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Antimicrobial Cytocompatible Pentaerythritol Triacrylate-co-Trimethylolpropane Composite Scaffolds for Orthopaedic Implants

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ABSTRACT: As of 2010, 5.3 million orthopedic surgeries are performed each year, and this number is expected to increase to 6.2 million by 2020. On average, 27.7% of all orthopedic surgeries result in infection which often leads to osteomyelitis and the loss of supporting bone. In this study, we describe two synthetic bone grafts, or augmentation methods, for a biodegradable, silver nanoparticle (SNPs) containing antimicrobial scaffolds composed of pentaerythritol triacrylate-*co*-trimethylolpropane tris (3-mercaptopropionate) (PETA) and hydroxyapatite (HA). This osteoinductive and degradable material is designed to stimulate proliferation of bone progenitor cells, and provide controlled release of antimicrobial components. The first method, denoted as the "incorporating method," involves dissolving SNPs in ethanol, butanol, or isopropanol and directly incorporating the particles into the scaffold prior to polymerization. The second method, "coating method," involves submerging fabricated scaffolds into their respective SNPs-solution and mixing for 24 h. The coating method allowed better distribution and release of SNPs from the surface of the composites when exposed to extracellular media. The *in vitro* release of silver for both methods was quantified by inductively coupled plasma optical emission spectroscopy (ICP-OES). The scaffolds made by means of the coating method showed increased release of silver with respect to time; no silver leached from the scaffolds formed by the incorporating method. Use of Alamar Blue assay demonstrated that the SNPs incorporation did not affect cell viability when tested with hASCs. The scaffolds formed by the coating method inhibited the proliferation of *Staphylococcus aureus* 99.5% and *Escherichia coli* by 99.9% within 24 h. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 41099.

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INTRODUCTION

Complications following internal fixation in orthopedic trauma cause significant setbacks and have devastating consequences. Once the surgical site is infected, microbial colonies and biofilms are formed that are often resistant to treatment, limiting therapeutic options. Surgical site infections often result in multiple surgeries ranging from tissue debridement and bone excision to soft tissue flaps.[1] Many infections are not only related to environmental organisms but are also hospital-acquired bacteria with infections rates as high as 30% in type 3 open fractures.[2–4] Traumatic injuries can involve significant soft tissue defects with exposed fractures in which there is a 22 to 66% rate of osteomyelitis.[5,6] If uncontrolled, the mortality rates from osteomyelitis can be as high as 2%.[7]

Musculoskeletal implants are well-known risk factors for infection especially in large open traumatic wounds. Infected implants can be removed at the expense of fracture stability and mobility or left in place with the risk that the infection may never resolve and resulting in chronic problems. The implant materials provide additional surfaces within the wound for microbial colonization and are often the source of persistent infection due to the formation of bacterial colonies and biofilms.[8,9] Management of these complications provides a tremendous strain on health care resources. The cost of direct care for these injuries has been estimated at $65 million and $169 million in disability costs.[10]

It is clear that preventing or mitigating the formation of bacterial colonies will help improve patient outcomes in all types of musculoskeletal surgery as well as reduce the financial burden on strained health care resources. To address this issue, many synthetic scaffolds are being synthesized as potential therapeutic modalities designed to deliver bioactive materials such as drugs, cells, genes, peptides, and/or proteins concomitantly with a degradable scaffold. By introducing the optimum porosity in

the synthetic structure, it can perform a critical function of providing temporary mechanical stability, maintain and support tissue regeneration, and most importantly, deliver bioactive/antimicrobial payload to reduce the bacteria associated with healthcare acquired infections.[11–16] Some of the widely used materials for biocompatible and bioresorbable synthetic scaffolds include aliphatic esters, chitosan, alginates, polyhydroxy acids, and poly(tyrosine-carbonates).[17] The large surface area and high reactivity of nanoparticles enables them to exhibit extraordinary chemical, physical, and biological properties as well as making them ideal candidates for antimicrobial agents. Silver, in various forms, is among the material-based, safe, broad-spectrum efficacious antimicrobial solutions that are currently commercially available. Silver nanoparticles (SNPs) allow for increased penetration and faster rates of fusion with cellular membranes compared to larger particles.[18–20]

Herein we describe a new class of *in situ* polymerizing compounds polymerized via an amine-catalyzed Michael addition resulting in a porous, interconnected, antimicrobial pentaerythritol triacrylate-hydroxyapatite-silver nanoparticle (PETA-HA-SNPs) scaffold. Two synthesis methods are compared and the *in vitro* contact times required for the elimination of known titres of two model organisms, *Staphylococcus aureus* and *Escherichia coli*, are investigated. Scaffold microstructure and viscoelastic mechanical properties are assessed as a function of composition. Additionally, the cytotoxicity of the released silver and hydrolysed product of PETA-HA-SNPs materials are tested against hASCs to determine the suitability for use in stem cell-based regenerative therapies. The results demonstrate how PETA-HA-SNPs combinations can best be implemented in nanocomposites to create engineered, active, antimicrobial tissue scaffolds.

EXPERIMENTAL

Antimicrobial Biocompatible PETA-HA Composite Scaffolds Fabrication by Incorporating and Coating Method

The chemicals utilized consisted of the following: trimethylolpropane tris(3-mercaptopropianate) (TMPTMP), diethylamine (99% purity)(DEA), pentaerythritol triacrylate(PETA), and hydroxyapatite(HA). The scaffold components were prepared by formulating PETA with 16.1% DEA relative to acrylate functionality. The PETA and 16.1% DEA were stirred for 3 h.

Two methods (incorporating and coating) were developed to integrate SNPs into the polymeric foam. The incorporating method involved dissolving HPC-SNPs (size distribution from 25 to 75 nm[21]) in 9.3% butanol at concentrations of 100, 1000, and 1500 ppm. The mixture was then directly added to the PETA/DEA stock solution. An excess of 20% TMPTMP along with HA (20%) were thoroughly stirred into the stock solution-SNPs mixture using an overhead mixer. The blend was then poured into a 250 mL pressurized spray canister and expelled into cylindrical molds using 7 gram-compressed nitrous oxide canisters as the gas foaming agent. Any remaining butanol in the scaffolds was removed using a Freeze-drying method.

The coating method followed the same polymer foaming method with the exception of adding the SNP blend to the standard stock solution. Instead, polymerized scaffolds foamed with 20% excess TMPTMP stock solution were soaked in SNPs solutions (dissolved in ethanol, isopropanol or butanol) at concentrations of 1000 ppm for 24 h. Each scaffold was then rinsed with DI water and freeze-dried to remove any remaining solvent or SNPs from the scaffolds.

Mechanical Testing

Each foamed scaffold was first sculpted into a cylinder shape (10 mm diameter × 5 mm height), then subjected to compression testing. A universal testing machine (Instron Model 5696, Canton, MA) was used at an extension rate of 0.5 mm min^{-1} to a maximum compression strain of 90%. Young's modulus and compressive strength were calculated based on the dataset obtained from the Bluehill computing system.

Determination of Crosslink Density

The crosslinking density was determined for the control and 20% excess thiol scaffolds according to previously described methods[22] by finding the average molecular weight between crosslinks (M_c)

$$M_c = \frac{3\rho RT}{E'}$$

where T is the temperature, E' is the tensile modulus, ρ is the polymer density, and R is the gas constant.

Tensile testing was conducted according to the ASTM D638, and the modulus was calculated based on a plot of stress versus strain.

SEM Analysis of PETA-HA-SNPs Scaffolds and hASC Morphology

All scaffolds were carefully cut into rectangular shape (10 mm × 10 mm × 5 mm) and mounted onto SEM stages. Each sample was then placed in a EMS550X sputter coater for two minutes, forming a conductive platinum coating. Standard SEM analysis was performed thereafter.

The antimicrobial scaffolds cultured with hASCs were transferred to a standard, sterile 96-well plate after 21 days. Each composite was fixed with 2% glutaraldehyde (GA) (made with 2 parts Cocadylate, 1 part 8% GA and 1 part distilled H_2O) for 30 min. The samples were subjected to a dehydration procedure involving dilutions of 30 : 70–100 : 0 ethanol : water ratios. The percent of ethanol was increased by 10% every 30 min. After 100% ethanol was added to the samples, all solution was removed and replaced with 100% HDMS (Hexamethyldisilazane). The HDMS was exposed to the samples overnight to remove all remaining ethanol. Each sample was then removed from solution and mounted on SEM stages. EMS550X sputter coater was run for 2 min to apply a conductive platinum coating to each sample. This procedure was followed by standard SEM analysis.

Micro-CT Analysis

Samples were sliced into 1–2 mm approximate cuboids of 10–15 mm height. Samples were imaged with 11 keV monochromatic X-rays at 2.5 μm/px resolution by means of the tomography beamline at the Center for Advanced Microstructures and Devices (Louisiana State University, Baton Rouge, LA). Projections numbered 720 corresponding to $\Delta\theta = 0.25$;

projection exposure time varied from 2 to 4 s, but reconstruction algorithms ensured normalized data. The two different datasets were directly comparable, both as an aggregate dataset and as orthoslices. Reconstruction data were 16-bit signed integer with mean air intensity scaled to zero. Pore size was measured using ImageJ 64.

Volume renderings were generated from the three foamed samples 3D data using Avizo 7.0.1 (Visualization Services Group). Two overlapping sub-volumes were rendered simultaneously, one with a red-orange-white colormap corresponding to trithiol-triacrylate foam, and the other with a blue-green colormap corresponding to hydroxyapatite inclusions.

In Vitro Release of Silver from Scaffolds

PETA scaffolds containing varying amounts of SNPs were exposed to 5 mL of PBS solution for 3 days ($n = 3$) at RT. About 100 µL of phosphate-buffered saline (PBS) solution was collected from each sample every 24 h, without replacement, for ICP-OES analysis. Prior to analysis, solutions were acidified with 1 mL of nitric acid, transferred to weighed ICP-OES tubes, agitated for 2.5 h and diluted to a final volume of 10 mL with DI water. The final vials were massed and run on the Varian Vista MPX. A normalization control sample was also included in the dataset.[23]

Microbiology Test Organisms

E. coli (ATCC 29522) and *S. aureus* (ATCC 6538) were inoculated and grown in sterile 15 mL centrifuge tubes (BD Falcon, Franklin Lakes, NJ) containing BHI broth and incubated at 37°C overnight.

Antimicrobial Activity

Quantitative antimicrobial testing with *S. aureus* and *E.coli* were conducted according to a modified ISO 22196 test method, which has a detection range of 30–300 colony forming units (CFUs). Nearly 300 mL of the inoculated bacterial cultures were exposed to cylindrical sections (diameter 4 mm × height 4 mm) of each test group run in triplicate and incubated for 24 h. The nanocomposites were then washed with PBS to extract *S. aureus* and *E. coli*. The bacteria were plated on mannitol salt agar and MacConkey agar plates (BD Falcon), respectively. A 1% triple-antibiotics (Penicillin-Streptomycin-Amphotericin B) was used as a control group to inhibit bacteria proliferation. The percentage reduction and CFU values were calculated according to previously published work.

Cell Culture

Liposuction aspirates from subcutaneous adipose tissue were obtained with informed consent under a clinical protocol reviewed and approved by the Institutional Review Board at the Pennington Biomedical Research Center and utilized under the exempted LSU protocol HE 11–17. Isolation of hASC was performed as described elsewhere.[24] The initial passage of primary cell culture was referred to as "Passage 0" (p0). The cells were passaged by means of trypsinization and plated at a density of 5,000 cells cm^{-2} ("Passage 1") for expansion on T125 flasks in order to attain 80%. Passage 2 was used for cell viability testing after acute exposure to scaffold extractives medium and loaded onto scaffolds using a spinner flask.

Cytotoxicity of Extracted Materials

All the samples (0.02 g per sample) were incubated on an orbital shaker in 5 mL of stromal media at 37°C and 150 rpm min^{-1} for a week. The extracts were centrifuged, filtered (0.22 µm pore size) and pipetted (100 µL $well^{-1}$) into a 96-well plate previously sub-cultured with hASCs (25,000 cells $well^{-1}$). The plate was then incubated in a CO_2 incubator (5% CO_2) at 37°C for 24 h. The cellular viability on each scaffold culture was determined using the Alamar blue assay. About 10 µL of Alamar Blue reagent was added to each well and incubated at 37°C in 5% CO_2 for 2 h. The fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 595 nm using a fluorescence plate reader. The tissue culture treated plastic 96-well plate served as a control substrate. hASCs exposed to 70% ethanol functioned as a dead control group.

Osteogenic Differentiation of hASCs

To verify that silver does not inhibit the osteogenic potential of the composites, hASC mineralization (cells alone and cells-scaffolds) was assessed after 7, 14 and 21 days of culture in control and osteogenic medium. Alizarin Red was chosen as the assay to measure calcium deposition. Cells and scaffolds were washed a total of four times with 0.9% NaCl (1 mL $well^{-1}$) and fixed with 70% ethanol (1 mL $well^{-1}$). All chemicals were then removed from the surface of the scaffolds by aspiration to prepare for staining with 2% Alizarin Red. Each sample was exposed to the assay for 10 min; the stain was then removed and each sample was washed with DI water six times. The samples were destained by means of 400 mL of 10% cetylpyridinium chloride monohydrate followed by shaking for 4 h at room temperature. The calcium deposition expressed by the optical density of the aliquots was then measured with a plate reader at an absorbance of 540 nm. Scaffolds cultured without cells were used as controls to normalize the results.

Statistical Analysis

All results were expressed as mean ± SEM. Data was analyzed with one-way analysis of variance (ANOVA), followed by Tukey's minimum significant difference (MSD) *post hoc* test for pairwise comparisons of main effects. For all comparisons, a P value < 0.05 was considered significant.

RESULTS AND DISCUSSION

Mechanical Properties of Scaffolds

Scaffolds formed by the incorporating method at concentrations of 100, 1000, and 1500 ppm did not show significant differences in young's modulus or ultimate compressive strength (Figure 1). The same trends were found in the mechanical properties of the scaffolds fabricated using the coating method, and the data for each solvent was then combined for graphical comparison. Both the scaffolds made by the incorporating method and those made by the coating method were shown to have lower Young's modulus and ultimate compressive strength values than the control group, PETA-*co*-TMPTMP-20%HA scaffolds containing no silver and no excess thiol. This trend is believed to be linked to the additional 20% TMPTMP in the stock solution used for polymerization. While aiding in the attachment of silver to the surface of the scaffold, the excess thiol reduced the cross-linking density, resulting in decreased mechanical strength. The decrease

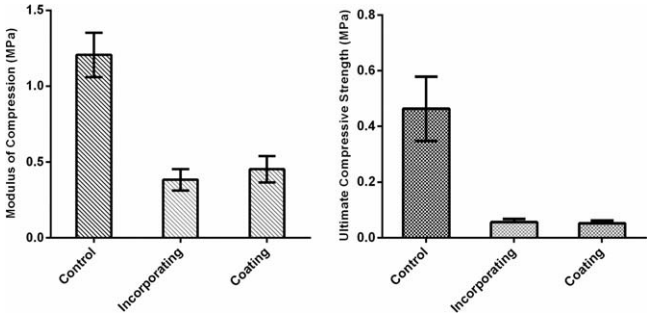

Figure 1. Young's modulus and ultimate compressive yield strength of the PETA : HA copolymer with SNPs inclusions. Control group was using PETA-*co*-TMP TMP scaffolds without 20% excess thiol. Both incorporating and coating group were using PETA-*co*-TMP TMP scaffolds with 20% excess thiol.

in crosslinking density for the antimicrobial scaffolds compared to the control scaffolds was verified by calculating M_c. The M_c for the excess thiol polymer and the control polymer was 1480 and 799 g mol^{-1}. As the molecular weight between junction points within the polymer decreases, the crosslinking density increases proving the excess thiol polymer had lower crosslinking density due to the higher M_c value.

Morphological Analysis

Each test group was analyzed using SEM. The surfaces of the scaffolds formed by the incorporating method [Figure 2(C)] were found to be identical to those formed by the coating method [Figure 2(D)]. Because there is no increased porosity in the scaffolds fabricated by the coating method, the proposition that the decreased mechanical strength is linked to increased thiol is further supported. The porosity of both antimicrobial scaffolds was also similar to the PETA:HA control scaffolds used in our previous study.[25] This indicates that the morphology did not change with the addition of SNPs and the exposure to polar solvents.

The scaffolds were also analyzed by means of micro-CT housed at the center for advanced microstructures and devices (Louisiana State University, Baton Rouge, LA). The scaffolds were found to have a well interconnected void space, and HA appears to be well dispersed throughout the polymer. SNPs were not detectable using this method of analysis. The rendered micro-CT data reconstructed using AVIZO 7.0 revealed that the scaffolds made by the incorporating method [Figure 2(A)] had a larger aggregation of HA in the middle of the scaffold compared to those formed by the coating method [Figure 2(B)], which presented a more even dispersion of HA throughout the composite. The scaffolds fabricated by means of the coating method more closely resembled the ceramic distribution found in the control PETA : HA scaffolds. This suggests that the addition of polar organic solvents to the stock solution resulted in the agglomeration of HA in the center of the structure. This may be a result of reduced solution viscosity allowing for increased migration of the nanoHA phase and aggregation as these compounds polymerize.

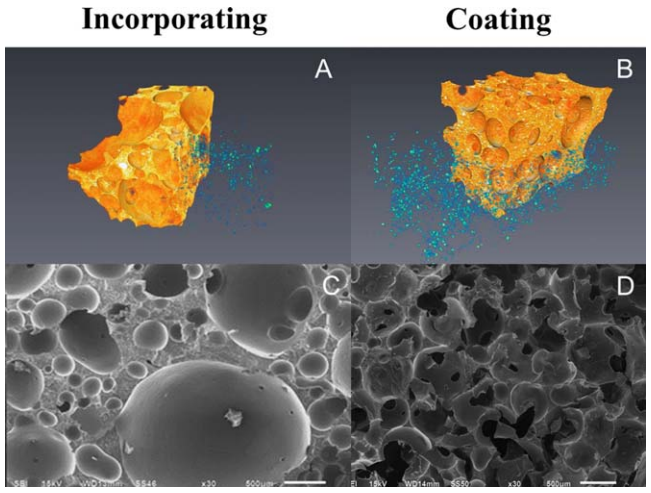

Figure 2. Morphology characterization of PETA-SNPs scaffolds by micro-CT and SEM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In Vitro Silver Release from Scaffolds

For the coating method, SNPs were dissolved in ethanol, butanol, or isopropanol to form a 1000 ppm solution; the PETA : HA scaffolds were then soaked in SNPs solution for 60 h. For the scaffolds made by the incorporating method, SNPs solution was added to the polymer stock solution prior to polymerization. The antimicrobial scaffolds fabricated by each method were then soaked in PBS for 3 days. Samples were extracted and analyzed every 24 h using inductively coupled plasma optical emission spectrometry (ICP-OES). The scaffolds made by the incorporating method were not shown to release any silver during the 3-day experiment. Scaffolds were completely dissolved to calculate total silver content of each scaffold. This data is shown in Figure 3(B), which shows that no silver is released into solution, but there was an increase of silver content in the scaffolds as the concentration of SNPs-stock solution increased from 100 to 1500 ppm. This trend is accredited to low molecular weight of the monomers and the high crosslink density of the PETA : HA polymer. Conversely, the scaffolds made by the coating method released a significant amount of silver. This is attributed to more SNPs being found at the interface and the reduced crosslink density achieved through the addition of 20% excess thiol to the polymerization process. The amount of silver in the extracellular solution increased after every 24-h interval [Figure 3(A)]. There was no statistical difference in the amount of silver released from each of the different treatments (ethanol, butanol, isopropanol). However, there did prove to be significantly more silver in the scaffolds coated in the ethanol-SNPs solution than in those submerged in the butanol-SNPs or isopropanol-SNPs solutions. This suggests that the scaffolds coated in the ethanol-SNPs solution would release silver for longer periods than the other test samples due to the greater reservoir. The most silver was leeched during the first 24 h, providing increased initial antimicrobial activity. There was a significant reduction in the amount of silver released during the second 24-h period as compared to the first 24 h. The concentration of silver released during the third 24-h interval was significantly increased when compared to the second 24-h period but reduced in comparison to the first 24 h. This trend is hypothesized to be related to the nature of diffusion. The SNPs

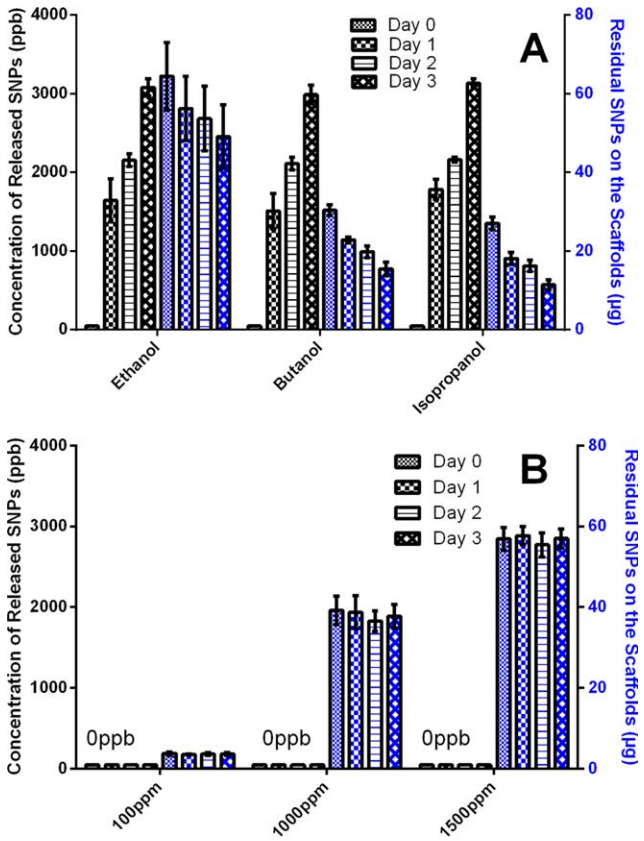

Figure 3. *In vitro* silver release from and residual silver on PETA-SNPs scaffolds made by incorporating and coating methods over a period of 3 days, as measured by ICP-OES. Bars with black pattern: Concentration of released SNPs; Bars with blue pattern: Residual SNPs on the scaffolds. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

on the surface (Figure 4) of the material were released during the first 24-h period. The second and third intervals represent the release of silver trapped below the surface of the material as the PBS penetrated deeper over time.

Antimicrobial Activity of SNPs

The antimicrobial properties of the scaffolds were tested against the two microorganisms most commonly associated with infection from healthcare acquired infections: *Escherichia coli* and *Staphylococcus aureus*. These strains of bacteria are also commonly known to show resistance to a number of antibiotics, including amoxicillin and methicillin.[26]

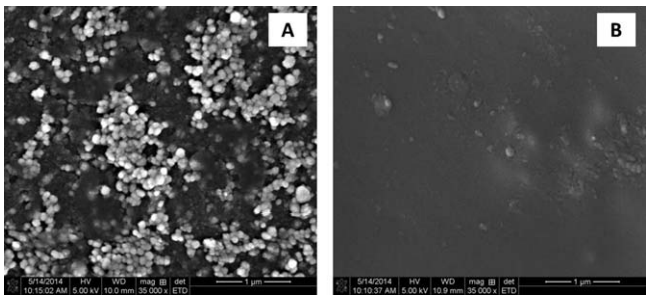

Figure 4. Localization of SNPs on both incorporating and coating scaffolds. (A) Localization of SNPs on scaffolds using coating method; (B) Localization of SNPs on scaffolds using incorporating method.

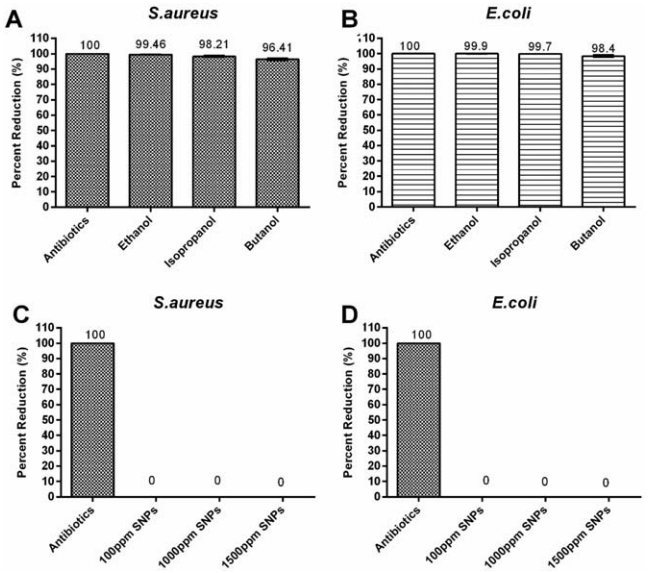

Figure 5. Antimicrobial Activity of scaffolds fabricated by incorporating method and coating method. (A) Antimicrobial activity of scaffolds using coating method against *S. aureus*; (B) Antimicrobial activity of scaffolds using coating method against *E. coli*; (C) Antimicrobial activity of scaffolds using incorporating method against *S. aureus*; (D) Antimicrobial activity of scaffolds using incorporating method against *E. coli*.

The scaffolds made by the incorporating method were exposed to varying dilutions of *S. aureus* and *E. coli*. As a direct correlation to the ICP-OES results (Figure 3) for this method, no reduction in viable colony levels was found [Figure 5(C,D)]. This is likely related to the low silver release from these highly crosslinked scaffold.[27]

Triplicates of the scaffolds made by the coating method were exposed to *S. aureus* and *E. coli*. The highest reduction in viable *S. aureus* colony levels was seen in the scaffolds coated with SNPs-isopropanol (98.2%) and SNPs-ethanol (99.5%) solutions [Figure 5(A,B)]. The scaffolds coated in SNPs-butanol solution showed reduction in viable colony levels of *S. aureus* (96.4%), but the levels were lower than those achieved by scaffolds coated in isopropanol and ethanol solutions. Collectively, the antimicrobial scaffolds made by the coating method reduced the viable colony levels of *S. aureus* by 99.5%. One percent antibiotics (Penicillin-Streptomycin-Amphotericin B) was used as a positive treatment control and reduced the viable colony levels of *S. aureus* by 99.9%. The scaffolds coated in SNPs- ethanol solution showed reduction in viable colony levels of *E.coli* (99.9%), the same effectiveness as the antibiotic control group. Scaffolds coated in SNPs-isopropanol and SNPs-butanol solutions reduced *E. coli* viable colony levels by 99.7 and 98.4%, respectively. The results indicate that PETA-SNPs scaffolds were effective in the reduction of both Gram-positive and Gram-negative bacterial colony forming units. Therefore, scaffolds fabricated by coating method were used for a further cytocompatibility study.

Cytotoxicity of Extracted Materials

A 7-day extraction study was conducted and the extract was tested against hASCs to assess the impact on viability. Because of the negative results acquired from the scaffolds made by the

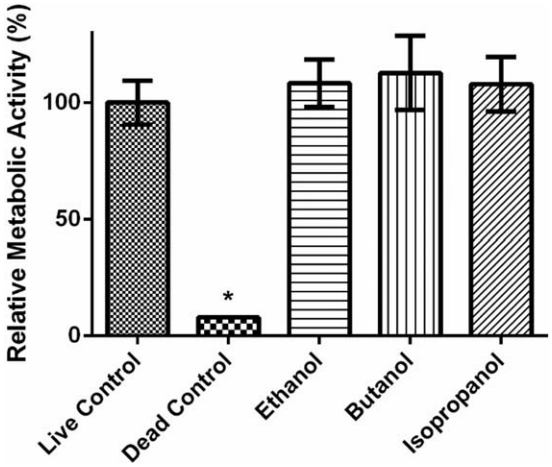

Figure 6. Metabolic activity of hACS cultured with extractives from scaffolds made with coating method measured by AlamarBlue®.

incorporating method, only the scaffolds made by the coating method were chosen for continued analysis. The scaffolds were submerged in 5 mL of stromal media for 7 days and then removed from the media and analyzed for weight loss. The scaffold coated in the ethanol-SNPs solution showed the highest weight loss of 1%. The scaffold coated in the butanol-SNPs solution was reduced by 0.5%. There was 0% loss of the scaffold coated in the isopropanol-SNPs solution. There was no significant difference in the metabolic activity of the live control of hASCs in stromal medium and the experimental hASCs exposed to the extracts from the antimicrobial scaffolds (Figure 6). The fluorescence data indicates high levels of metabolic activity on each of the test groups, suggesting that the concentration of silver that leeched from the antimicrobial scaffolds during the 7-day trial was antimicrobially active but not high enough to cause any significant harm to the cells.

In support of the fluorescence data, the scaffolds were analyzed using SEM to examine the hASC growth on PETA-HA-SNPs composite after 21 days (Figure 7). Extracellular matrix (ECM) deposition and well spread, apparently healthy cells, were found on all antimicrobial scaffold compositions. The samples cultured in stromal media showed more similarities between testing groups than those cultured in osteogenic media. The cell and ECM layers on the osteogenic samples showed more surface roughness than those in the stromal media. This may be due to calcium deposits on the ECM and cell surface. Overall, the matrices were thick and showed cell migration across the surface of the scaffolds. The existence of this healthy cellular structure provides further support for the claim that SNPs incorporated into the scaffold was not substantially cytotoxic.

Osteogenic Differentiation of hASCs

The mineralization of scaffolds coated with different solvent groups was assessed using Alizarin Red assay, which stains calcium-rich deposits. hASCs cultured on each scaffold were tested against a control to assure that silver did not inhibit ostegeogenic differentiation (Figure 8). Alizarin Red staining was significantly higher in hASCs cultured in osteogenic media for the control group compared to samples cultured in stromal media. There was not, however, statistically different absorbance levels in the test groups for the two media. hASCs cultured in both media showed significant increases in staining between the 7 and 14 day time points. Past 14 days, the levels of calcium deposition were not statistically different. The samples coated with SNPs were not shown to be statistically different than the control group without SNPs. This suggests that the addition of silver in small amounts had no evident effect on the potential for osteogenic differentiation.

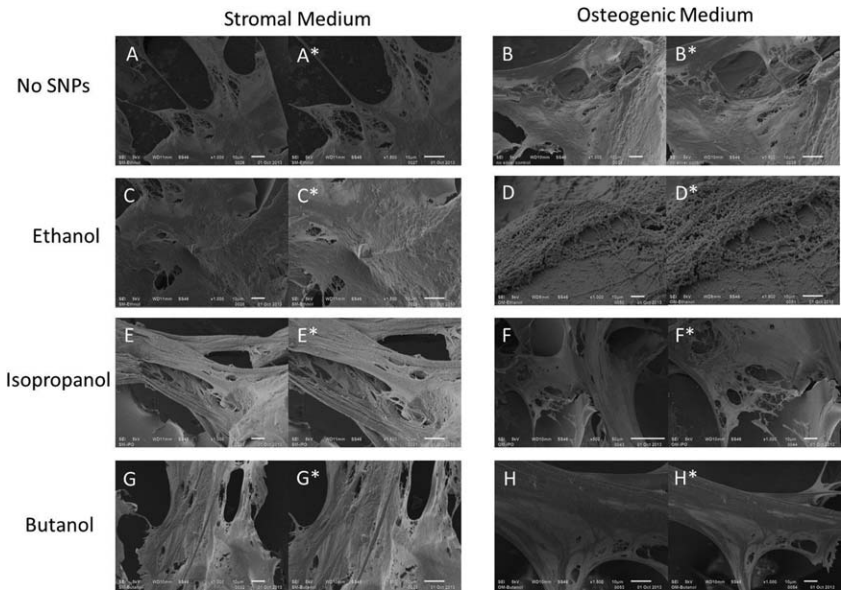

Figure 7. SEM images of hASCs 21 days after initial seeding on bioscaffolds with different solvents for coating method: (A,B) No SNPs added to scaffolds cultured in stromal medium and osteogenic medium respectively; (C,D) Scaffolds coated with SNPs dissolved in ethanol and cultured in stromal medium and osteogenic medium respectively; (E,F) Scaffolds coated with SNPs dissolved in isopropanol and cultured in stromal medium and osteogenic medium respectively; (G,H) Scaffolds coated with SNPs dissolved in butanol and cultured in stromal medium and osteogenic medium respectively.

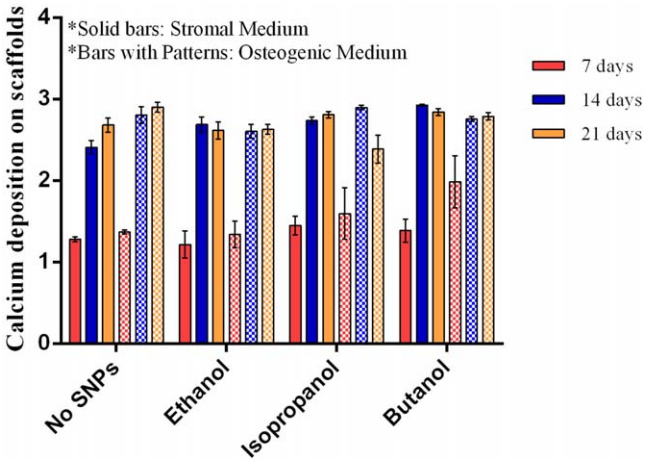

Figure 8. Calcium deposition as a function of Alizarin red staining of SNPs-coated scaffolds made by different solvents (ethanol, isopropanol, butanol), as well as a control group with scaffolds containing no SNPs additives. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CONCLUSION

The results of this study revealed that the coating method improved the release of SNPs from the scaffolds. The surface-bound SNPs were readily oxidized resulting in silver ion release over the 3 days of the study. The antimicrobial testing demonstrated that the coated nanocomposites reduced the *Staphylococcus aureus* and *Escherichia coli* viable culture levels by 99.5% and 99.9%, respectively. Silver content analysis by ICP-OES verified that no silver was released from the scaffolds fabricated by the incorporating method. Therefore, these incorporating scaffolds had no appreciable antimicrobial activity and were not able to reduce the *Staphylococcus aureus* or *Escherichia coli* viable culture levels.

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REFERENCES

1. Rajasekaran, S.; Dheenadhayalan, J.; Babu, J. N.; Sundararajan, S. R.; Venkatramani, H.; Sabapathy, S. R. *J. Bone Joint Surg. Br.* **2009**, *91*, 217.
2. Weitz-Marshall, A. D.; Bosse, M. J. *J. Am. Acad. Orthop. Surg.* **2002**, *10*, 379.
3. Templeman, D. C.; Gulli, B.; Tsukayama, D. T.; Gustilo, R. B. *Clin. Orthop. Relat. Res.* **1998**, *350*, 18.
4. Patzakis, M. J.; Bains, R. S.; Lee, J.; Shepherd, L.; Singer, G.; Ressler, R.; Harvey, F.; Holtom, P. *J. Orthop. Trauma* **2000**, *14*, 529.
5. Khatod, M.; Botte, M.; Hoyt, D.; Meyer, R.; Smith, J.; Akeson, W. *J. Trauma Injury Infect. Crit. Care* **2003**, *55*, 949.
6. Yun, H. C.; Branstetter, J. G.; Murray, C. K. *J. Trauma Injury Infect. Crit. Care* **2008**, *64*, S163.
7. Bhattacharyya, T.; Iorio, R.; Healy, W. *JBJS* **2002**, *84*, 562.
8. Schmidt, A. H.; Swiontkowski, M. F. *J. Am. Acad. Orthop. Surg.* **2000**, *8*, 285.
9. Petty, W.; Spanier, S.; Shuster, J. J.; Silverthorne, C. *J. Bone Joint Surg. Am.* **1985**, *67*, 1236.
10. MacKenzie, E. J.; Jones, A. S.; Bosse, M. J.; Castillo, R. C.; Pollak, A. N.; Webb, L. X.; Swiontkowski, M. F.; Kellam, J. F.; Smith, D. G.; Sanders, R. W.; Jones, A. L.; Starr, A. J.; McAndrew, M. P.; Patterson, B. M.; Burgess, A. R. *J. Bone Joint Surg. Am.* **2007**, *89*, 1685.
11. Vacanti, J. P.; Langer, R. *Lancet* **1999**, *354*, SI32.
12. Yang, S. L. K. F.; Du, Z.; Chua, C. K. *Tissue Eng.* **2001**, *7*, 679.
13. Agrawal, C. M.; Athanasiou, K. A.; Heckman, J. D. In Porous Materials for Tissue Engineering; Transtec Publications Ltd: Zurich-Uetikon, **1997**.
14. McKnight, N. L.; Frangos, J. A. *Ann. Biomed. Eng.* **2003**, *31*, 239.
15. Li, M. G. J. D.; Green, H.; Mills, D. K.; McShane, M. J.; Gale, B. K. Proc.1st Annu. Int. IEEE-EMBS Spec. Top. Conf. Microtechnologies in Medicine and Biology, Lyon, France, **2000**, p 531.
16. Hollister, S. *Nat. Mater.* **2005**, *4*, 518.
17. Middleton, J.; Tipton, A. *Biomaterials* **2000**, *21*, 2335.
18. Saravanan, S.; Nethala, S.; Pattnaik, S.; Tripathi, A.; Moorthi, A.; Selvamurugan, N. *Int. J. Biol. Macromol.* **2011**, *49*, 188.
19. Madhumathi, K.; Kumar, P. S.; Abhilash, S.; Sreeja, V.; Tamura, H.; Manzoor, K.; Nair, S.; Jayakumar, R. *J. Mater. Sci. Mater. Med.* **2010**, *21*, 807.
20. Mandal, A.; Meda, V.; Zhang, W. J.; Farhan, K. M.; Gnanamani, A. *Colloids Surf. B Biointerfaces* **2012**, *90*, 191.
21. Qureshi, A. T.; Monroe, W. T.; Lopez, M. J.; Janes, M. E.; Dasa, V.; Park, S.; Amirsadeghi, A.; Hayes, D. J. *J. Appl. Polym. Sci.* **2011**, *120*, 3042.
22. McNair, O. D.; Janisse, A. P.; Krzeminski, D. E.; Brent, D. E.; Gould, T. E.; Rawlins, J. W.; Savin, D. A. *ACS Appl. Mater. Interfaces* **2013**, *5*, 11004.
23. Qureshi, A. T.; Terrell, L.; Monroe, W. T.; Dasa, V.; Janes, M. E.; Gimble, J. M.; Hayes, D. J. *J. Tissue Eng. Regen. Med.* **2012**, *8*, 386.
24. Gimble, J. M.; Guilak, F.; Bunnell, B. A. *Stem Cell Res. Ther.* **2010**, *1*, 9.
25. Garber, L.; Chen, C.; Kilchrist, K. V.; Bounds, C.; Pojman, J. A.; Hayes, D. *J. Biomed. Mater. Res. A* **2013**, *101*, 3531.
26. Moumile, K.; Carbonne, A.; Rouquet, M.; Gamard, M.; Bornand-Rousselot, A.; Jarlier, V.; Cambau, E. *Pathol. Biol.* **2004**, *52*, 557.
27. Bounds, C. O.; Goetter, R.; Pojman, J. A.; Vandersall, M. *J. Polym. Sci. Part A Polym. Chem.* **2012**, *50*, 409.