 3.75% (15PMMA) was needed for PMMA bone cement. Therefore, the impregnated bone cements with PS-AgsboxNPs may be further explored as an alternative antimicrobial therapy for the treatment of infected bone defects.

ARTICLE HISTORY

Received 22 May 2019
Accepted 23 August 2019

KEYWORDS

Bone cement; polystyrene; pre-osteoblastic MCT3T3-E1 cells; silver nanoparticle; soybean oil

Introduction

The estimated total number of patients who has complained of surgical site infections (SSI) related to orthopaedic procedures ranges from 31,000 to 35,000 [1]. The emergence of SSI results in increased total healthcare costs depends on prolonged hospitalisation, reduced their health-related quality of life and infectious complications ranging from superficial to deep infections. Thus, infection could result in a fatality. Therefore, orthopaedic and trauma device-related infections (ODRIs) are very unwelcome outcome in modern trauma and orthopaedic surgery and still remain as a formidable clinical complication [2]. Numerous factors such as bacteria concentration on device, microorganism host type and performed surgical procedure are efficient on the occurrence of ODRIs. In order to eliminate trauma device-related infection formation, antibiotics were used either parentally or in particularly, were doped into the bone cements. Prophylactic parenteral antibiotic therapies are not very cost-effective because of the requirement of repetitive administration in order to maintain their concentration in blood and the therapeutic level at the target area. Moreover, the therapeutic approach remains incapable of the eradication of the infection caused by microorganisms and orthopaedic implants are still vulnerable to bacterial or fungal contamination. This inevitable situation generally results in persistent device-

related infection. Therefore, the prevention of infection in long term is a major public health concern and novel treatment methods must be tailored to the requirements.

The use of metals, predominantly silver, in all forms such as ionic (Ag^+), silver-containing compounds or the production of nanosilver by the advance of nanotechnology has shown significant potential [3]. As is known, silver has a broad-spectrum antimicrobial action against antibiotic-resistant strains as well as fungal species [4]. Although it is known that silver fragments caused a considerable brain tissue damage, nanosilver at lower doses is safe for mammalian cells [5] and thereby, there is a broad application area such as creams [6], wound wrappings [7], fabrics [8] and eluting surfaces [9]. Two approaches have been attempted for the synthesis of AgNPs: 'top-down' and 'bottom-up'. In the bottom-up approach, during the synthesis, the chemicals used are toxic and non-ecofriendly byproducts occur [10]. In the top-down approach, evaporation-condensation is the frequently used method for synthesis of metal NPs. Furthermore, this method causes some defects in the surface structure of the product [11]. Therefore, the development of green synthesis (using bacteria, fungi or plant and their extracts) of AgNPs could be an alternative to chemical and physical methods with a lot of benefits such as cost-affectivity, eco-friendliness and being an easily scale-up synthesis [12]. Recently, the use of

vegetable oils as stabilising agents for the synthesis of NPs attracted increasing attention from the polymer community [13]. For instance, Zamiri et al. prepared Ag-NPs in virgin coconut oil using the laser ablation (LA) technique. They obtained nanoparticles in size of 4.84, 5.18 and 6.33 nm, which were well dispersed and uniform [14]. Kumar et al. investigated an environmental-friendly chemistry approach to synthesise metal nanoparticles embedded paint through the use of vegetable oil. The synthesised paint displayed very strong antimicrobial activity against Gram-positive and negative bacteria species [15]. In this context, vegetable-oils are the most attractive option owing to their properties such easy-processability, chemical functionality and cost-effectivity [16]. Soybean oil is one of the popular vegetable oils because of its low price and high availability. In this manner, as a new approach, oxidised polyunsaturated fatty acid, i.e., soybean oil has been used as a macroperoxide initiator in the free radical polymerisation of vinyl monomers to prepare for vegetable oil-based block/graft polymers [17]. The vegetable oil-based graft/block copolymers have been successfully used in biomedical applications such as osteogenic differentiation and mineralisation, cell proliferation and cell growth [18].

In this study, we focused on the development of one such ideal bone replacement material containing eco-friendly oxidised polymeric soybean oil, and AgNPs, which are capable of releasing the silver ions at a tunable rate ranging from hours to weeks. In literature, many different antibacterial bone cement impregnated with AgNPs were tested as a replacement for traditional antibiotics. Although these bone replacement materials show antibacterial effect, they may produce hazardous byproducts and consequently cause toxic effects, which might lead to even greater healthcare problems. Therefore, PS-AgsboxNPs at different concentrations were doped in PMMA and β -TCP bone cements, their antimicrobial activity against *S. aureus* and *E. coli* commonly encountered in trauma device-related infections were tested. The concentration of these nanoparticles doped into the bone cements was varied to compare the mechanical, cytotoxic and osteointegration properties of the bone cements.

Materials and methods

Materials

Soybean oil used for polymer synthesis was provided by Çotanak/Altaş Yağ Su ve Tarım Ürünleri Gıda İnşaat Otomotiv Nakliyat San. ve Tic. AŞ Ordu, Turkey. AgNO₃ and dichloromethane (DCM) were obtained from Sigma-Aldrich (Germany). PMMA and β -TCP-based bone cements were supplied by Oliga Ltd., Cement Oliga1® and Gelişim Medikal, Neocement®, respectively. Calcium Colorimetric Assay Kit (MAK022) and Alkaline Phosphatase

Diethanolamine Activity Kit (AP0100) were provided by Sigma-Aldrich Chemical Company, St Louis, MO, USA.

Cell culture and bacterial strains

Pre-osteoblastic, MCT3T3-E1 cells were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in α -MEM (Minimum Essential Medium), supplemented with 10% fetal bovine serum and 1% UI ml⁻¹ penicillin–streptomycin in tissue culture flasks, at 37°C, in an incubator with 5% CO₂. The bacterial strains *E. coli* ATTC25922 and *S. aureus* ATTC25923 were supplied from ATCC. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl tetrazolium bromide (MTT) and live/dead cell imaging kit (Life Technologies LIVE/DEAD Cell Double Staining Kit) were obtained from Sigma-Aldrich (USA). All other chemicals and solvents were used as received without further purifications.

Synthesis of autoxidised polymeric soybean oil (Psbox) with AgNPs (Agsbox)

Autoxidation of Agsbox was performed according to the previously established protocol [19]. Briefly, 2.7 g of soybean oil was spread out into a Petri dish (Φ = 7 cm, oil thickness: 0.7 mm) and 0.50 g of AgNO₃ was mixed with this oil using a glass rod. The oil layer was exposed to daylight in the air at room temperature for 40 days. A brown viscous polymer was formed due to the presence of AgNPs. The soluble part of the polymeric soybean oil was removed by the chloroform extraction method and was dried under a vacuum oven at room temperature for 24 h.

Copolymerisation of styrene with Agsbox (PS-Agsbox)

Polymerisation procedure of styrene was initiated according to procedures previously cited in literature [19]. Firstly, 4.52 g of styrene and 0.05 g of Agsbox was dissolved in toluene and transferred into the Schlenk tube. Then, argon gas was passed into the flask for about 3 min in order to remove reactive species like oxygen and nitrogen. The tightly closed bottle was incubated into a water bath at 95°C for 6 h. After that, the content of the tube was treated with methanol to precipitate the polymer and, the precipitated copolymer sample was dried under vacuum at 40°C for 24 h. Finally, the sample was dissolved in CHCl₃, and the polymer film approximately the size of a petri dish (Φ = 5 cm) was casted from solution.

¹H-NMR spectra in CHCl₃ solution of the polymer was obtained at a temperature 25°C with a Bruker DPX-400, 400-MHz High-Performance Digital FT-NMR Spectrometer (Karlsruhe, Germany). Attenuated total reflectance–Fourier transform infrared spectroscopy

(ATR-FTIR) in a spectral range of 550–4000 cm^{-1} spectra was recorded using a Perkin-Elmer SpectrumOne (Nicolet 520, USA) instrument.

Synthesis of PS-Agsbox NPs

Synthesis of PS-Agsbox NPs was carried out by double-emulsion solvent evaporation technique and Tween 80 (6%) was used as a surfactant to obtain an emulsion. Briefly, PS-Agsbox (1 mg/mL) was dissolved in DCM as an organic solvent to give a clear yellow solution. Then, the polymer phase was added dropwise to an aqueous phase using a probe sonicator (T 125 Digital Ultra Turrax Homogeniser; IKA, Germany) under ice water bath. The emulsion formed was sonicated for another 2 h to allow evaporation of the organic solvent. Following evaporation step, the resulting nanoparticles were collected by ultracentrifugation (Centrifuge 5810R; Eppendorf, Germany) at $\sim 12,000$ rpm for 30 min, and the obtained nanoparticle pellet was washed three times and finally, the pellet was freeze-dried to obtain free-flowing powder for further experiments.

To determine optimum conditions for nanoparticle synthesis during the emulsification-solvent evaporation process, several variables such as polymer concentration, Tween-80 concentration and homogenisation rate were tested and the effects of process variables on the preparation of PS-Agsbox NPs were studied first in this investigation. Particle size, polydispersity index (PDI), and zeta potential (ZP) analysis was carried out using a dynamic light scattering (DLS; model 3000 HAS; Malvern Instruments, UK). After the selection of homogenisation speed and polymer/Tween-80 concentration for small particles size, the formulation optimised was used in all experiments.

A Fourier transform infrared spectrophotometer (Perkin-Elmer SpectrumOne, Thermo Nicolet) was used to investigate the chemical structure of the PS-Agsbox NPs. The surface structure of NPs was evaluated by field emission scanning electron microscopy using a SEM Quanta FEG 450 (Quanta FEG 450, Germany) operated at a 15.0 kV accelerating voltage. To prepare a sample for SEM, a drop of the NPs solution was placed on a grid, and the particles were then coated with a gold/palladium layer using a sputter coater for 30 s. Furthermore, PS-Agsbox NPs were characterised using AFM (Nanomagnetics Instruments, Ankara, Turkey) in tapping mode.

Bone cement preparation and characterisation

PMMA-based bone cement was fabricated by mixing of PMMA (Cement Oligal®, Ankara, Turkey) (0.4 g),

benzoyl peroxide (150 μL) and PS-Agsbox NPs at various ratios [1.25%, 2.5% and 3.75% (w/w)]. The PS-Agsbox NPs-containing cements were prepared manually by mixing all the compounds until the NPs and PMMA powder was completely wetted. The final mixture sample was poured into a mould and formed in a cylindrical shape by hardening (1 cm in diameter and 0.5 cm height), and this took approximately 1 min. After completely dried, the samples were extracted and stored in dark, sterile conditions prior to use. β -TCP-based bone cements were also prepared by the same method. Briefly, PS-Agsbox NPs [1.25%, 2.5% and 3.75% (w/w)] were mixed with β -TCP of 0.4 g (Neocement®, Portugal), chitosan of 0.07 g, and liquid phase (water, citric acid and glucose) of 150 μL , and the bone cements were allowed to harden.

Scanning electron microscopy (SEM, Quanta FEG 450, Germany), and mercury intrusion porosimetry (Quantachrome Corporation, Poremaster 60) were used to evaluate the morphology of the PMMA- and β -TCP-based bone cements. SEM images were collected at the accelerating voltage at 10 kV.

Water uptake

The PMMA- and β -TCP-based bone cements were weighed (W_{dry}) and placed in 10 mL phosphate-buffered saline solution (PBS), pH 7.4. The specimens were then incubated in an oven at 37°C for 19 days. Following the first day, the bone cements were taken out of PBS at daily intervals and dried on filter paper to remove excess water and weighed (W_{wet}). The samples were returned to the PBS and the solution was renewed after every measurement. The water uptake percentage (W) of the bone cements in PBS was then calculated with the following equation,

$$W = [(W_{\text{wet}} - W_{\text{dry}}) / (W_{\text{dry}})] \times 100$$

The values were expressed as the mean of three independent bone cement samples.

Compression testing of bone cements

Compression testing of bone cements was performed using a universal testing-machine (Zwick Roell, 250 kN, Ulm, Germany). The tests were conducted at a constant compression rate of 10 mN/min and at room temperature. Cylindrical samples, which are 0.5 heights with a diameter of 1 cm, were tested 24 h after the end of mixing. The stress versus deformation responses of cements was recorded, and three samples for each group were used to calculate mean and standard deviation values of compressive strength.

Silver-ion release from bone cements

Silver released from the bone cement samples were determined by Inductively Coupled Plasma – Mass Spectrometer (ICP-MS, ThermoFischer). Prior to testing, the samples were placed in 10 mL of sterile fresh PBS at pH 7.4 and were kept at 37°C for 9 days with continuous shaking. After incubation for different periods of time, supernatant from the bone cement was collected (1 mL) and replaced with the same amount of fresh PBS. For analysis, the samples were diluted 10 times with PBS. Triplicate analyses of three independent samples were performed and mean $\pm$ SD values were reported for each defined time.

Determination of antimicrobial activity in vitro

In vitro antibacterial activities of bone cements were investigated by plate counting method using the previously mentioned bacteria [e.g. *E. coli* (Gram-negative bacteria) and *S. aureus* (Gram-positive bacteria)]. Microbial species were cultivated in a nutrient broth for 24 h at 37°C in a shaking incubator at a speed of 250 rpm. According to the method, bacteria suspensions were adjusted to a 0.25 McFarland standard (approximately 10^4 CFU/mL) and the samples were cultured in 2 mL of the bacteria-containing medium for 24 h. To remove the unattached bacteria on the samples, all of them were rinsed with PBS twice and the supernatant was removed. After 2 mL of PBS buffer was added to the samples, they were gently vortexed to detach of adherent bacteria at the maximum power for 0.5 min. The solutions were serially diluted and the aliquots of 10 μ L were gently spread on the agar plates, and incubated at 37°C overnight. After 24 h incubation, the colonies on the agar plates were counted and the number of CFU was determined.

To determine whether any biofilm formation occurred on the surface of the bone cements after treatment with the microorganisms, a tetrazolium/formazan test was performed. The TTC (2,3,5-triphenyltetrazolium chloride) assay, which reduces to red formazan in the presence of bacteria, was performed in order to indicate the activity and viability of the bacteria cells. Prior to performing this test, the bone cements (1x0.5 cm) were sterilised with UV-irradiation for 30 min. After that, the samples were placed in the well plate using sterile forceps and incubated 2 mL of nutrient broth medium containing 10^4 CFU/mL microorganisms (*E. coli* and *S. aureus*). Sample-free solution was also represented as the control. After the well plates were incubated at 37°C for 3 h, the inoculum solution of 1 mL from each well was transferred to sterilised eppendorf tubes containing 100 μ L TTC solution (0.5%, w/v). The tubes were then incubated in an oven at 37°C for 20 min. The appeared red

formazan solution was centrifuged at 6000 $\times$ rpm for 3 min, the pellets were resuspended in ethanol 50% and then, centrifuged again. Finally, the formazan absorbance, which indicates the activity and viability of the bacteria cells, was read at 480 nm using a microplate reader (Biochrom Asys Expert Plus. Microplate Reader, Holliston, MA).

In vitro cytotoxicity of bone cements

As a model of osteoblast cell behaviour, pre-osteoblastic MCT3T3-E1 cell line (MC3T3-E1) was used for the biocompatibility of the bone cements. Bone cement samples were sterilised with UV prior to use and washed three times with sterile PBS. MC3T3-E1 cells were incubated for 24 h in α -MEM supplemented with 10% fetal bovine serum and 1% UI mL^{-1} penicillin–streptomycin as the culture medium at 37°C, with 5% CO₂ in an incubator. After that, when the cell line reached about %80 confluence, MC3T3-E1 cells were seeded in 96-well plates at 9×10^3 cells per well and 200 μ L of the medium treated with bone cements per well was pipetted and the plate was incubated at 37°C, 5% CO₂ overnight. After 24 h, an MTT assay was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt. As in a typical MTT assay, 200 μ L of 3 mg/mL MTT solution was pipetted to each well after the removal of extraction medium and the well plate was incubated at 37°C, 5% CO₂. At the end of 4 h, the medium was removed and 200 μ L of isopropanol/HCl mixture was added to each well to solubilize the purple formazan crystals. The liquid was read at 570 nm using a microplate reader (Biochrom Asys Expert Plus. Microplate Reader, Holliston, MA). The percentage of cytotoxicity was calculated with the following equation:

$$\text{Cell viability(\%)} = [A_{570} (\text{sample}) / A_{570} (\text{control})] \times 100$$

Cell viability graph was drawn using the mean and standard deviation of three parallel measurements.

In addition to that, on the 3rd day of culture, the MC3T3-E1 cells were fixed with a paraformaldehyde solution (4%, w/v), and SEM was applied to evaluate cell morphology and growth.

Live/dead assay

To visualise the effect of bone cements on cell viability, a live/dead-assay was performed following manufacturer's instructions. Briefly, MC3T3-E1 cells were cultured in 24-well plate at a density of 5×10^3 cells per well and grown overnight. In the meantime, the bone cements were incubated in the cell culture medium at 37°C overnight, and then the samples were removed and the extraction medium of 1 mL was added to the

each well. After the incubation of 3 h, the cell culture medium was removed, and gently washed with PBS. The cells were incubated with calcein-AM/ethidium homodimer-1 solution (100 mL per well) in a 37°C, 5% CO₂ incubator and the MC3T3-E1 cells were observed by a fluorescence microscope (Leica, Germany) including Texas Red and fluorescein (FITC) filters.

ALP activity assay

Alkaline phosphatase assay (ALP) was performed to determine the activity of MC3T3-E1 cultured on bone cements according to the kit instructions for 7 days. Briefly, the cells at a density 2×10^4 cells were seeded onto the samples, and the samples were then washed with PBS. Cells were detached from the bone cements using 0.25% trypsin and cell lysates were centrifuged for 10 min at 5000 rpm, then washed twice with PBS. 20 μ l of the supernatant was put into the each well of 96 well plates containing 20 μ l of *p*-nitrophenol phosphate (pNPP) (0.67 M) and 960 μ l buffer solution. Thereafter, 20 μ l of alkaline phosphatase solution was put into the plate and the absorbance was measured at 405 nm every 5 min. The ALP activity was calculated with following formula;

$$\frac{(\Delta A_{405\text{nm}} / \text{min Test} - \Delta A_{405\text{nm}} / \text{min Blank}) (d_f) (V_f)}{(18.5)(V_e)}$$

where A_{405} is the absorbance of blank and test solution at 405 nm, V_f is the total assay volume, V_e is the volume of the sample solution and β is the extinction coefficient for *p*-nitrophenol (18.5 at 405 nm).

Calcium assay

Calcium Colorimetric Assay was used to determine the mineralise matrix synthesis of MC3T3-E1 cells

cultured on PMMA- and β -TCP-based bone cements at day 7. This assay is based on the chromogenic complex formed between calcium ions and o-cresolphthalein. The manufacturer's protocol was followed to calculate the concentration of Ca⁺² in the samples.

Furthermore, calcium deposition on the cell-bone cement constructs was examined by Alizarin Red-S staining. Cell-bone cement constructs were fixed by cold methanol for 10 min and subsequently washed twice with distilled water and stained with 2% Alizarin Red (Sigma-Aldrich, USA) solution for 3 min. Stained constructs were washed six times and images were captured for observation of calcium deposition.

Statistical analysis

One way ANOVA was used to determine statistical significances between groups. Results were presented as mean $\pm$ SD. For all analyses, a p-value of <0.05 and <0.005 was considered statistically significant.

Results and discussions

Characterisation of PS-Agsbox

Initially, AgNO₃ into soybean oil was reduced by the effect of daylight during the autoxidation process for the synthesis of graft copolymer. PS-Agsbox was synthesised by free radical polymerisation of styrene from Agsbox. ¹H NMR spectroscopy and FT-IR techniques were used to analyse the composition of the PS-Agsbox graft polymer. Figure 1 shows ¹H NMR spectra obtained for PS-Agsbox. The spectrum displayed characteristic signatures of autoxidised soybean oil: (i) a peak located at 1.6 ppm coming from (-CH₂-CH₂-COO-), (ii) a peak at 1.2 ppm from -(CH₂)_n-, (iii)

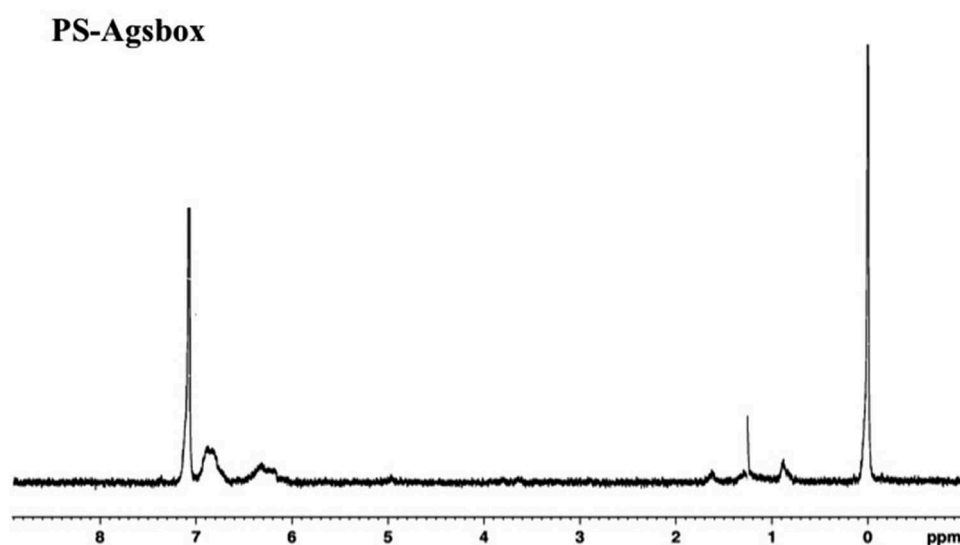


Figure 1. ¹H NMR spectrum of the PS-Agsbox graft copolymer.

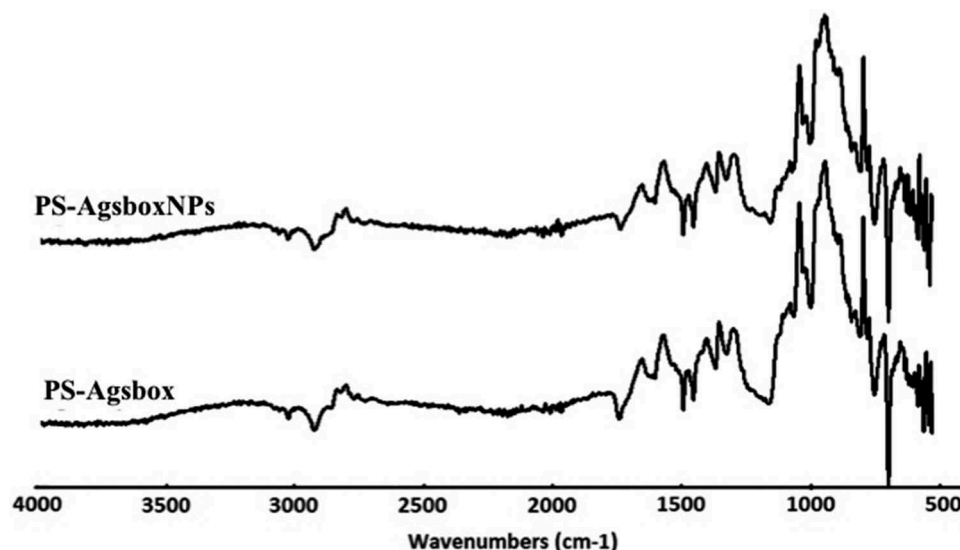


Figure 2. FT-IR spectrum of the PS-Agsbox graft copolymer and PS-AgsboxNPs.

Table 1. The characterisation of PS-AgsboxNPs. The different parameter effects on the size and size distribution of nanoparticles.

Polymer concentration (mg/mL DCM)	Tween 80 concentration (mL/mL)	Homogenisation rate (% amplitude)	Size (nm)	Polydispersity index (PDI)	Zeta Potential (mV)
5	0.06	90	402 ± 7	0.119 ± 0.045	-26.5 ± 0.43
3	0.06	90	364 ± 5	0.098 ± 0.063	-28.1 ± 0.020
1	0.06	90	193 ± 2	0.020 ± 0.010	-31.9 ± 0.15
1	0.10	90	252 ± 7	0.115 ± 0.032	-29.2 ± 0.22
1	0.06	90	193 ± 2	0.020 ± 0.010	-31.9 ± 0.15
1	0.03	90	327 ± 6	0.101 ± 0.005	-15.8 ± 0.31
1	0.06	90	193 ± 2	0.020 ± 0.010	-31.9 ± 0.15
1	0.06	70	299 ± 4	0.070 ± 0.077	-30.1 ± 0.25
1	0.06	50	410 ± 1	0.129 ± 0.024	-10.7 ± 0.08

a peak located at 0.9 ppm arising from ($\text{CH}_3\text{-CH}_{2\text{-n}}$), and (iv) a broad peak centered around 7.2 ppm assigned to phenyl from polystyrene [19].

Collected FT-IR spectra in Figure 2 showed characteristic peaks coming from soybean oil and styrene. The most prominent peak occurred at 1748 cm^{-1} , which corresponds to $\text{C}=\text{O}$ stretching of Psbox blocks. The observation clearly demonstrated the autoxidation of soybean oil. The characteristic phenyl group of polystyrene was observed with the broad bands at 1602 cm^{-1} [19].

Characterisation of PS-AgsboxNPs

A systematic study was carried out by changing of polymer and surfactant concentrations, homogenisation rate during synthesis process. Varying synthesis parameters were used to obtain PS-Agsbox nanoparticles in different sizes. The main parameters and the particle size distribution are shown in Table 1. The amount of polymer was varied among 1, 3 and 5 mg under the experimental condition while the concentration of surfactant and the homogenisation rate was kept constant. The particle size increased with increasing polymer concentration as observed in Table 1. As is known, increasing amounts of polymer

led to an increase of particles size because of the polymer precipitation [20]. As another parameter, the particle size is generally affected by the homogenisation process, because more energy released with increasing homogenisation rate applied in the process, which provides rapidly distribution of the polymer phase. As can be seen from Table 1, the nanoparticles having with larger particle sizes (299 ± 4 and 410 ± 1 nm, respectively) were obtained at lower homogenisation speeds (70% and 50%, amplitude) while the particle size was 193 ± 2 nm at a homogenisation rate of 90%.

For nanoparticle preparation step, Tween-80 was selected as a surfactant and the effect of varying its concentration on nanoparticle size and zeta potential were assessed. It was observed that increasing concentration from 0.03 to 0.1 mL (v/v, in distilled water) resulted in a decrease in the particle sizes from 327 ± 6 to 252 ± 7 nm, respectively. The concentration of the surfactant used in the process had a crucial role, because the production of smaller nanodroplets, which had a larger surface area and their formation required more surfactant [21]. The nanoparticle size decreased with an increase in the surfactant concentration. Similarly, an increase in surfactant concentration from 0.03 to 0.06 mL resulted in a nanoparticle formation of smaller size. The decrease

in particle size could have been contributing to the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory, which is based on the agglomeration of nanoparticles if Coulomb repulsion had not been effective enough to keep the NPs apart [22]. Consequently, it is shown in Table 1 that the optimised nanoparticle size was selected as 193 ± 2 nm with a polydispersity index of 0.020 ± 0.010 and zeta potential of -31.9 ± 0.15 mV.

To understand the surface morphology and to provide more information about the size of PS-AgsboxNPs, AFM and SEM investigations were conducted. Examples of SEM and AFM images of the PS-AgsboxNPs are shown in Figure 3. It can be observed that the shapes of NPs were mostly spherical and they had well-distributed particle morphology with a size around 200 nm.

Bone cements characterisation

In this investigation, two types of cements, β -TCP as a bioactive bone cement and PMMA bone cement-containing PS-AgsboxNPs were prepared. SEM images of the 15PMMA and 15 β -TCP bone cement are shown in Figure 3(c,e), respectively. The PS-AgsboxNPs were uniformly distributed in the dry cement matrix. The particles were also completely integrated into the

PMMA matrix. However, they did not separate out in the β -TCP bone cement sample.

The porosity percent and distribution were calculated from the mercury intrusion porosimetry data and given in Table 2. To gain a better understanding, Table 2 summarises the results obtained for the bone cements and the average porosity of 10- and 15 PMMA was found to be $60.03 \pm 6.3\%$ and $71.42 \pm 5.2\%$, respectively, and 15 PMMA exhibited a maximum porosity in all the cement samples. The average porosity for β -TCP was determined to be approximately 50%. The addition of PS-AgsboxNPs particles increased the mean porosity for PMMA bone cements while it did not have any influence on β -TCP.

Table 2. Porosity percent of PMMA and β -TCP bone cements.

Formulation	Porosity%
PMMA	12.36 ± 1.7
5PMMA	27.05 ± 2.5
10PMMA	60.03 ± 6.3
15PMMA	71.42 ± 5.2
β -TCP	45.73 ± 3.2
5 β -TCP	50.71 ± 5.6
10 β -TCP	50.64 ± 4.8
15 β -TCP	55.70 ± 4.1

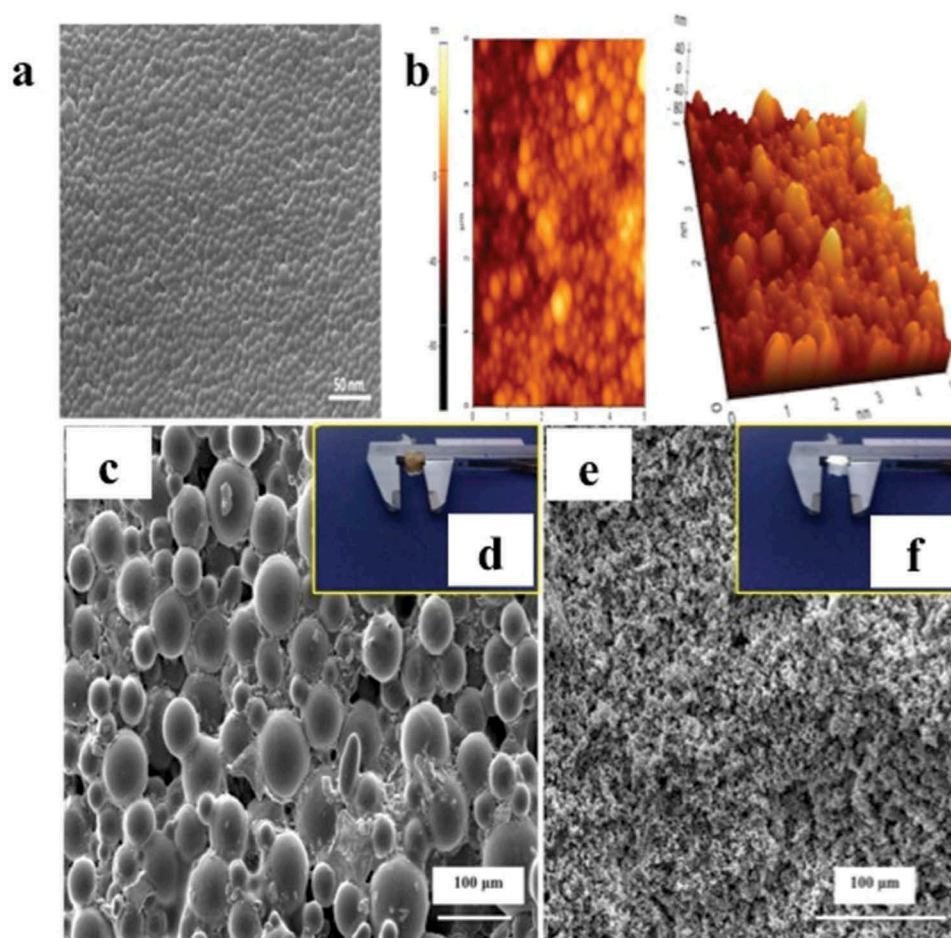


Figure 3. The SEM (a) and AFM (b) images of PS-AgsboxNPs, and SEM images of the 15PMMA (c) and 15 β -TCP (e) containing PS-AgsboxNPs bone cements freshly prepared. Scale bars are 50 nm and 40 nm, and equal to 100 μ m for PS-AgsboxNPs and bone cements, respectively. Inset: Photograph of 15PMMA (d) and 15 β -TCP (f) bone cements.

Commercially PMMA bone cements are incapable of transferring homogenously the load to the surrounding bone tissue because of their high elastic modulus. Furthermore, it has been reported that their mechanical endurance is closely related to the composition of acrylic bone cements [23]. In order to bear load and offer immediate stability, various techniques for PMMA enhancement have been recommended and the compounding of PMMA with other biomaterials is best-known alternative. Tai et al. reported that the mechanical properties of PMMA were considerably diminished with increasing porosity when PMMA was modified with castor oil [24]. Furthermore, He et al. reported porous PMMA bone cements prepared by doping of ingredients such as cellulose and gelatin microparticles to improve bone growth [25].

Water uptake

Figure 4 displays the percentages of water absorption of PMMA and β -TCP containing PS-AgsboxNPs bone cements versus incubation time. The results showed that the water uptake of β -TCP containing PS-AgsboxNPs bone cement formulations at the end of 19 days was higher compared the other counterparts and it clearly showed that their water uptakes profiles were changed dramatically after the encapsulation of PS-AgsboxNPs into β -TCP cements, and the reinforcement of PS-Agsbox nanoparticles to the β -TCP cements enhanced their water uptake capacity. Furthermore, from Figure 4, one can notice that β -TCP containing PS-AgsboxNPs significantly absorbed more water than PMMA containing PS-AgsboxNPs at all time periods. This certified that β -TCP bone cements showed superior water absorbability than PMMA because of PMMA's hydrophobic nature, and the results of the study agree well with the findings obtained by others [26,27].

Compression testing of bone cements

Some physical characteristics of PMMA and β -TCP containing PS-AgsboxNPs bone cements are given in Table 3. Table 3 shows the tensile stress and elongation of break values of the β -TCP and PMMA bone cements. It can be seen that the β -TCP bone cements had a lower initial tensile stress than the PMMA bone cements. Initially, the PMMA bone cement had a tensile stress of 146.68 ± 3 MPa and bone ranged between 80 and 200 MPa. Following the embedding of PS-AgsboxNPs into the cements, the stress of the 5PMMA bone cement increased to 236.79 ± 2 MPa. Similarly, Hamed-Rad et al. have stated that the compression strength of PMMA reinforced with 5% wt AgNPs increased from 102.16 to 121.19 MPa [28].

Further, the experimental results revealed that the mechanical strength did not proportionally increase with the rise of PS-AgsboxNPs content. According to the results, 5PMMA showed the highest compression strength. Ghaffari et al. in their study demonstrated that the content of AgNPs in PMMA significantly improved the mean compressive strength compared to the unmodified PMMA. Further, they did not find a statistically significant difference between the groups containing 0.2% and 2% AgNPs [29]. This argument was supported by Gopalakrishnan et al. [30]. They synthesised silver nanoparticle (AgNPs) based on poly(methyl methacrylate) by a compression-moulding technique. Mechanical performance results showed that a loading beyond 5 wt%, the tensile strength decreased, when adding AgNPs up to 5 wt% led to statistically significant improvement in tensile strength.

According to the results, the compressive strength of β -TCP bone cements was lower compared to the PMMA. In all β -TCP bone cement samples, the compressive strength of 5 β -TCP was found to be 8.28 ± 0.2 MPa. This value was four-fold greater than pure β -TCP bone cement, and higher than the other β -TCP bone cements, likely PMMA bone cements. Similarly, Zhang

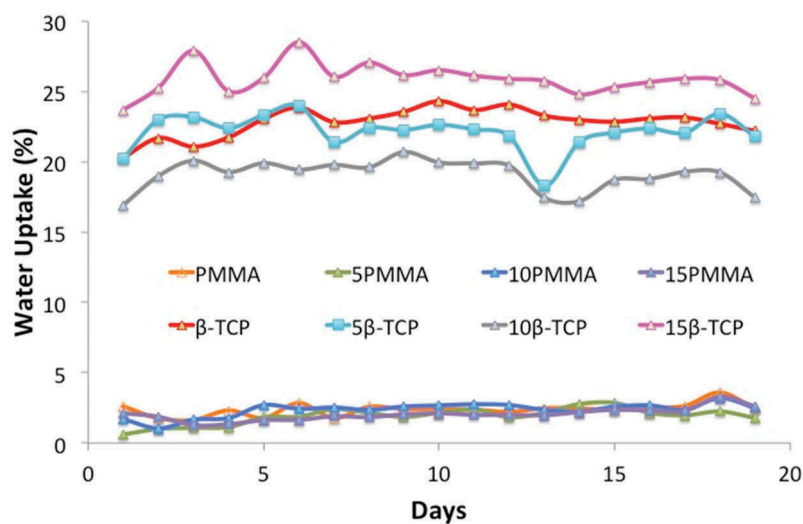


Figure 4. Water uptake of PS-AgsboxNPs-reinforced PMMA and β -TCP bone cements in PBS over time (days).

Table 3. Mechanical properties of PS-AgsboxNPs-reinforced PMMA and β -TCP bone cements.

Sample	Tensile Stress (MPa)	Deformation (%)
PMMA	146.68 $\pm$ 3	67.37
5PMMA	236.79 $\pm$ 2	71.69
10PMMA	164.98 $\pm$ 6	64.14
15PMMA	166.51 $\pm$ 9	61.99
β -TCP	2.57 $\pm$ 0.3	30.18
5 β -TCP	8.28 $\pm$ 0.2	19.30
10 β -TCP	2.82 $\pm$ 0.04	6.05
15 β -TCP	2.60 $\pm$ 0.01	6.94

et al. reported the bacterial cellulose (BC)-reinforced CPC (calcium phosphate cement) composites [31]. BC-incorporated CPC cements displayed two-fold higher compressive strength compared with pure CPC. Furthermore, BC-content was not directly proportional to the compressive strength.

The compressive strength of CP bone cements showed a typical value between 20 and 40 MPa, in agreement with reports stating that it varied from 4 to 120 MPa [32]. Further, Rau et al. prepared Ag-doped calcium phosphate bone cements (CPC-Ag) and the CPC-Ag 0 wt % cement displayed with a compressive strength of 6.5 ± 1.0 MPa, yet CPC-Ag 0.6 wt % and CPC-Ag 1.0 wt % exhibited a compressive strength diminished to 4.0 ± 1.0 and 1.5 ± 1.0 MPa, respectively [33]. These results demonstrated that the mechanical properties of β -TCP bone cements were not directly proportional to the content of PS-AgsboxNPs.

Silver ion release from bone cements

The silver-ion concentrations released from the bone cements into the PBS were measured at previously determined time intervals and is shown in Figure 5. The silver release curve of the bone cements with the same nanoparticles composition showed that the β -TCP bone cements significantly released more silver ions into the PBS than PMMA during the incubation period. Similarly, Perni et al. reported that propylparaben nanoparticles embedded in different bone cement types such as PMMA, hydroxyapatite and brushite. As a result, the whole of the paraben was released from the hydroxyapatite, whereas only about 5% of the initial amount of propylparaben was released from PMMA [34]. Results obtained for PMMA and β -TCP bone cements indicated continuously released Ag^+ ions into the PBS in a desired and controlled manner. Furthermore, it was observed that the concentration of Ag ions released changed proportionally with increases in concentration of PS-AgsboxNPs from β -TCP and PMMA bone cement samples.

Determination of antimicrobial activity in vitro

The antimicrobial properties of the bone cement samples against gram positive (*S. aureus*) and negative

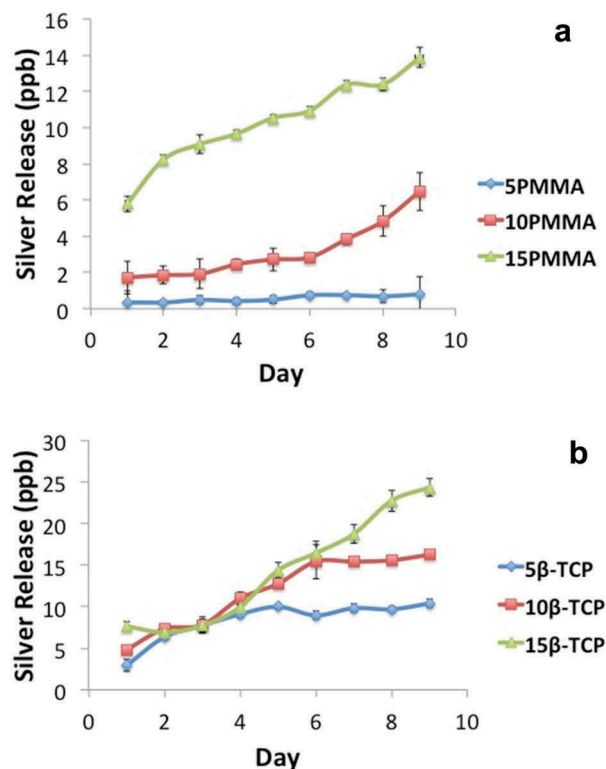


Figure 5. Ag^+ cumulative releases from PS-AgsboxNPs-reinforced PMMA (a) and β -TCP (b) bone cements during 9 days (SD shown as error bars, $n = 3$).

(*E. coli*) bacteria were assessed qualitatively/quantitatively at the first 24 h using plate counting method, and the results are presented in Figure 6. As can be seen in Figure 6, 10 β -TCP and 15 β -TCP caused a significant reduction in the amount of viable *S. aureus*, as compared to the PMMA and 5PMMA group (35 , 37.3 , 4.6 and 3.3 $\text{CFU} \cdot 10^6 \text{mL}^{-1}$ for PMMA, 5PMMA, 10 β -TCP and 15 β -TCP, respectively, $p < 0.005$); whereas those of *E. coli* are 12.6 , 8.8 , 1.9 and 0.6 $\text{CFU} \cdot 10^{11} \text{mL}^{-1}$, demonstrating that these bone cements improved with PS-AgsboxNPs were able to reduce the proliferation of both bacteria. However, the amounts of viable *E. coli* adhered on bone cements samples were much higher than those of *S. aureus*, and β -TCP bone cements containing PS-AgsboxNPs had more significant bactericidal effect against *S. aureus* than against *E. coli*. The mechanism of inhibitory action against gram positive and negative bacteria closely associated with the surface charge [35]. PS-AgsboxNPs have negative charge; they could easily attach with the positive-charged bacteria, i.e. *S. aureus* due to the effect of the electrostatic interaction. Herein, the antibacterial effect of our synthesised PS-AgsboxNPs towards *S. aureus* was greater compared to *E. coli*, and it was confirmed by the aforementioned report.

Additionally, the antibacterial activity of PMMA- and β -TCP bone cement reinforced with different concentrations of PS-AgsboxNPs was evaluated against *S. aureus* and *E. coli* via TTC assay. Figure 7 showed the absorbance of red formazan solution,

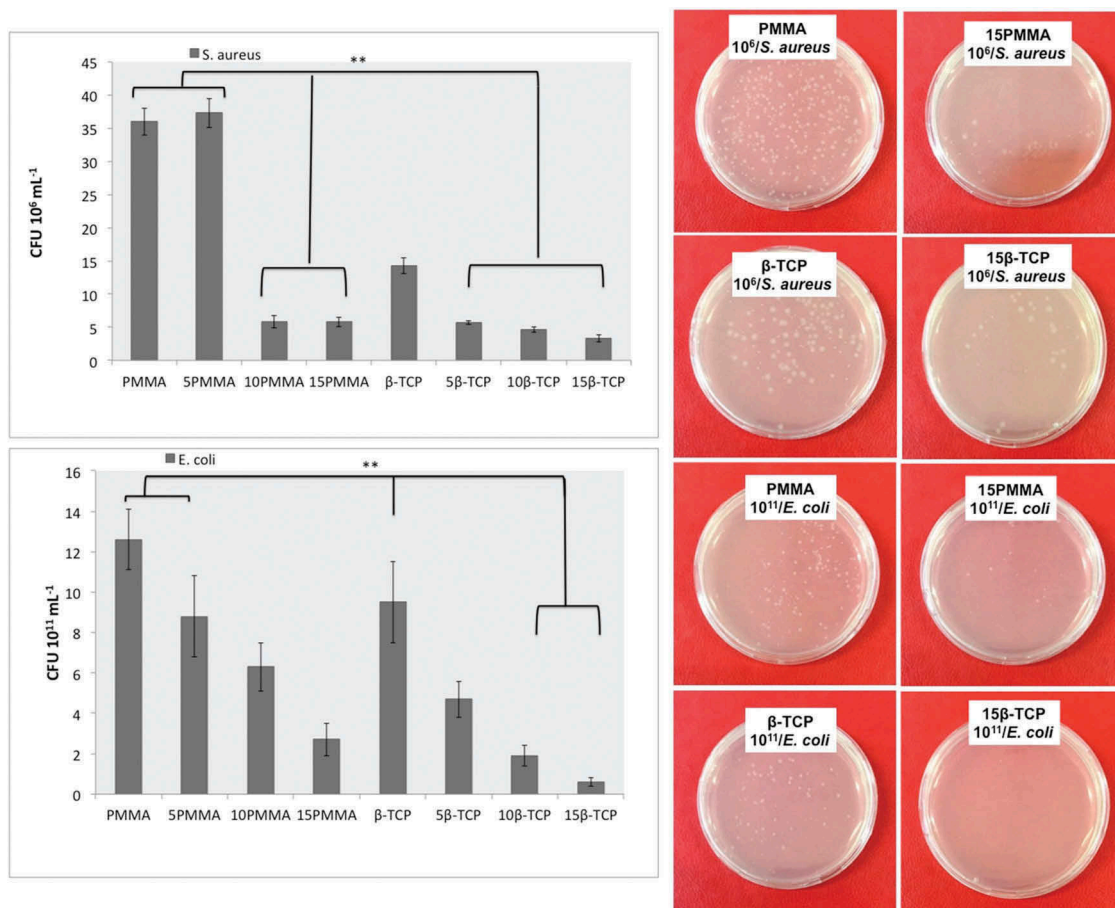


Figure 6. Microbial colonies on PS-AgsboxNPs-reinforced PMMA and β -TCP bone cements were assayed by the plate counting method after treatment of with bacterial strains. Values are mean $\pm$ SEM; $n = 3$. ** $p < 0.005$ compared to PMMA, 5PMMA and β -TCP.

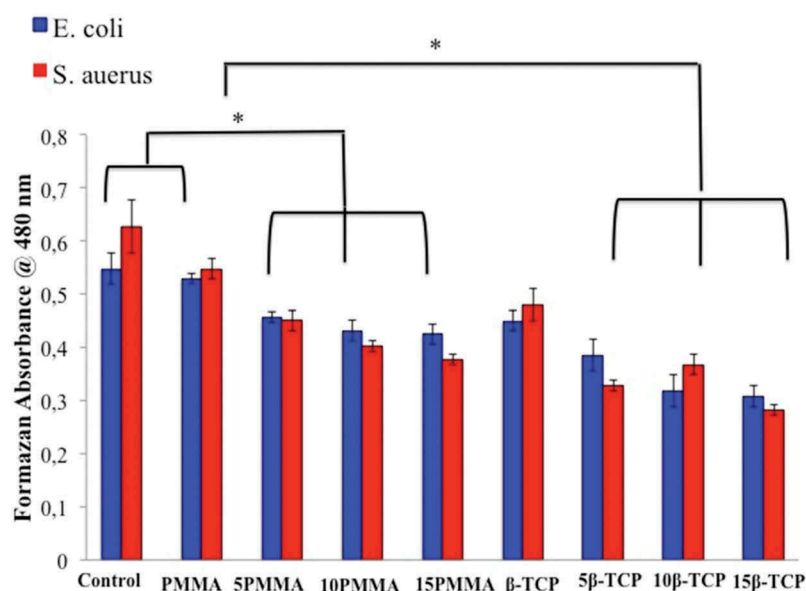


Figure 7. Determination of formed formazan after treatment of bacterial strains with PS-AgsboxNPs-reinforced PMMA and β -TCP bone cements. Values are mean $\pm$ SEM; $n = 3$. * $p < 0.05$ in comparison with control and PMMA samples.

which was formed by the bacteria *E. coli* and *S. aureus* adhered on the surface of the bone cements. It could be observed that the vitality of the bacteria cells reduced depends on the presence of PS-

AgsboxNPs into all PMMA- and β -TCP bone cement samples. Consequently, the antibacterial activity increased with an increase in its concentration. Similary, Ipekoglu and Altıntas synthesised nanosized

calcium deficient hydroxyapatite (CDHA) and nano-sized calcium deficient hydroxyapatite with silver substitution (SCDHA) by a rapid microwave-assisted synthesis method, and they demonstrated that the antibacterial activity increases with the increasing amount of silver substitution [36]. Rau et al. demonstrated that the inhibition zone diameter of silver-doped CPCs (CPCs-Ag) enlarged with effect of increasing Ag^+ concentration [33].

Following treatment with bacteria strains, a reduced biofilm formation was seen after treatment with the reinforced β -TCP bone cement. For example, the obtained absorbance values for pure PMMA and 15PMMA against *S. aureus* were 0.548 ± 0.02 and 0.378 ± 0.01 , respectively, while the absorbance of formed formazan colour at 480 nm were 0.48 ± 0.03 for pure β -TCP and 0.283 ± 0.01 for 15 β -TCP. β -TCP bone cements demonstrated a decrease in biofilm, especially against *S. aureus*. Perni et al. determined the antimicrobial activity of PMMA, hydroxyapatite and brushite bone cements containing paraben nanoparticles. Their results demonstrated that the nanoparticle concentrations with a 5% w/w in hydroxyapatite bone cement were inhibited the growth of microorganisms, whereas performance in the same level for PMMA bone cement needed 7% w/w. In summary, this study showed both the significance of nanoparticle concentration on the antibacterial property and the affectivity of calcium phosphate compared to acrylic (PMMA) bone cements [34].

As a result, a good correlation was found between antibacterial activity behaviours, which were observed from the TTC assay and plate counting method performed against gram-positive and gram-negative bacteria.

In vitro cytotoxicity of bone cements

Cell viability on PS-AgsboxNPs-containing PMMA- and β -TCP-based bone cements were analysed using MTT assay (Figure 8). The data from MTT assay showed that all of the bone cement formulations did not cause significant toxicity to cells at the end of 24 h related to PS-AgsboxNPs-doping. For example, the viability of cells incubated with 15PMMA bone cement remained at about $73 \pm 0.3\%$ while this value for PMMA was $76 \pm 0.5\%$; this indicated that PS-AgsboxNPs-containing PMMA bone cements were not cytotoxic to the cells. β -TCP-based bone cements showed similar results with PMMA counterparts; 6% increase in the proportion of viable cells was observed at concentration of 15 mg/cement compared to the bare β -TCP, at which, the percentage of viable cells is $93 \pm 0.6\%$. This revealed that soybean oil-modified AgNPs incorporated in bone cements were biocompatible and the cells incubated with the different concentration of PS-AgsboxNPs did not reveal significant changes in cell viability.

Pauksh et al. also investigated the biocompatibility with bone-forming cells of PMMA bone cement functionalised with AgNP and/or gentamicin. According to the MTT results, a decrease was not found in cell viability and a differentiation for all three PMMA variants. Furthermore, when they compared the different experimental groups, PMMA functionalised with AgNP showed the highest cell viability at day 21 [37].

When the effect of bone cement formulations on cell compatibility was evaluated, the cell viabilities of β -TCP-based bone cements reached around 100% (%)

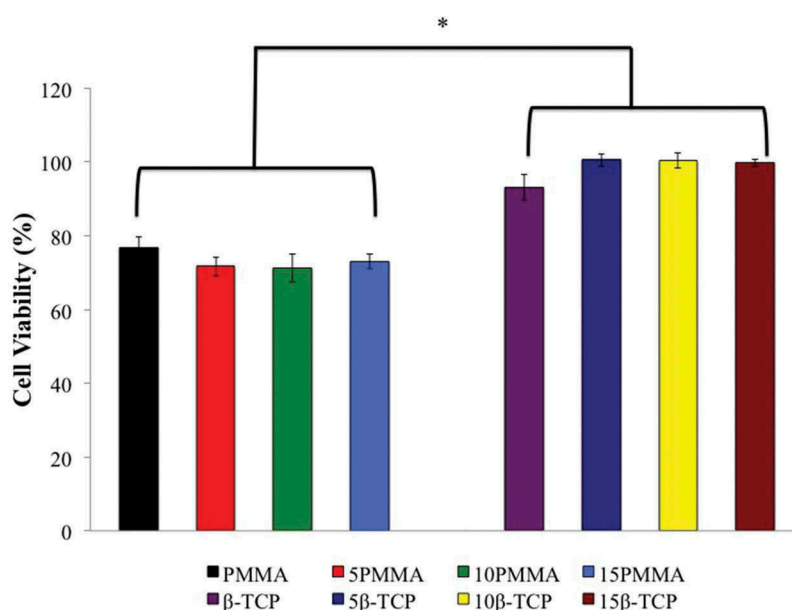


Figure 8. In vitro cytotoxicity results (MTT assay) for bare, PS-AgsboxNPs-containing β -TCP- and PMMA-based bone cements after 24 h treatment. Results were expressed as the percentage and represent the mean $\pm$ standard deviation of triplicate assays. * $p < 0.05$ in comparison with PMMA samples.

99 ± 0.1 , 15β -TCP). The cell viabilities with β -TCP-based bone cement formulations were even higher compared to PMMA-based with a $76 \pm 0.5\%$ of cell viability ($p < 0.05$), suggesting that β -TCP doped with PS-AgsboxNPs could improve the cytocompatibility.

Kang et al. used hydroxyapatite (HA) microspheres as an additive to improve cell-PMMA-based bone cements interaction. While the bare PMMA revealed a limited biocompatibility, the PMMA/HA composites permitted to adhere and spread of cells and they showed a filopodia-like extension. These results demonstrated that the bioactive HA microspheres could be used as an additive and could play an active role to enhance in biocompatibility and osteoconductivity of PMMA bone cements [38]. Similarly, another study reported that the decorated HA nanoparticles using polydopamine (pDA) and BMP2 mimicking peptide (serine-serine-valine-proline-threonine, SSVPT) was doped into PMMA-based cement. The cell viabilities towards hFOB in the presence of extract of PMMA and BaSO_4 /PMMA cements were 66% and 72%, respectively, while that of HA/PMMA reached 80% [39].

The presented SEM images in Figure 9 showed MC3T3-E1 cells attached and spread on PMMA and β -TCP bone cements. The cells were fully attached at multiple focal points and spread on the surface of bone cements with filopodia-like extensions, and created cell-cell interactions. There was no considerable effect of the content of PS-AgsboxNPs on the morphology and distribution of cells. Furthermore, the number of cells attached to PS-AgsboxNPs-containing bone cements, especially β -TCP-based bone cements were greater during the incubation period than that on bare β -TCP- and PMMA-based bone cements (Figure 9(a,d); Figure 9(e,h)). The differences in the cellular response on bone cements were attributed to grain size with ranging from micrometres to several nanometres. It is well known that the surface properties influence cell

attachment and growth [40]. Misra et al. investigated the effect of grain structure on osteoblasts functions and reported that nanograined structures have a positive influence on protein adsorption, preosteoblasts growth and attachment [41,42].

Live-dead assay

In parallel, cellular viability was quantitatively studied using Live/Dead staining methods, as shown in representative images in Figure 10. In the Live/Dead staining technique, calcein-AM penetrates healthy, viable cell membranes and produces green fluorescence while EthD-1 is a notable marker for measuring of bright red fluorescence intensity in dead cells. The obtained images confirmed an improved cellular viability and reduced cytotoxicity for MC3T3-E1 treated with β -TCP- and PMMA-based bone cements. Furthermore, a nonsignificant cytotoxicity in the cell viability was observed for all bone cements in the whole range of NPs concentrations. These findings revealed that the increase of concentration of nanoparticles did not have a direct influence on the viability of osteoblast cells, and this circumstance was clearly related to the modification of AgNPs with soybean oil. A novel copolymer produced by auto-oxidation of soybean oil containing AgNO_3 preserved the all properties of AgNPs as well as creating a cell-friendly nanomaterial.

Similarly, Gonzalez-Sanchez et al. reported new acrylate base nanocomposite hydrogels as bone graft materials. In this study, the silver nanoparticles were embedded into the composite matrix to provide non-antibiotic based antimicrobial activity. According to MTT assay, which performed to check the toxicity of Ag nanoparticles, silver nanoparticles did not exhibit a detrimental effect on the viability of osteoblast cells irrespective of the cross-linking and nanoparticles concentrations [43].

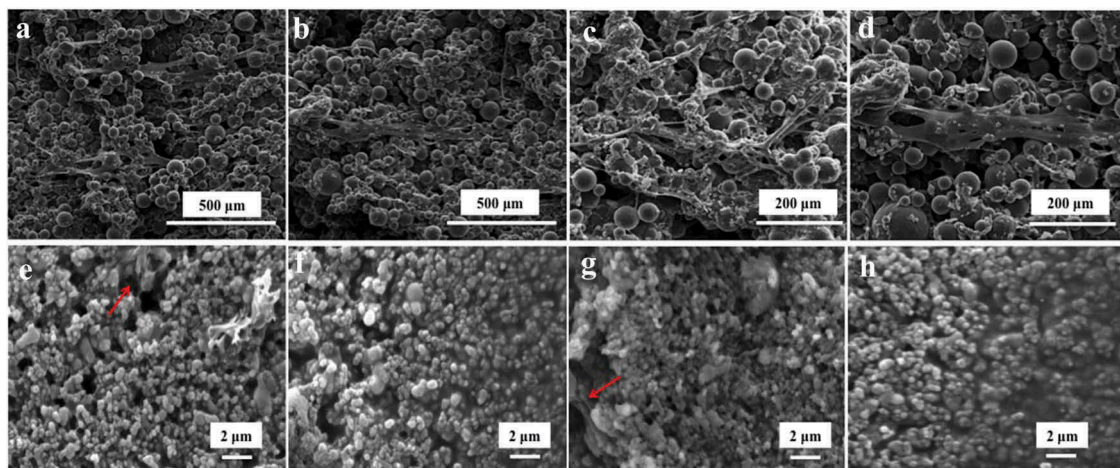


Figure 9. Representative SEM images of cell attachment on the pure PMMA (a), 5PMMA (b), 10PMMA (c), 15PMMA (d) and pure β -TCP (e), 5 β -TCP (f), 10 β -TCP (g), 15 β -TCP (h). Red arrows indicate the seeded MC3T3-E1.

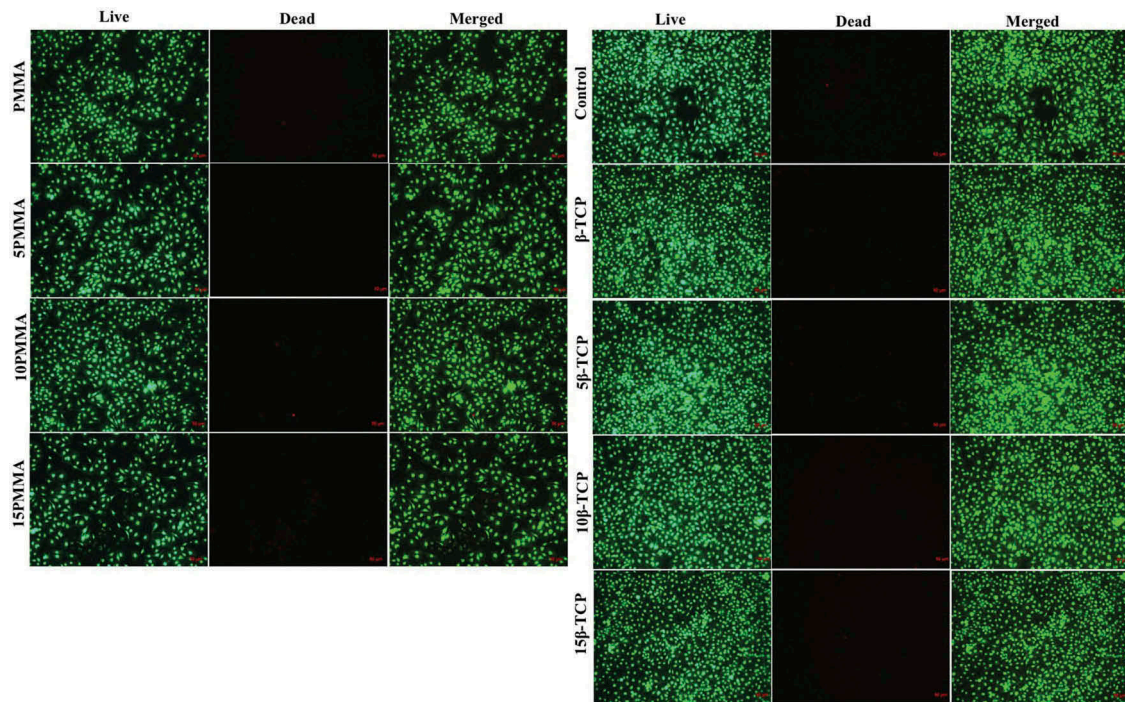


Figure 10. Fluorescence microscopy images of MC3T3-E1 cells after 24 h incubation with bare, PS-AgsboxNPs-containing β -TCP- and PMMA-based bone cements. Scale bars are 10 μ m.

Yu et al. synthesised nanosized silver colloids using polyanionic Na(+)-poly(γ -glutamic acid) (PGA) as a stabiliser under chemical reduction by dextrose. The in vitro cytotoxicity of L929 fibroblasts was assessed by microscopy and the observed images exhibited a damage in the integrity of the cell monolayer as well as 70% cell lysis at Ag^+ ion concentrations more than 2 ppm. L929 cultured on PGA/ Ag^0 generated numerous filopodia-like formations as a marker of cell-substrate and cell-cell interactions and the surface was covered with a continuous sheet of cells. According to the results, PGA/ Ag^0 did not cause to cell lysis or toxicity. This suggested that the existence of cytocompatible PGA could improve the proliferation of L929 fibroblasts [44].

Consequently, the analysis results both MTT and fluorescence microscopy in this study demonstrated that the PS-AgsboxNPs-containing β -TCP- and PMMA-based bone cements did not cause any toxicity on MC3T3-E1 cells related to the concentration of PS-AgsboxNPs.

Calcium and ALP assay

Additionally, Alizarin Red assay was performed as a marker of the cell mineralisation. After 7 days in culture, the calcium deposition formed by cells on the PMMA- and β -TCP- based bone cements revealed significant difference, as shown in Figure 11(a,b). The staining for β -TCP samples was much more intensive than that of PMMA. Velu et al. reported the in vitro osteoblast functions of the sintered PMMA/ β -TCP

composites using MG-63 cells and they obtained better staining with the higher the β -TCP content [45]. Finally, we concluded that the osteoblast differentiation and mineralisation on the β -TCP bone cements was significantly higher compared to those on PMMA samples after 7 days and there was no cytotoxic response related to PS-AgsboxNPs.

Calcium assay was performed to assess the mineralised matrix formation on the bone cements. The synthesised calcium amount as an index of extended osteogenic maturation of MC3T3-E1 is presented in Figure 11(c). Qualitative analysis showed that the average calcium deposition on β -TCP-based bone cements was significantly higher than that on PMMA bone cements ($p < 0.005$). At the end of day 7, the calcium deposition on 10PMMA was $3.73 \pm 0.2 \mu\text{g}/\text{cement}$ while on 10 β -TCP it reached to $6.14 \pm 0.4 \mu\text{g}/\text{cement}$. 15 β -TCP had the highest amount of calcium accumulation with a calcium deposition of $7.30 \pm 0.5 \mu\text{g}/\text{cement}$ and calcium deposition profiles for all β -TCP- based bone cements were at least twice higher compared to PMMA. The difference in calcium deposition was not statistically significant with varying PS-AgsboxNPs content in the PMMA- and β -TCP-based bone cements.

ALP activity was evaluated as an indicator of osteoblastic activity. Figure 11(d) presents the expression of ALP by the samples at day 7. At the end of day 7, ALP level in all β -TCP groups were significantly more than that of PMMA ($p < 0.05$). It was found that the ALP activities were directly related to the composition of bone cement. Our study

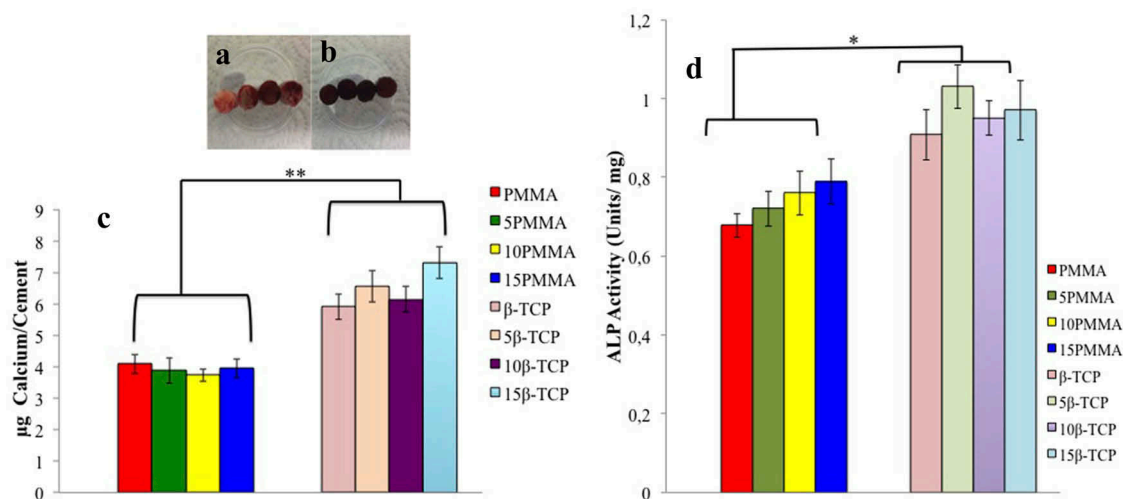


Figure 11. Photographs of bone cements stained with Alizarin Red to visually follow the biomineralisation (a-b). Calcium deposition (c) and ALP activity (d) assay on bone cements after 7 days in culture. *p < 0.05 and **p < 0.005 in comparison with PMMA samples.

coincided with the report of Palmer et al.; the investigators assessed the biocompatibility properties of the CPC and collagen-CPC composites using human bone marrow stromal cells and found that the ALP activities of cells on the CaP bone cement were higher than those of cells on the PMMA bone cement [46].

Zhang et al., in their study, in which combined PMMA with biphasic calcium phosphate (BCPx) showed that ALP was expressed higher at day 14 compared to day 7 by all the BCPx/PMMA samples. This suggested that the ALP activity was directly proportional to content of β-TCP [47]. The results suggested that the presence of β-TCP in the cement could have significantly induced the growth and differentiation of preosteoblastic bone cells.

Finally, the results obtained from the in vitro cytotoxicity test are in good agreement with ALP activity data and calcium deposition rate.

Conclusions

In the proposed study, we aimed to fabricate bioactive PMMA or β-TCP bone cements reinforced with PS-AgsboxNPs, which were synthesised using a soybean-oil derived graft polymer, as an alternative to meet the surface and mechanical requirements of the bone cements commonly used in bone tissue applications. Results showed that, PS-AgsboxNPs can be easily doped into bone cement and presented a concentration-dependent antimicrobial activity, especially high efficiency at low concentration in β-TCP than in PMMA cements without influencing their mechanical, surface properties and cytocompatibility. The mineralisation and ALP activity assays indicated that β-TCP cements could induce more of

the deposition of Ca^{+2} mineral and the content of β-TCP would promote cell adhesion, proliferation and osteogenesis without side effects depending on NPs concentration, which is a promising result in bone tissue engineering. Finally, this study demonstrated the feasibility of developing PMMA- and β-TCP bone cements through their impregnation with PS-AgsboxNPs for the treatment of orthopaedic surgery-related infections.

Acknowledgments

The authors would like to thank Murat Demirbilek for helping with antibacterial assays. Our special thanks go to Gelişim Medikal and Oliga Ltd., Turkey for providing us with the PMMA and β-TCP samples used in this study. This work was supported by grant from the Bulent Ecevit University (BEU-2016-33496813-02).

Disclosure statement

No potential conflict of interest was reported by the authors.

- [1] Greene LR. Guide to the elimination of orthopedic surgery surgical site infections: an executive summary of the association for professionals in infection control and epidemiology elimination guide. *Am J Infect Control*. 2012;40:384–386.
- [2] Tsaras G, Osmon DR, Mabry T, et al. Incidence, secular trends, and outcomes of prosthetic joint infection: a population-based study, olmsted county, Minnesota, 1969-2007. *Infect Control Hosp Epidemiol*. 2012;33:1207–1212.
- [3] Pathak SP, Gopal K. Evaluation of bactericidal efficacy of silver ions on *Escherichia coli* for drinking water disinfection. *Environ Sci Pollut Res Int*. 2012;19:2285–2290.
- [4] Sambhy V, MacBride MM, Peterson BR, et al. Silver bromide nanoparticle/polymer composites: dual

- action tunable antimicrobial materials. *J Am Chem Soc.* **2006**;128:9798–9808.
- [5] Galiano K, Pleifer C, Engelhardt K, et al. Silver segregation and bacterial growth of intraventricular catheters impregnated with silver nanoparticles in cerebrospinal fluid drainages. *Neurol Res.* **2008**;30:285–287.
 - [6] Keck CM, Schwabe K. Silver-nanolipid complex for application to atopic dermatitis skin: rheological characterization, in vivo efficiency and theory of action. *J Biomed Nanotechnol.* **2009**;5(4):428–436.
 - [7] Luan J, Wu J, Zheng Y, et al. Impregnation of silver sulfadiazine into bacterial cellulose for antimicrobial and biocompatible wound dressing. *Biomed Mater.* **2012**;7(6):065006.
 - [8] Sataev MS, Koshkarbaeva ST, Tleuova AB, et al. Novel process for coating textile materials with silver to prepare antimicrobial fabrics. *Colloid Surf A.* **2014**;442:146–151.
 - [9] Alt V, Bechert T, Steinrücke P, et al. An in vitro assessment of the antibacterial properties and cytotoxicity of nanoparticulate silver bone cement. *Biomaterials.* **2004**;25(18):4383–4391.
 - [10] Tran QH, Nguyen VQ, Le AT. Silver nanoparticles: synthesis, properties, toxicology, applications and perspectives. *Adv Nat Sci Nanosci Nanotechnol.* **2013**;4(3):033001.
 - [11] Hurst SJ, Lytton-Jean AK, Mirkin CA. Maximizing DNA loading on a range of gold nanoparticle sizes. *Anal Chem.* **2006**;78:8313–8318.
 - [12] Ahmed S, Ahmad M, Swami BL, et al. A review on plants extract mediated synthesis of silver nanoparticles for antimicrobial applications: a green expertise. *J Adv Res.* **2016**;7:17–28.
 - [13] Wu N, Fu L, Aslam M, et al. Interaction of fatty acid monolayers with cobalt nanoparticles. *Nano Lett.* **2004**;4:383–386.
 - [14] Zamiri R, Azmi BZ, Sadrolhosseini AR, et al. Preparation of silver nanoparticles in virgin coconut oil using laser ablation. *Int J Nanomed.* **2011**;6:71–75.
 - [15] Kumar A, Vemula PK, Ajayan PM, et al. Silver-nanoparticle-embedded antimicrobial paints based on vegetable oil. *Nat Mater.* **2008**;7(3):236–241.
 - [16] US Department of Agriculture. World agricultural supply and demand estimates. Washington, DC: US Department of Agriculture; **2015**. p. 2–5.
 - [17] İnce Ö, Akyol E, Sulu E, et al. Synthesis and characterization of novel rod-coil (tadpole) poly (linoleic acid) based graft copolymers. *J Polym Res.* **2016**;23(5):1–10.
 - [18] Çakmaklı B, Hazer B, Açıkgöz S, et al. PMMA-multigraft copolymers derived from linseed oil, soybean oil, and linoleic acid: protein adsorption and bacterial adherence. *J Appl Polym Sci.* **2007**;105:3448–3457.
 - [19] Hazer B, Kalaycı ÖA. High fluorescence emission silver nano particles coated with poly (styrene-g-soybean oil) graft copolymers: antibacterial activity and polymerization kinetics. *Mater Sci Eng C.* **2016**;74:259–269.
 - [20] Iler RK. Relation of particle size of colloidal silica to the amount of a cationic polymer required for flocculation and surface coverage. *J Colloid Interface Sci.* **1971**;37:364–373.
 - [21] Sharma N, Madan P, Senshang L. Effect of process and formulation variables on the preparation of parenteral paclitaxel-loaded biodegradable polymeric nanoparticles: A co-surfactant study. *Asian J Pharm Sci.* **2016**;11:404–416.
 - [22] Hotez EM, Phenrat T, Lowry GV. Nanoparticle aggregation: challenges to understanding transport and reactivity in the environment. *J Environ Qual.* **2010**;39:1909–1924.
 - [23] Baroud G, Nemes J, Heini PF, et al. Load shift of the intervertebral disc after a vertebroplasty: a finite-element study. *Eur Spine J.* **2003**;12:421–426.
 - [24] Tai CL, Lai PL, Lin WD, et al. Modification of mechanical properties, polymerization temperature, and handling time of polymethylmethacrylate cement for enhancing applicability in vertebroplasty. *BioMed Res Int.* **2016**;901562. DOI:10.1155/2016/7901562
 - [25] He Q, Chen H, Huang L, et al. Porous Surface Modified Bioactive Bone Cement for Enhanced Bone Bonding. *PLoS ONE.* **2012**;7(8):e42525.
 - [26] Varaprasad K, Mohan YM, Ravindra S, et al. Hydrogel–silver nanoparticle composites: a new generation of antimicrobials. *J Appl Polym Sci.* **2009**;115:1199–1207.
 - [27] N'Diaye M, Pascaretti-Grizon F, Massin P, et al. Water absorption of poly(methyl methacrylate) measured by vertical interference microscopy. *Langmuir.* **2012**;28(31):11609–11614.
 - [28] Hamed-Rad F, Ghaffari T, Rezaii F, et al. Effect of nanosilver on thermal and mechanical properties of acrylic base complete dentures. *J Dent (Tehran, Iran).* **2014**;11(5):495.
 - [29] Ghaffari T, Hamedirad F, Ezzati B. In vitro comparison of compressive and tensile strengths of acrylic resins reinforced by silver nanoparticles at 2% and 0.2% concentrations. *J Dent Res Dent Clin Dent Prospects.* **2014**;8(4):204–209.
 - [30] Gopalakrishnan S, Mathew AT, Mozetič M, et al. Development of biocompatible and biofilm-resistant silver-poly(methylmethacrylate) nanocomposites for stomatognathic rehabilitation. *Int J Polym Mater PO.* **2019**. DOI:10.1080/00914037.2018.1552863
 - [31] Zhang Q, Leia Z, Peng M, et al. Enhancement of mechanical and biological properties of calcium phosphate bone cement by incorporating bacterial cellulose. *Mater Tech.* **2019**;1–7. DOI:10.1080/10667857.2019.1630951
 - [32] Roy E, Patra S, Saha S, et al. Shape-specific silver nanoparticles prepared by microwave-assisted green synthesis using pomegranate juice for bacterial inactivation and removal. *RSC Adv.* **2015**;5:95433–95442.
 - [33] Rau JV, Fosca M, Graziani V, et al. Silver-doped calcium phosphate bone cements with antibacterial properties. *J Funct Biomater.* **2016**;7(2):10.
 - [34] Perni S, Thenault V, Abdo P, et al. Antimicrobial activity of bone cements embedded with organic nanoparticles. *Int J Nanomedicine.* **2015**;10:6317–6329.
 - [35] Vanaja M, Annadurai G. Coleus aromaticus leaf extract mediated synthesis of silver nanoparticles and its bactericidal activity. *Appl Nanosci.* **2012**;3:217–223.
 - [36] Ipekoglu M, Altintas S. Silver substituted nanosized calcium deficient hydroxyapatite. *Mater Tech.* **2010**;25:5:295–301.
 - [37] Pauksch L, Hartmann S, Szalay G, et al. In vitro assessment of nanosilver-functionalized PMMA Bone cement on primary human mesenchymal stem cells and osteoblasts. *PLoS One.* **2014**;9(12):e114740.

- [38] Kang IG, Park CI, Lee H, et al. Hydroxyapatite microspheres as an additive to enhance radiopacity, biocompatibility, and osteoconductivity of poly(methyl methacrylate) bone cement. *Materials (Basel)*. 2018;11(2):E258.
- [39] Kang T, Hua X, Liang P, et al. Synergistic reinforcement of polydopamine-coated hydroxyapatite and BMP2 biomimetic peptide on the bioactivity of PMMA-based cement. *Compos Sci Technol*. 2016;123:232–240.
- [40] Mali SA, Nune KC, Misra RDK. Biomimetic nanostructured hydroxyapatite coatings on metallic implant materials. *Mater Tech*. 2016;31:782–790.
- [41] Misra RDK, Nune C, Pesacreata TC, et al. Interplay between grain structure and protein adsorption on functional response of osteoblasts: ultrafine-grained versus coarse-grained substrates. *J Biomed Mater Res A*. 2013;101(1):1–12.
- [42] Misra RDK, Mali S, Thein-Han WW, et al. Cellular activity of bioactive nanograined/ultrafine grained materials. *Acta Biomater*. 2010;6(7):2826–2833.
- [43] González-Sánchez MI, Perni S, Tommasi G, et al. Silver nanoparticle based antibacterial methacrylate hydrogels potential for bone graft applications. *Mater Sci Eng C Mater Biol Appl*. 2015;50:332–340.
- [44] Yu DG. Formation of colloidal silver nanoparticles stabilized by Na^+ -poly(γ -glutamic acid)-silver nitrate complex via chemical reduction process. *Colloids Surf B Biointerfaces*. 2007;59(2):1171–1178.
- [45] Velu R, Kamarajan BP, Anantahsubramanian M, et al. Post-process composition and biological responses of laser sintered PMMA and b-TCP composites. *J Mater Res*. 2018. DOI:10.1557/jmr.2018.76
- [46] Palmer I, Nelson J, Schatton W, et al. Biocompatibility of calcium phosphate bone cement with optimized mechanical properties. *J Biomed Mater Res B Appl Biomater*. 2016;104(2):308–315.
- [47] Zhang X, Kang T, Liang P, et al. Biological activity of an injectable biphasic calcium phosphate/PMMA bone cement for induced osteogenesis in rabbit model. *Macromol Biosci*. 2018;18:3.