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Novel heart valve prosthesis with self-endothelialization potential made of modified polyhedral oligomeric silsesquioxane-nanocomposite material

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In the cardiovascular system, the endothelial layer provides a natural antithrombogenic surface on the inner portion of the heart and associated vessels. For a synthetic material therefore, the ability to attract and retain endothelial or endothelial progenitor cells (EPCs), ultimately creating a single endothelial layer on its surface, is of prime importance. The authors have developed a nanocomposite polymer, based on a combination of polyhedral oligomeric silsesquioxane nanoparticles and polycarbonate urea urethane (POSS-PCU), which is biocompatible and has been used in human for the world's first synthetic trachea, tear duct, and bypass graft. In this study, the authors modified the surface of this casted nanocomposite by grafting fibronectin derived bioactive peptides [glycine-arginine-glycine-aspartic acid-glycine (GRGDG) and lauric acid conjugated GRGDG (GRGDG-LA)] to enhance the endothelialization for using heart valves leaflets from circulating EPCs. Human peripheral blood mononuclear cells were separated using Ficoll–Paque centrifugation, with harvested EPCs purified using CD34 microbead labeling and magnetic-activated cell sorting. Cells were seeded onto 96 well plates coated with POSS-PCU, GRGDG/GRGDG-LA modified POSS-PCU and PCU polymers, for a period of 21 days. Cells were studied under light, confocal, and scanning electron microscope (SEM). Fluorescence-activated cell sorting was used to analyze cell surface markers. Cell attachment and proliferation was observed in all POSS-PCU samples, significantly higher than the activity seen within the control PCU polymers ($p < 0.05$). Microscopic examination revealed clonal expansion and morphological changes in cells seeded on POSS-PCU. The cells expressed increasing levels of mature endothelial cell markers over time with a concurrent reduction in hematopoietic stem cell marker expression. SEM showed a mixed population of morphologically differentiated endothelial cells and EPCs. These results support the use of heart valve made with the POSS-PCU polymer and demonstrate that suitable chemical modification of this nanocomposite could increase self-endothelialization potential and reduce associated thrombotic events. © 2016 American Vacuum Society. [http://dx.doi.org/10.1116/1.4939036]

I. INTRODUCTION

Heart valve prostheses, mechanical and bioprosthetic, are among the most widely used biomedical devices. The need

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for these prostheses is rising due to the increasing incidence of age related degenerative valve disease across both developed and developing nations.[1] Although current mechanical and bioprosthetic heart valves have been subject to considerable improvements over past decades, they are still prone to a number of life-threatening complications. Mechanical valves are inherently thrombogenic, resulting in patients' spending lengthy periods on side-effect laden thrombolytic medications, while bioprostheses display poor durability over the human lifespan. These are problems which advancements in modern science have not been able to rectify yet.[2] Alternative options include synthetic leaflet heart valves and tissue engineered cardiac valve prostheses, or a combination of both in a hybrid prosthesis—the latter incidentally being the focus of our research.

Despite the disappointing progress with the use of synthetic polymeric materials in the experimental years of valve replacement therapy, there has been renewed interest in the application of newly emerged advanced materials in engineering cardiovascular devices including synthetic leaflet heart valves, bypass grafts, and stents. It is now thought that synthetic leaflet heart valves based on enhanced polymeric materials and advanced trileaflet valve designs can potentially serve as a breakthrough in heart valve replacement therapy.[2]

In the cardiovascular system, normal endothelial cell function is crucial for all aspects of vascular homeostasis. In particular, endothelial cells play an important role in the continuous adjustment of vascular tone, and hence the control of blood pressure; in the physiological regulation of leukocyte traffic from blood to tissues; and perhaps most importantly, the maintenance of an antithrombotic and anticoagulant balance in circulating blood. Utilization of this natural protective measure improves long-term survival of cardiovascular implants. This has been evidenced in studies where bioprosthetic valve degeneration, calcification, and thromboembolic events have been reduced by surface coverage of valves with autologous endothelial cells.[3]

Tissue engineering is a revolutionary methodology for the generation of viable valve prostheses inherently capable of repair and remodeling; both processes that current mechanical and bioprosthetic heart valves do not undertake. *In vivo* tissue engineering is an interesting concept, which utilizes the natural regenerative capacity of the human body to create partial or complete tissue engineering organ. Development of innovative materials with smart surfaces to interact with multipotent progenitor cells circulating in peripheral blood is a key factor in this innovative approach. In particular, endothelial progenitor cells (EPCs) are multipotent cells circulating in peripheral blood with natural regenerative capacity in the cardiovascular system. They have preserved stemness capacity with potential of proliferation and differentiation into a variety of cell lines including endothelial cells. Engineering bioresponsive smart polymers will enable these autologous regenerative cells circulating in blood to recognize and adhere to the polymer surface. This can be facilitated by various surface modification techniques to enhance

cell affinity and attachment. The main aim of these techniques is to mimic extracellular matrix (ECM), which is a natural host for cells in the body.

Elements of ECM such as peptide sequences have been suggested to affect cell response by directly interacting with cell receptors, including recognition sequences derived from laminin, IKLLI, and IKVAV;[4] collagen type I derived equence;[5] and fibronectin derived sequences, RGD. RGD is probably the most widely studied modification agent in polymeric cardiovascular implants in order to increase EC and EPC adhesion.[6,7] These cells can then proliferate and differentiate into mature endothelial cells, eventually forming a layer on the polymer surface.[8] The potential of *in situ* endothelialization could dramatically enhance blood compatibility and long-term durability of heart valves and other cardiovascular implants, because it is now known that circulating endothelial progenitor cells are inversely correlated with in-stent restenosis in patients treated with EPC-capture stents.[9]

Our group has engineered a novel nanocomposite polymer potentially suitable for this purpose. Polyhedral oligomeric silsesquioxane–polycarbonate urea urethane (POSS-PCU) is a novel nanocomposite based on POSS and PCU.

POSS $(RSiO_{3/2})_n$ due to a unique cagelike structure, nanoscale dimensions (1.5 nm core), and the potential to tune chemical interactions through multiple reactive functionalities was an ideal candidate to incorporate into PCU and create nanostructured hybrid material. Nanocomposite materials can be produced by reacting POSS as a pendant group attached to the polymer backbone, as an end-group, or as a cross-linking agent in polymer matrix forming a reinforced network. In the present study, reaction of the silanol (-SiOH) groups of cyclohexanechlorohydrine-functionalized POSS with isocyanate gave segmented PCU with POSS incorporated as an end group to the hard segment (Fig. 1). Therefore, POSS gives rise to a linear organic–inorganic hybrid nanocomposite, which reacts with a single (or double) polymerizable functional group when grafted onto PCU forming covalent bonds. Synthesized nanocomposite can offer unique structural properties compared to conventional microcomposites due to the dispersion of nanoscale inorganic components in the polymeric matrix. POSS improves hemocompatibility of PCU due to surface characteristics and more mobile surface moieties.

POSS-PCU nanocomposite has been demonstrated to have antithrombogenic potential in conjunction with a proven resistance to degradation.[10] This novel polymer also demonstrates excellent biocompatibility, biostability, and advanced mechanical properties. Previous studies have also indicated that POSS-PCU nanocomposite can sustain human umbilical vein endothelial cell seeding.[11,12] Our results have also evidenced the anticalcification potential of this nanocomposite compared with other polyurethane polymers and bioprosthetic tissues such as bovine pericardium, making this nanocomposite a significant material of choice for engineering synthetic leaflet heart valves. In this study, the ability of POSS-PCU nanocomposite to retain EPCs extracted from human peripheral blood and umbilical cord blood was assessed *in vitro*. Surface modified POSS-PCU using
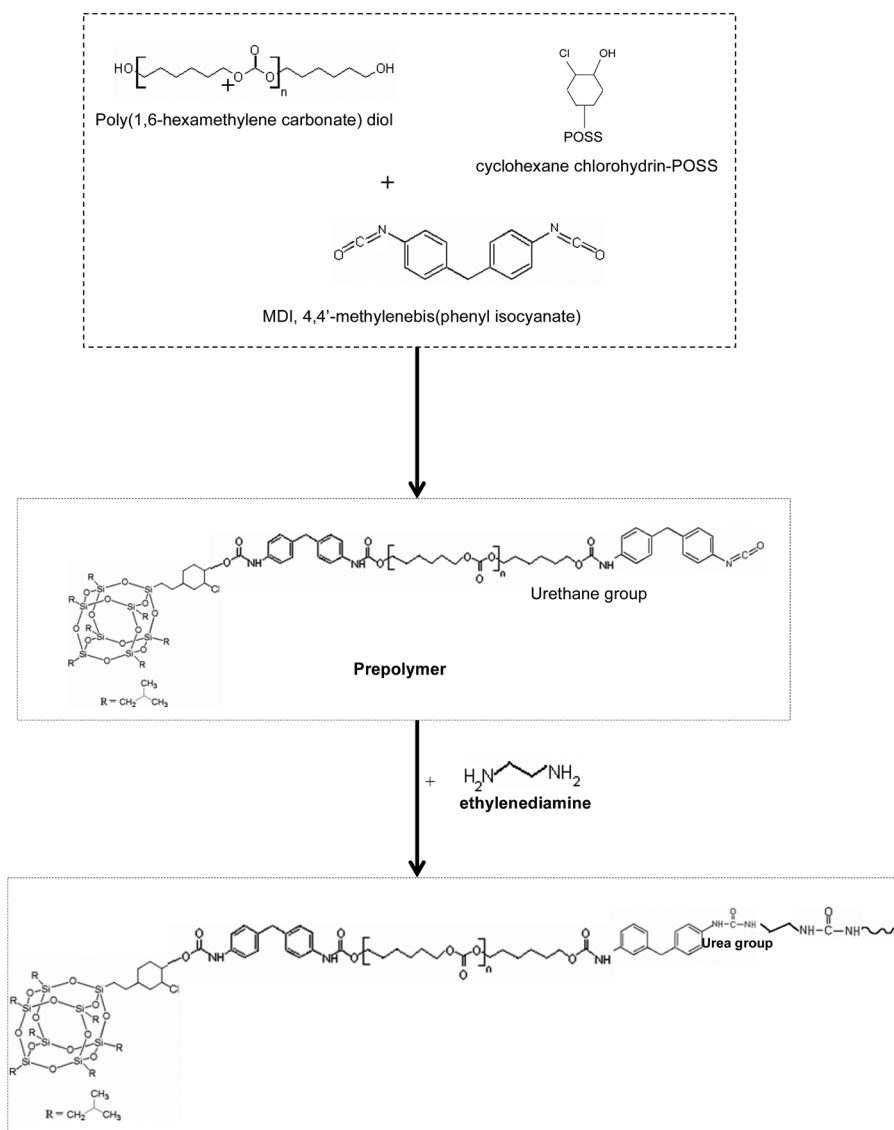
FIG. 1. Simplified reaction scheme of nanocomposite synthesis.

fibronectin derived sequences, glycine-arginine-glycine-aspartic acid-glycine (GRGDG) and lauric acid conjugated GRGDG (GRGDG-LA) were also assessed to investigate the effect of these modifications on the cells' behavior. In particular, the morphological changes, proliferation, and differentiation capacity of seeded EPCs were monitored during the culture period.

II. MATERIALS AND METHODS

A. Preparation of polymers

Synthesis of the POSS-PCU nanocomposite has been described in detail previously.[13] In brief, the inorganic urethane is made from 4,4′-methylene-bis(phenylisocyanate) (MDI), poly(hexamethylene carbonate) diol, and silsesquioxane dissolved in tetrahydrofuran, here, bis[3-(trimethoxysilyl)propyl]amine, and a chain extended with ethylene diamine in N,N'-dimethylacetamide. 1-butanol dimethylacetamide was added to form a 20% (w/v) solution of POSS-PCU with the POSS moiety, therein being at a concentration of 2%. All chemicals were purchased from Aldrich Limited (Gillingham, UK).

PCU polymer was synthesized using commercial methods as follows: Dry polycarbonate polyol (2000 mwt) was placed in a 250 ml reaction flask equipped with a mechanical stirrer and nitrogen inlet. The polyol was heated to 60 °C, and then, flake MDI was added and reacted with the polyol, under nitrogen, at 70–80 °C for 90 min to form a prepolymer. Dry dimethylacetamide was added slowly to the prepolymer to form a solution; the solution was cooled to 40 °C. Chain extension of the prepolymer was carried out by the dropwise addition of a mixture of ethylenediamine and diethylamine in dry dimethylacetamide. After completion of the chain extension, 1-butanol in dimethylacetamide was added to the polymer solution. In this study, PCU was used as a control.

Using 18% POSS-PCU and PCU polymers, films of nanocomposite were casted with a thickness of 100 μm, which is the thickness of the proposed heart valve leaflets.[14] The

resulting films were then thoroughly washed with sterile phosphate-buffered saline (PBS) and were sterilized by immersion in 70% ethanol followed by air-drying.

B. Synthesis of GRGDG and LA-GRGDG

The synthesis of a 5-residue peptide Gly-Arg-Gly-Asp-Gly-hexanoic-COOH was carried out manually by a stepwise solid phase method on a Rink acid resin in which 0.15 mmol of hydroxyl groups are present. The Rink resin was washed with dimethylformamide (DMF; 10 ml/g; 5 × 1 min). A solution of Fmoc-Gly (1 mmol) in dichloromethane (DCM; 5 ml) was stirred at room temperature and activated by adding diisopropylcarbodiimide in dry DCM to the amino acid solution. The mixture was stirred for 15 min at 0 °C, and then, 5 ml of DMF was added. The mixture was then added to the Rink acid resin. Dimethylaminopyridine was dissolved in DCM (0.1 eq. relative to resin loading) and then was added to the resin/amino acid mixture. The mixture was agitated for 1 h with oxygen free nitrogen gas. Sequential addition of the protected amino acids was carried out to complete sequence. The Rink-acid solid phase was finally treated with 95% trifluoroacetic acid to completely detach and deprotect the peptide from the solid phase. The final product was precipitated using cold diethylether. The peptide was purified by HPLC using a semipreparative C16 column. In the case of LA-GRGDG, the same process was followed by the conjugation of LA prior to the detachment of the peptide. Rink-amide resin and F-moc amino acids were supplied by Novabiochem, Beeston, UK, and all other chemicals by Rathburn, Walkerburn, Scotland.

C. Biofunctionalization of the POSS-PCU surface with peptides

Cast films of POSS-PCU nanocomposite were used to graft GRGDG and GRGDG-LA using a modified direct grafting method.[15] Three different concentrations (1, 2, and 10 mg/g of polymer) of GRGDG and GRGDG-LA peptides were prepared and coated onto polymer films in a clean fume cupboard and air dried. Once dried, the samples were exposed to 2% (v/v) glutaraldehyde in PBS solution and incubated for 2 h. The samples were washed with PBS three times and were rinsed with water containing 0.1% Triton X-100, then with water, and stored dry until further use.

D. Characterization of the surfaces

The samples of modified and nonmodified polymers were characterized using atomic force microscopy (AFM) and attenuated total reflectance Fourier transform infrared (ATR-FTIR). The surface morphology was investigated using AFM in a semicontact mode using a Nanoscope III multimode scanning probe microscope (Digital Instruments, Veeco Instruments, Inc., New York). The AFM micrographs of the surfaces were collected over a scan area of $100 \times 100 \,\mu$m for all samples. At least four randomly selected areas of each sample were analyzed for surface roughness values. ATR-FTIR spectra of the polymers were obtained using a Nicolet

8700 FTIR spectrometer (Thermo Electron Corporation, UK), in conjunction with an ATR accessory at room temperature. Data were collected on germanium crystal using a variable-angle ATR unit at a nominal incident angle of 45°. The samples were cut randomly from polymer films, cut to ATR crystal size, and mounted on trapezoid crystal. Spectra were recorded in the midinfrared region (4000–400 cm^{-1}) at 4 cm^{-1} resolution and averaging 128 numbers of scans. The spectra data were acquired using OMNIC 7.2 software.

E. Contact angle measurement

The contact angle is a measure of the ability of a liquid to spread on a solid surface. Water contact angle provides information on the affinity of water to a solid surface, or hydrophilicity and hydrophobicity of the material in other words. Measurements of static water contact angle were taken according to the standard procedure. In brief, a small drop of deionized water (20 μl) was put onto the flat sample surfaces using a syringe such that the drop volume at the surface expands. The resulting contact angle was evaluated by optical methods using a digital camera. The measurements were taken in an air-conditioned laboratory at 25 °C. For each sample, the measurement was repeated six times.

F. Isolation of EPC from peripheral and umbilical cord blood

With consent from healthy volunteers, fresh whole blood (50 ml) was obtained using a Vacutainer system (BD Bioscience), containing preservative free heparin. With consent, human umbilical cord blood was collected from placental cord of volunteers undergoing cesarean section delivery immediately after delivery of placenta to avoid clot formation. Peripheral blood mononuclear cells were isolated by Ficoll-Paque (GE Health care Bio-science, Sweden) density gradient centrifugation ($400 \times g$, 40 min at 20 °C) and resuspended in PBS containing 2 mM EDTA (autoMACS Rinsing Solution, Miltenyi Biotec, Germany). The cells were washed three times and resuspended into 300 μl autoMACS buffer for magnetic labeling. To block nonspecific/specific binding via fragment crystallisable region (FcR), 100 μl of FcR blocking human IgG was added followed by adding the same amount of MicroBeads conjugated with antihuman CD34 antibodies (CD34 MicroBead kit, Militenyi Biotec, UK). The cell suspension was incubated in the fridge for 30 min at 4 °C and then loaded onto a magnetic-activated cell sorting (MACS) column, which was placed in the magnetic field of MACS separator. The magnetically retained CD34$^+$ cells were collected and washed.

G. Evaluation of EPC purity

The purity of the isolated CD34$^+$ cells was evaluated by flowcytometry. Following magnetic separation, the labeled cells were stained with monoclonal anti-CD34-PerCPCy5.5 (BD Bio-Science), anti-CD133-APC (Miltenyi Biotec GmbH), anti-VEGFR2-FITC, and anti-CD144-PE (R&D Systems) at 4 °C for 15 min. After additional washing, the

cells were fixed in 4% paraformaldehyde and immediately sorted using a florescence-activated cell sorting (FACS) Calibur flowcytometer in conjunction with CELL QUEST software. A minimum of 10 000 cells were acquired for each analyzed sample.

H. EPC culture

Isolated EPCs were seeded onto 96-well plates coated with POSS-PCU nanocomposite and GRGDG & lauric acid conjugated GRGDG modified POSS-PCU and PCU polymer as a control. A separate control was EPC culture in a petri dish. Endothelial cell growth medium (Provitro GmbH, Germany) was used supplemented with fetal calf serum 10%, heparin 22.50 μg, epidermal growth factor (EGF) 05.00 ng, basic fibroblast growth factor (bFGF) 10.00 ng, vascular endothelial growth factor (VEGF) 00.50 ng, arginine 3-insulin like growth factor-1 (R3-IGF-1) 20.00 ng, ascorbic acid 01.00 μg, hydrocortisone 00.20 μg, gentamicin 50.00 μg, and amphothericin B 50.00 ng (Provitro GmbH, Germany). The medium was changed weekly during the 21-day period.

I. EPC characterization

Immunostaining for cell surface markers was carried out on day 1, 7, 14, and 21 on different samples. The cells were collected from individual wells and stained for the surface markers, including CD34 PerCyP, VEGFR2 FITC, CD144 PE, CD133 APC, CD31 APC, and VW FITC. Surface staining was carried out at 4 °C for 15 min. The cells were then washed with PBS, resuspended in 100 μl of 4% paraformaldehyde, and were sorted using FACS Calibur system. The FLOWJO software (Tree Star, Inc., USA) was used for combination analysis of different subsets. Morphological studies were carried out using live light microscopy, scanning electron microscopy, and confocal microscopy.

J. Measurement of cell metabolism

Cell metabolism was assessed by Alamar blue[TM] (AB) (Serotec, Kidlington, UK) assay at day 1, 7, 14, and 21 as described in literature.[12] In brief, the medium was removed from the polymer film-coated dishes, and 4 ml of 10% AB in cell culture medium (CCM) was added. After 4-h incubation, the samples of AB/CCM were removed and measured on a Fluroskan Ascent FL (Thermo Labsystems, Basingstoke, UK) fluorescent plate reader (excitation 530 nm, emission at 620 nm). The unseeded polymer film-coated petri dishes were used as controls.

K. Statistical analysis

GRAPHPAD PRISM (version 5) was used for plotting graphs and for statistical analysis, Student's t test and two-way ANOVAS were used as indicated.

III. RESULTS

A. Surface characterization

The AFM results (Fig. 2) revealed that incorporation of POSS to PCU led to an increase in the roughness values for POSS-PCU (96.83 $\pm$ 3.2 nm) compared to PCU (49.7 $\pm$ 2.4 nm). The surface morphology and topography was dramatically changed by the inclusion of POSS into PCU, resulting in a specific micro-/nanoglobular texture of POSS-PCU. Surface modification of POSS-PCU films with GRGDG and GRGDG-LA peptides significantly increased the surface roughness (181.2 $\pm$ 4.6, 185.34 $\pm$ 3.7 nm, successively), indicating the effect of peptide grafting on the surface topography.

Further assessment of the surfaces was carried out using ATR-FTIR (Fig. 3). The results showed that surface modification with GRGDG and GRGDG-LA (fixing reaction) has created new chemistry on the surface. The IR peak at 1100 cm^{-1} confirmed the existence of Si-O-Si groups in the silsesquioxane cages on surface of all modified POSS-PCU films. Although the POSS related peaks appeared to be unchanged, the peaks at 1742 cm^{-1} correspond to C=O group on the surface of PCU and POSS-PCU disappeared due to GRGDG and GRGDG-LA deposition. Moreover, urethane amide groups at 1635, 1532, and 1253 cm^{-1} were affected in GRGDG and GRGDG-LA fixing process. This confirms that amide groups in both GRGDG and GRGDG-LA modified polymers were involved in cross-linking reaction (fixing process). It is also evident that the C-N group in POSS modified films at 1230 cm^{-1} did not change due to peptide deposition. Surface characterization results confirm the presence of peptide sequences in the modified polymers.

B. Contact angle measurement

Water contact angle measures the hydrophobicity/hydrophilicity of a material. Incorporation of POSS to PCU increases water contact angle and surface hydrophobicity. POSS-PCU displayed a contact angle of 115° ($\pm$5), suggesting a certain degree of hydrophobicity (Fig. 4). After conjugation with peptides, POSS-PCU displayed a reduced hydrophobicity, shown by a reduction in the contact angle to 87° ($\pm$6) and 84° ($\pm$7), for GRGDG and GRGDG-LA, respectively. The effect of water contact angle on hemocompatibility is still not clearly defined, but peptide grafting certainly changes the water contact angle of the material.

C. Isolation of EPC

Isolated peripheral blood mononuclear cells and CD34$^+$ labeled cells were counted by two independent observers after each cell extraction. The number of isolated mononuclear cells was 760.0 $\pm$ 105.8 $\times$ 10^3/ml for peripheral blood samples obtained from healthy adult volunteers while it was 1610 $\pm$ 187.4 $\times$ 10^3/ml in the samples extracted from umbilical cord blood, which was significantly higher
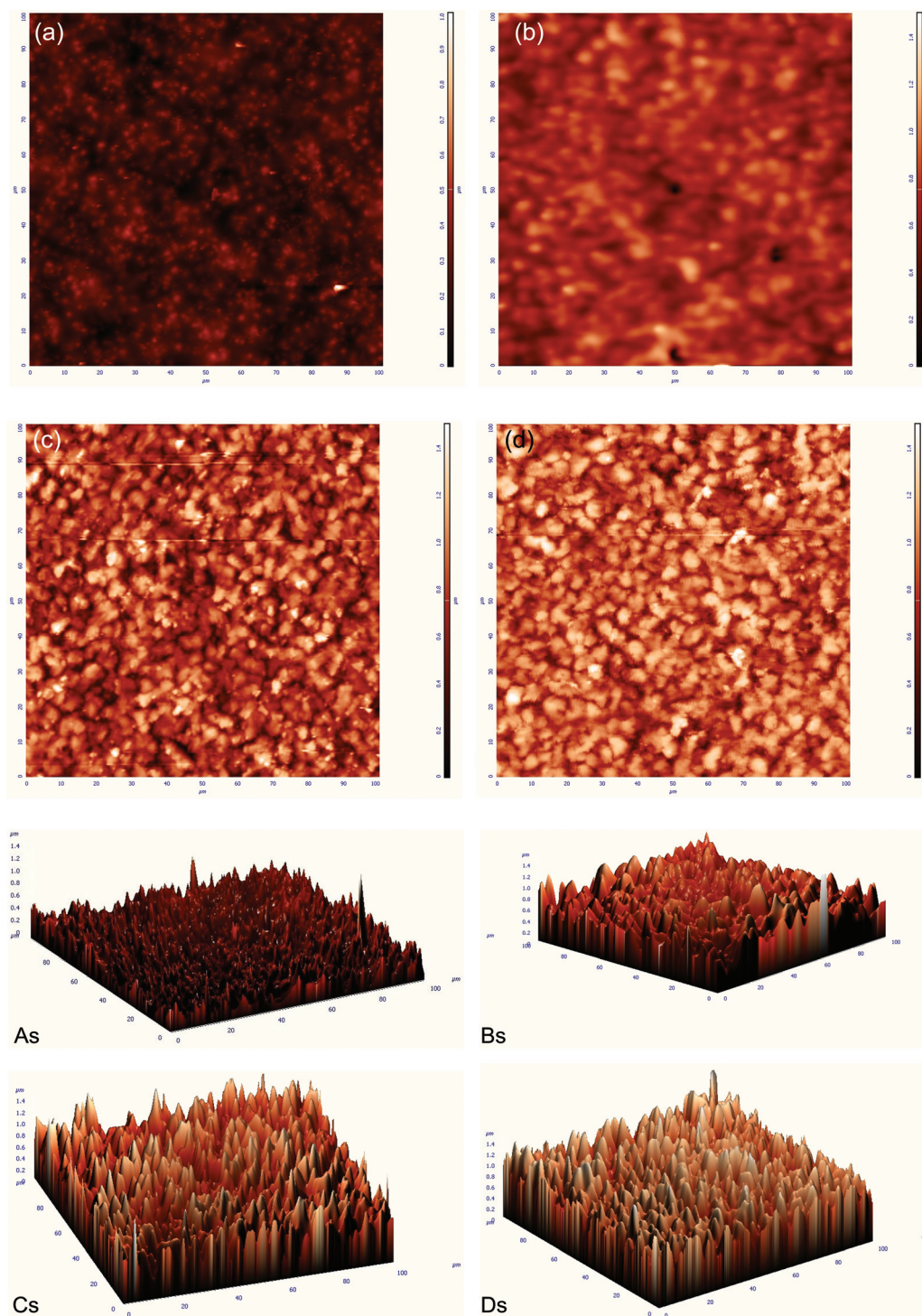
FIG. 2. AFM images of (a) PCU, (b) POSS-PCU, (c) GRGDG modified POSS-PCU, and (d) GRGDG-LA modified POSS-PCU (X, Y, Z $= 100 \times 100 \times 1.5 \, \mu$m). The surface morphology and roughness were dramatically changed by surface modifying agents compared to the unmodified PCU.

($p < 0.05$) compared to peripheral blood. After extracting CD34$^+$ cells using magnetic activated cell sorting method, the number of labeled cells extracted from peripheral blood ($18.13 \pm 1.92 \times 10^3$/ml) was significantly lower than the one for isolated from umbilical cord blood ($31.42 \pm 2.02 \times 10^3$/ml) ($p < 0.05$, n: 3).

D. Evaluation of EPC purity

In order to evaluate the purity of the labeled cells for the expression of CD34 surface marker, the samples of labeled and unlabeled cell population were stained with anti-CD34 FITC and acquired by FACS Calibur. In each set of the experiments, high levels of purification for CD34$^+$
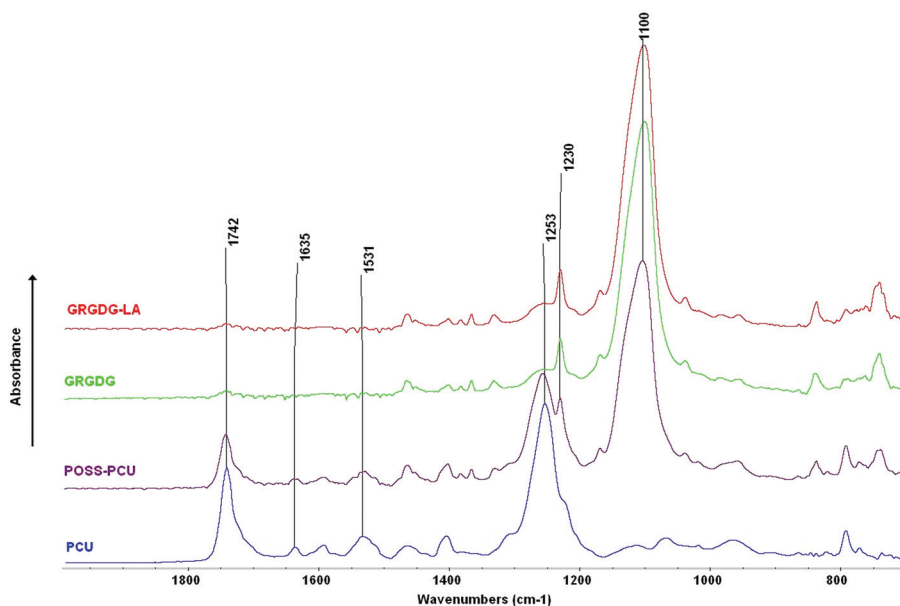
FIG. 3. ATR-FTIR spectra of PCU, POSS-PCU, and GRGDG and GRGDG-LA modified POSS-PCU used in the experiment, in the range of $2000–700\,cm^{-1}$. Induced changes in the surface spectra are the results of surface modification. The Si-C, Si-O-Si, Si-H, and CH bonds of the POSS nanocages revealed peaks at 1230, 1100, 834, and $738\,cm^{-1}$, respectively, on all POSS-PCU polymers. Peaks at $1742\,cm^{-1}$ correspond to C=O group and at 1635, 1532, and $1253\,cm^{-1}$ representing urethane amide groups were affected in GRGDG and GRGDG-LA modification.

cells were found using FACS immunostaining, as shown in Fig. 5.

E. EPC proliferation

The isolated peripheral blood mononuclear cells and $CD34^+$ labeled cells were counted by two independent observers after each cell extraction. The number of isolated mononuclear cells was $760.0 \pm 105.8 \times 10^3$/ml for peripheral blood samples obtained from healthy adult volunteers while it was $1610 \pm 187.4 \times 10^3$/ml in the samples extracted from umbilical cord blood, which was significantly higher ($p < 0.05$) compared to peripheral blood. After extracting $CD34^+$ cells using magnetic activated cell sorting method, the number of labeled cells extracted from peripheral blood ($18.13 \pm 1.92 \times 10^3$/ml) was significantly lower than the one
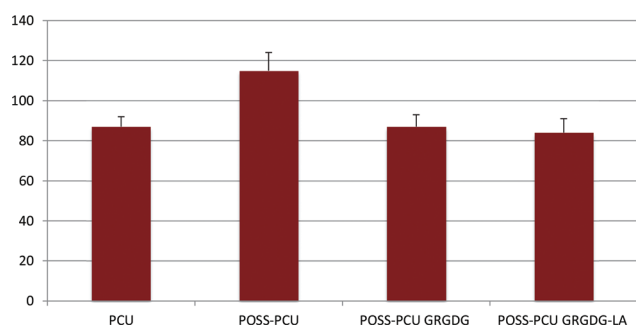

FIG. 4. Contact angle measurement results of PCU, POSS-PCU, POSS-PCU GRGDG, and POSS-PCU GRGDG LA samples. The water contact angle results show that the POSS incorporation significantly increased hydrophobicity of the surface of PCU, whereas peptide grafting renders the surface less hydrophobic. Data are presented as mean $\pm$ SEM.

for isolated from umbilical cord blood ($31.42 \pm 2.02 \times 10^3$/ml) ($p < 0.05$, n: 3).

F. EPC differentiation

The isolated $CD34^+$ cells were seeded on the 96-well plates coated with POSS-PCU, PCU, GRGDG-LA, and GRGDG modified POSS-PCU nanocomposite and cultured for 21 days. During the culture, the cells were monitored for morphological changes, the presence of EPC colony forming units, and the overall cell population and proliferation capability of the cells. Representative live microscopy images of the arrangement of colony forming units on days 7 and 14 are shown in Fig. 6. The colonies were defined morphologically as a central core of round cells with elongated cells at the periphery.[16,17] Confocal microscopy of the colonies revealed that the majority of the cells forming the colonies were positive for CD34 surface marker, as shown in Fig. 7. To confirm the identity of the cultured cells, the cells were studied by FACS for surface markers. In particular, randomly selected samples of the cells cultured on different polymer disks were collected and stained with antibodies directed to CD34, CD133, VEGFR2, and CD144, and acquired using FACS Calibur flowcytometer on days 1, 7, 14, and 21. Using the appropriate gating system, the frequency of a specific subset of cells, which were positive for CD34, CD133, and VEGFR2, was determined. This subset is considered as true EPC in literature.[18] As shown in Fig. 8, the frequency of triple positive cells (positive for CD34, CD133, and VEGFR2), which are considered as true EPC, was increased over time, thus showing the expansion of this cell subset within the colonies. The GRGDG-LA modified POSS-PCU retained higher percentage of EPC compared to
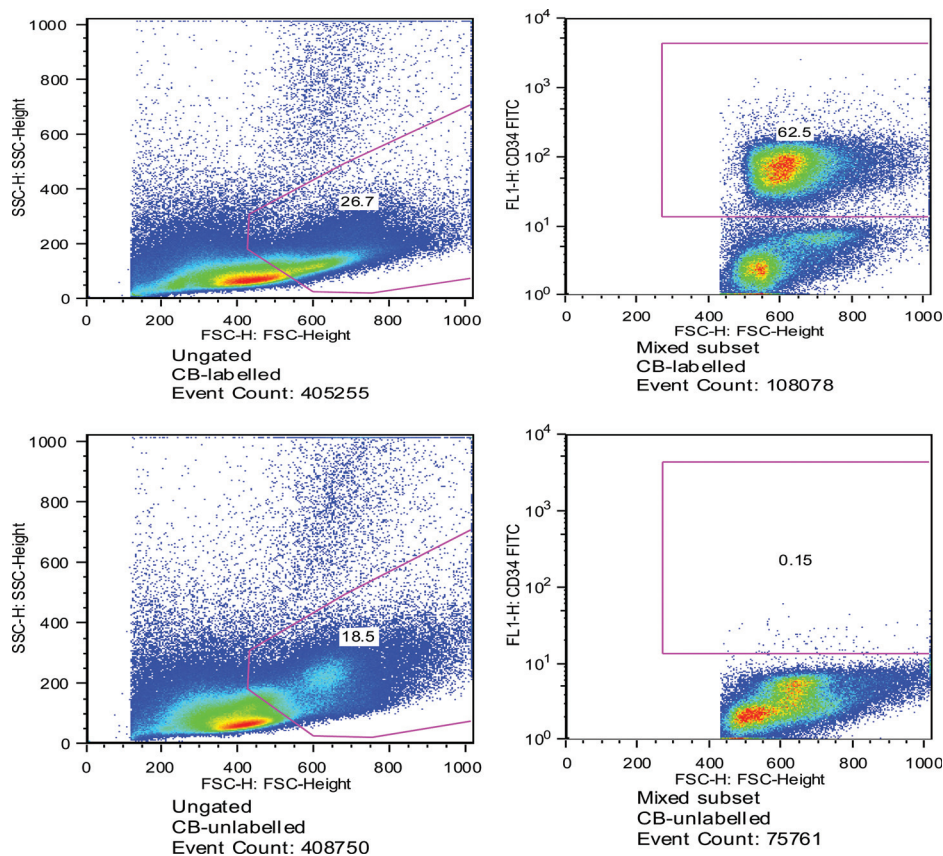
FIG. 5. Representative FACS plots of the labeled cells (top row) and unlabeled cells (bottom row) isolated using CD34 microbeads, showing high degree of purification achieved after separation by MACS.

the other polymers, as shown in Fig. 8, indicating that this novel functionalized nanocomposite polymer may offer better *in situ* endothelialization of heart valves.

G. EPC differentiation

Over the time of culture, cells changed morphologically from spindle-shape characteristic for early EPCs to cobble stone, indicating potential differentiation of the cells to mature endothelial cells [Figs. 6(c) and 6(d)]. FACS analysis confirmed the increase in the expression of endothelial specific cell surface markers, including VEGFR2, CD144, CD31, and von Willebrand factor (Figs. 9–11). FACS analysis revealed that the cells cultured on different polymer disk expressed CD144 with the increase in time. The cells isolated from adult human peripheral blood expressed this marker more frequently than the cells separated from umbilical cord blood. Since CD144 is a mature endothelial cell marker, it can be concluded that umbilical cord EPCs remain undifferentiated for longer than adult peripheral blood counterparts. Higher percentage of triple positive EPCs in cord blood samples is also in accordance with this finding. Similar results were found for VEGFR2 surface marker expression during the culture period (Fig. 11). Higher expression of VEGFR2 at day 7 on cells extracted from peripheral blood compared to the ones isolated from umbilical cord blood indicates more stemness feature of EPCs

originated from umbilical cord. However, EPCs from both sources could differentiate into endothelial cells cultured on all polymer surfaces after three weeks culture. Cultured cells were further analyzed for the expression of other endothelial cell surface markers, including CD31 and von Willebrand factor. The results showed that these markers were also highly expressed by the cultured cells after one week, as shown in Fig. 10. This again confirms differentiation of the cells into mature endothelial cells. In order to determine the expression of endothelial specific cell surface markers, the cultured cells were stained for von Willebrand factor and VEGFR2 at day 14 (Fig. 12). The results revealed that the cells were positive for these markers, indicating the presence of endothelial cells on the samples.

SEM of the samples taken at day 7 revealed adequate cell attachments on the POSS-PCU samples morphologically similar to endothelial cells (flat, spindle-shaped cells with numerous filopodia) (Fig. 13).

H. Cell metabolism

Cell metabolism was measured using Alamar Blue®, a vital dye which viable cells metabolize from blue (resazurin) to pink (resorufin), and the change in color was detected spectroscopically.[19] The cells remained highly viable during the experiment, and there was no significant reduction in cell metabolism in the samples during the culture compared to
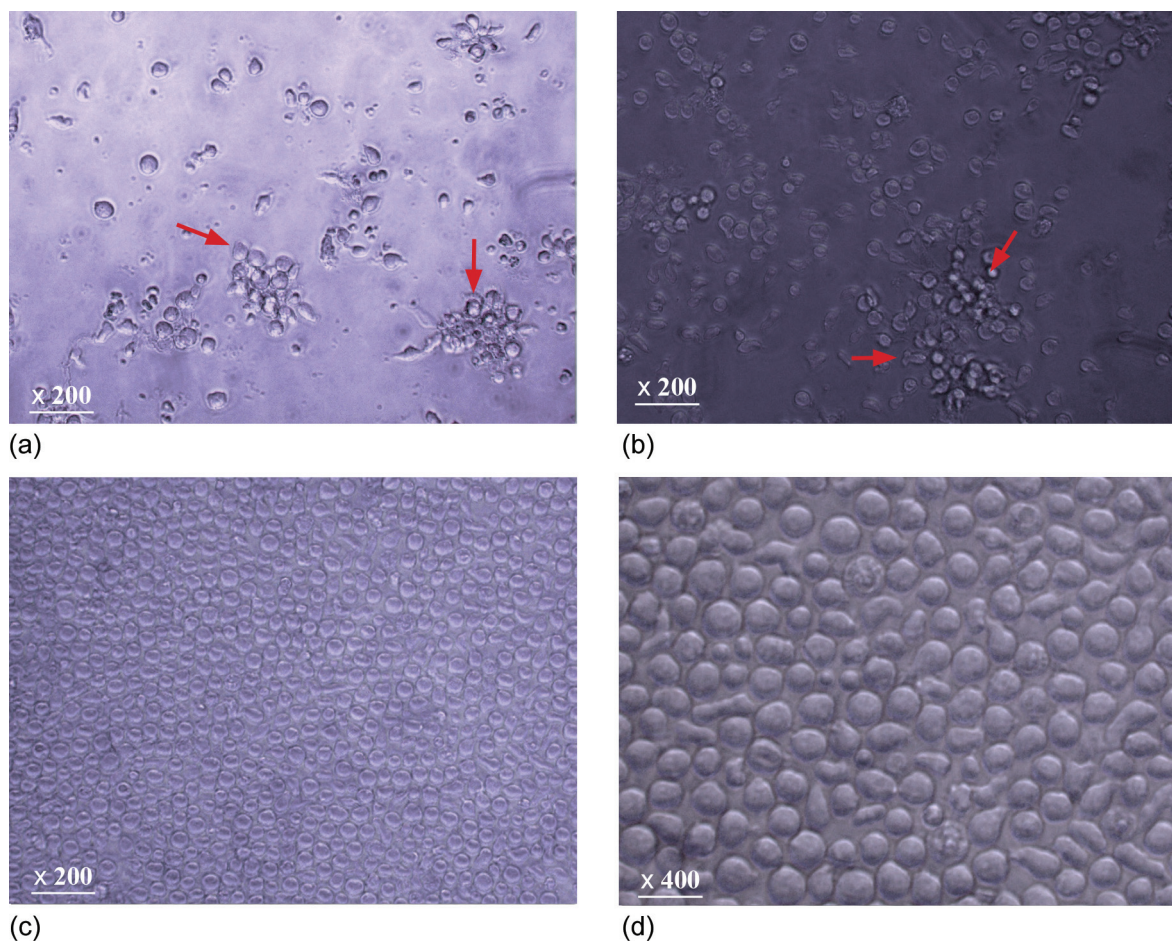
FIG. 6. Live microscopy images of the isolated cells cultured on POSS-PCU nanocomposite samples. Colonies of EPC (marked with arrows) undergoing proliferation resulted in an increased cell population over time of culture as shown on day 7 (a) and day 14 (b). The cells underwent morphological changes during the culture and spindle-shaped morphology of early EPCs on day 7 (a) was dominated by cobble stone-shaped confluent layer at day 21 (c), characteristic morphology of the endothelial cells. (d) Higher magnification of confluent layer of EC on day 21.
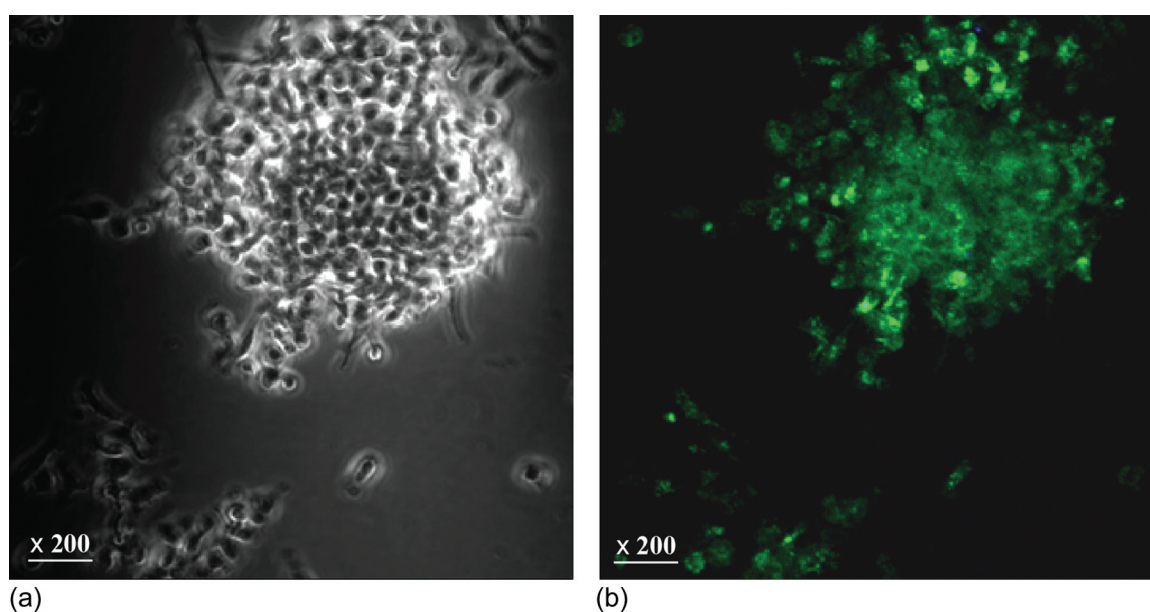

FIG. 7. (a) Representative image of EPC colonies at day 14, and (b) the confocal microscopy image of the same colony shows expression of CD34 surface markers on the colony forming cells.
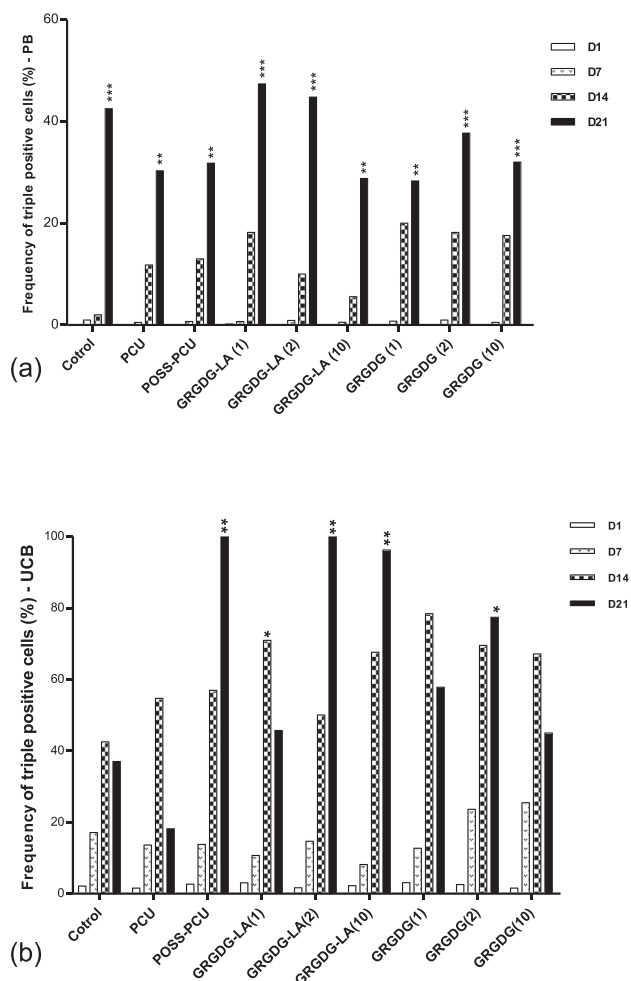
(a)

(b)

FIG. 8. Frequency of triple positive EPC (positive for CD34, CD133, and VEGFR2) on different samples over the time of experiment. (a) The cells extracted from peripheral blood (PB) and (b) cells isolated from umbilical cord blood (UCB). As shown, there was a significant increase in the frequency of this subset of cells at day 21 compared to day 1 on POSS-PCU and GRGDG-LA modified samples (two way ANOVA; *$p < 0.05$; **$p < 0.01$, and ***$p < 0.001$). UCB, umbilical cord blood; PB, peripheral blood; GRGDG, POSS-PCU modified with GRGDG peptide; GRGDG-LA, POSS-PCU modified with lauric acid conjugated GRGDG peptide; (numbers within brackets), concentration of peptide as mg/g polymer.

metabolism measured by Alamar Blue assay at day 1, as shown in Table I.

IV. DISCUSSION

Studies have shown that progenitor stem cells circulating in the blood have regenerative capacity and play a role in the natural reconstruction and remodeling process of cardiovascular system. It has been demonstrated[20] that bone marrow derived hematopoietic progenitor cells can give rise to ECs and contribute to endothelial recovery and new capillary formation after ischemia. These cells provide a reparative capacity for cardiovascular system by contribution in repair as well as the growth of new blood vessels. Potentially, this capacity can be used in re-endothelialization of the decellularized matrix or synthetic implants.[21] Several lines of evidence point to an important role of EPC in revascularization
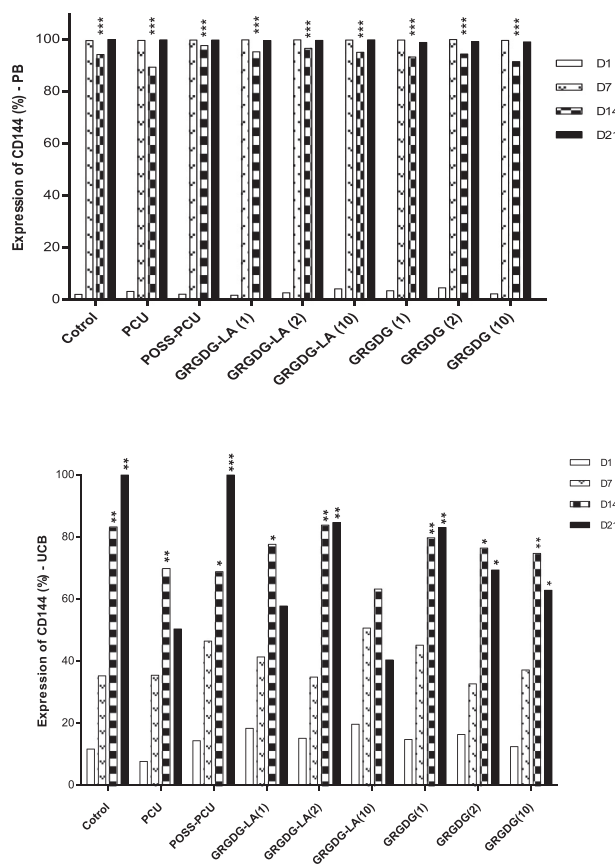

FIG. 9. Expression of CD144 (endothelial cell surface markers) on cells cultured on different polymer disks. The cells isolated from peripheral blood (a) differentiated more rapidly (at day 7), compared to the cells extracted from umbilical cord blood (b). As shown, there was a significant increase in the expression of CD144 on most of the samples compared to day 1 (two way ANOVA; *$p < 0.05$ and **$p < 0.01$, and ***$p < 0.001$).

process in an adult, but the exact definition of EPC still remains a controversy. Different surface markers have been used to characterize EPCs, and based on that, various isolation techniques have been used to extract these cells from peripheral blood. We used the most commonly accepted surface markers, i.e., CD34, CD133, and VEGFR2, as representative markers of true EPCs. The frequency of the cells being positive for these three markers was very low in the peripheral blood samples compared to those in the umbilical cord blood samples. However, in both cases, there was a significant increase in the frequency of triple positive EPCs, showing that this subset has been the subject to proliferation and expansion over the time of culture. The increase was higher in umbilical cord blood samples, and EPCs extracted from adult peripheral blood differentiated to ECs earlier than the ones extracted from cord blood, confirming higher stemness characteristics of these cells reported in other studies.[22] However, clinically peripheral blood sample results may be more relevant, and the aim of further work will be to increase endothelial cell coverage on used nanopolymers. As stimulation of EPC colony formation could be a double-edged sword because it can increase the risk of clotting and unwanted differentiation of EPCs into smooth muscle cells
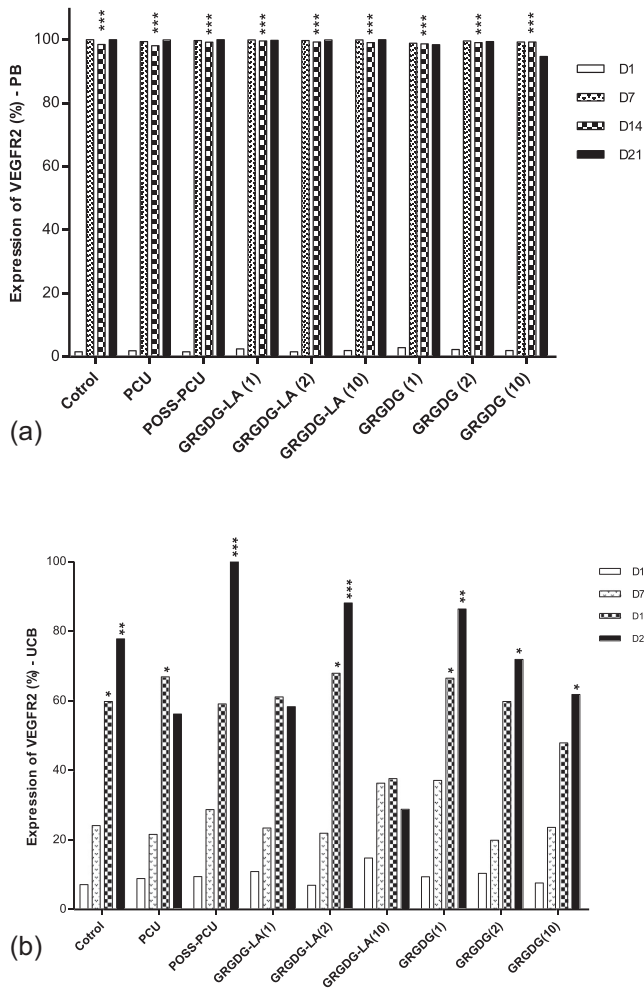
(a)

(b)

FIG. 10. Expression of VEGFR2 (an endothelial cell surface marker) on the cultured cells; Like CD144, the cells isolated from peripheral blood (a) expressed differentiation markers more rapidly (on day 7) compared to the cells extracted from umbilical cord blood (b). As shown, there was a significant increase in the expression of CD 144 on most of the samples compared to day 1 (two way ANOVA; *$p < 0.05$ and **$p < 0.01$, and ***$p < 0.001$).

may exacerbate intimal hyperplasia,[23] care must be taken to ensure EPCs differentiate into ECs. POSS-PCU nanocomposite and the GRGDG-LA modified nanocomposite revealed higher levels of EPC expansion compared to the PCU and GRGDG modified polymers ($p < 0.05$). The surface composition and its chemical and physical properties are determining factors in the cell affinity of a synthetic surface. We assume that the presence of POSS in nanocomposite surface promotes EPC proliferation by providing favorable environment in terms of surface topography and biochemical properties. It has been already shown that the POSS modification affects the nanotopography of the surface as well as hydrophobicity[24] and cell affinity,[12] although the exact mechanism is not completely known and is currently under investigation. Also, the GRGDG-LA modified polymers showed higher degree of EPC expansion, which was in accordance with previous findings.[6,7] It is clear from ATR-FTIR and AFM results that both POSS modification and peptide grafting have changed the surface chemistry and its texture topography. These might be the reasons behind enhanced cell attachment and proliferation in surface modified samples compared with the unmodified PCU sample.

Previous cardiovascular implants developed in our laboratories using POSS-PCU, including vascular grafts tested *in vivo* and currently in clinical trials,[25] have been developed using coagulation technique, left in a water-bath to allow phase-separation to occur and resulted in spongylike constructs, which posses elastic and pulsatile properties of blood vessels. We have also shown that with the increase in pore size in POSS-PCU, there is a reduction in tensile strain and strength.[26] In this study, POSS-PCU was fabricated using casting technique, which renders it mechanical strength and solid structure suitable for cardiac valves. This design would produce valves with optimized hemodynamic features and reduced stress distribution over the leaflets.

In order to facilitate cell attachment to the synthetic surface, various peptide sequences derived from ECM have been isolated and grafted onto materials to enhance
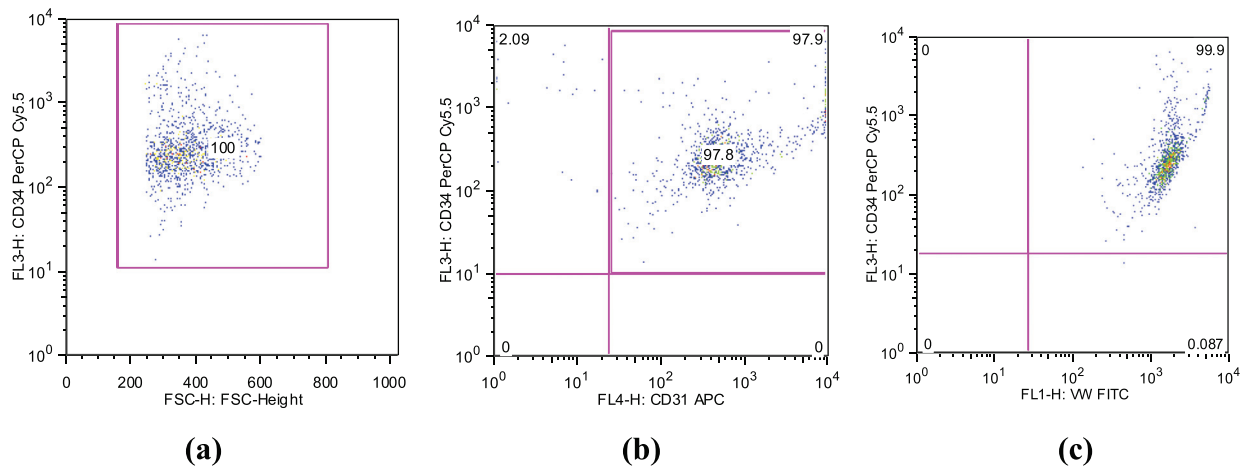

(a)   (b)   (c)

FIG. 11. Representative FACS plots of the cultured cells on POSS-PCU sample at day 7, indicating positive expression of CD31 and vWF by the cultured cells. Population of CD34$^+$ cells (a) were analyzed for the expression of CD31 (b) and vWF (c), showing that endothelial cell surface markers were highly expressed by the cultured cells.
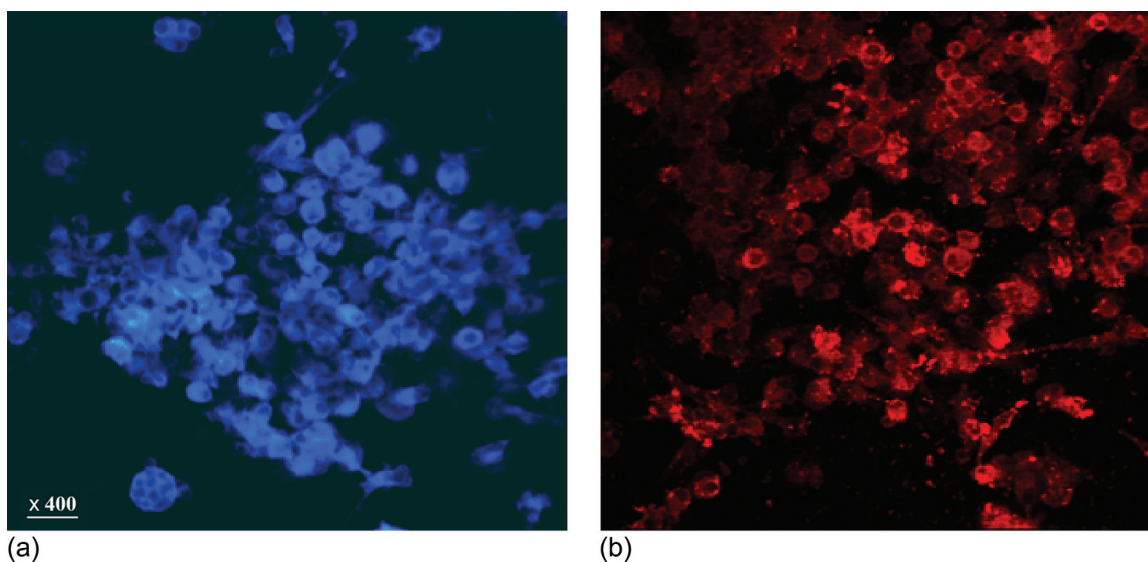
(a)

(b)

FIG. 12. Immunostaining results of the cultured cells on day 14; samples of the cultured cells were stained for vWF (a) and VEGFR2 (b), showing positive expression of these surface markers on the cells.
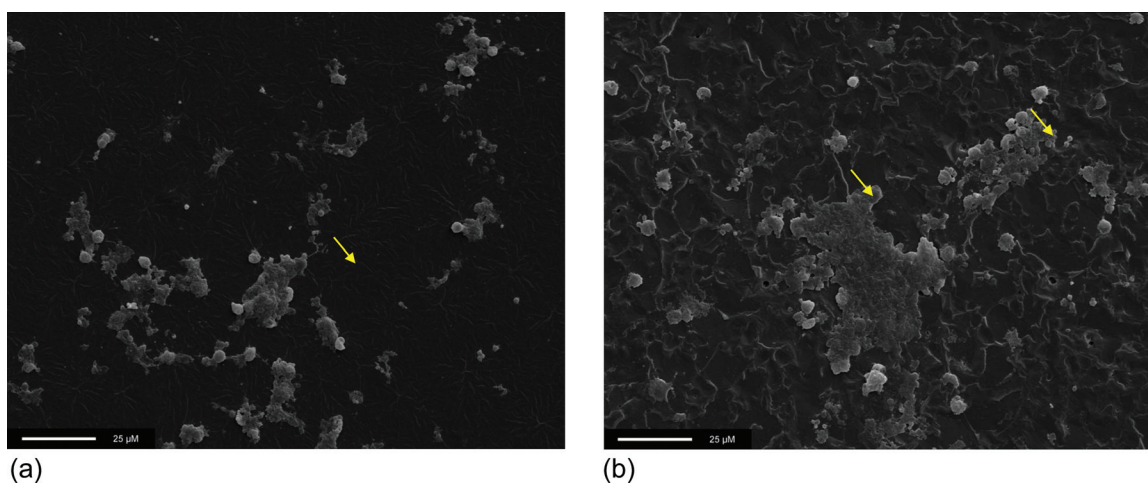

(a)

(b)

FIG. 13. Scanning electron microscopy of the cells cultured on: (a) PCU sample and (b) POSS-PCU samples. Colonies of the cells (marked with arrows) were observed by SEM at day 7, strongly attached to the polymer surface.

TABLE I. Cell metabolism determined by Alamar Blue^{TM} assay measuring fluorescence (excitation: 530; emission: 620). Data are presented as mean $\pm$ SEM (n: 3).

samples	Time Day 1	Day 7	Day 14	Day 21
Control	61.12 ± 2.69	55.01 ± 8.42	101.02 ± 1.83	73.88 ± 9.71
PCU	73.51 ± 7.40	50.12 ± 3.1	80.18 ± 7.64	67.16 ± 11.38
POSS-PCU	73.63 ± 4.65	60.07 ± 7.57	92.41 ± 2.57	81.30 ± 12.6
GRGDG-LA (1)	81.86 ± 10.73	52.13 ± 7.75	79.92 ± 13.46	62.39 ± 2.66
GRGDG-LA (2)	72.27 ± 2.92	67.42 ± 14.50	92.20 ± 3.84	62.73 ± 2.54
GRGD-LA (10)	73.78 ± 8.11	56.42 ± 6.75	91.79 ± 4.34	60.22 ± 7.75
GRGDG (1)	78.81 ± 13.54	58.63 ± 7.38	81.04 ± 2.51	50.19 ± 8.26
GRGDG (2)	80.24 ± 7.70	60.42 ± 4.79	96.56 ± 6.54	50.6 ± 6.47
GRGDG (10)	69.27 ± 15.51	63.61 ± 10.48	99.23 ± 3.91	60.26 ± 5.02

biological properties and influence cell behavior. Examples are REDV,[27] PHSRN,[28] RGD,[29] GRGDG, and GRGDSP from fibronectin;[30] stem cell homing factor SDF-1α;[31,32] and collagen type I derived sequence, laminin-derived recognition sequences, IKLLI, IKVAV;[33] DGEA.[4] Of the peptides investigated, the RGD peptide has featured in the largest number of biomaterials studies.[34] In cardiovascular bioengineering, biofunctionalization of polycarbonate polyurethane urea, and nanocomposite polymers with RGD/modified RGD and heparin, has been reported to increase EC and EPC adhesion.[6,7,35–37]

In this study, we used a sequence of Gly-Arg-Gly-Asp-Gly (GRGDG) peptide and GRGDG conjugated with lauric acid GRGDG-LA to modify the surface of nanocomposite in order to improve cell affinity of the surface. Lauric acid conjugation was used to facilitate incorporation of the peptide into the matrix of the polymer and has been shown to be suitable to enhance cell metabolism on the modified surfaces.[7] Different concentrations of the peptides (1, 2, and 10 mg/g of polymer) were used in order to assess their effect on cell attachment, proliferation, and differentiation. Microscopic observation revealed higher population of the cells on the POSS-PCU nanocomposite and the GRGDG-LA modified nanocomposites. FACS analysis showed that the frequency of triple positive EPCs in the POSS-PCU and GRGDG-LA modified polymers (concentrations of 2 and 10 mg/g) was significantly higher at day 21 compared to day 1 in both the peripheral blood and umbilical cord blood samples. Interestingly, the expression of CD144 and VEGFR2 was also significantly higher for the same polymers (POSS-PCU and GRGDG-LA, 2 and 10 mg/g) at day 21 compared to day 1 in the umbilical cord blood samples. However, in peripheral blood series, they were high in all samples regardless of the type of the polymer. According to the results, we assume that the GRGDG-LA modification was better than the GRGDG modification and POSS-PCU itself, and provides preferred environment for EPC proliferation and differentiation. The cells remained highly viable during the culture, and there was no significant change over the time and between the samples compared to day 1, even though there was some fluctuation in Alamar Blue assay results.

The POSS-PCU nanocomposite has been shown to have antithrombogenic potential, improved biocompatibility, and calcification resistance, but in order to achieve optimal endothelialization of cardiovascular implants and form antithrombotic and anti-platelet barrier, novel techniques of coating POSS-PCU to maximize EPC capture must be developed. One possibility to increase EPC capture is by covalent binding of antibodies against EPC cell-specific biomarkers to cardiovascular implants.[38] *In vivo* study of pulmonary valves conjugated with antibodies against CD133 showed no evidence of calcification and thrombi formation three months postimplantation into sheep model.[39] Preliminary *in vitro* studies in our laboratory with POSS-PCU nanocomposite polymer functionalized with antibodies against CD133 showed minimal increase in EPCs capture,[40] but significant improvement in endothelialization and hemocompatibility

was observed with immobilized anti-CD34 antibodies on POSS-PCU.[41] Nevertheless, the use of antibodies carries drawbacks: it involves delicate chemical conjugation and more complex manufacturing process. Also, it poses a new challenge to maintain the viability of the antibody in order to have a long shelf life. Thus, this approach would increase the cost of the implant, making it less likely to find large-scale clinical application. Therefore, several issues need to be addressed in order to find optimal surface covering of cardiovascular implants, and in this study, we tested a different set of peptides. Our results suggest that the POSS-PCU nanocomposite could be modified by GRGDG-LA and used in the development of new generation synthetic leaflet heart valves with *in situ* endothelialization potential by attracting endothelial progenitor stem cells from circulating blood. However, more experiments are required to test hemocompatibility *in vitro*[42] before commencing *in vivo* testing. For further evaluation, the next step is dynamic EPCs culture on heart valve prototypes in a specifically developed bioreactor system.

V. CONCLUSIONS

In an *in vitro* setting, EPCs were extracted from adult peripheral blood and umbilical cord blood and cultured on the POSS-PCU nanocomposite in comparison with PCU, GRGDG, and GRGDG-LA modified polymers. EPCs' proliferation and differentiation was noticed over the time of culture. The POSS-PCU nanocomposite revealed an enhanced cell affinity and capability to provide a cell friendly environment for EPC proliferation and differentiation. According to the results, this nanocomposite material can be used for the development of synthetic leaflet heart valves, but modification with suitable peptides could result in superior in-situ endothelialization capability.

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