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Effect of endothelium mimicking self-assembled nanomatrices on cell adhesion and spreading of human endothelial cells and smooth muscle cells

Adinarayana Andukuri, B. Tech.[#], **Will P. Minor, B.S.**[#], **Meenakshi Kushwaha, M.S.**, **Joel M. Anderson, M.S.**, and **Ho-Wook Jun, PhD.**[*]
Department of Biomedical Engineering, University of Alabama at Birmingham, Birmingham, AL, 35294, USA

Abstract

The goal of this study is to develop unique native endothelium mimicking nanomatrices and evaluate their effects on adhesion and spreading of human umbilical vein endothelial cells (HUVECs) and aortic smooth muscle cells (AoSMCs). These nanomatrices were developed by self-assembly of peptide amphiphiles (PAs) through a solvent evaporation technique. Three PAs, one containing the Tyr-Ile-Gly-Ser-Arg (YIGSR) ligand, second containing the Val-Ala-Pro-Gly (VAPG) ligand, and a third without cell adhesive ligands were developed. Cell adhesion and spreading were evaluated by a PicoGreen-DNA assay and Live/Dead assay respectively. Our results show that PA-YIGSR significantly enhances HUVEC adhesion (26704±2708) spreading (84 ±8%), and proliferation (50 ±2%) when compared to other PAs. PA-VAPG and PA-YIGSR showed significantly greater AoSMC adhesion when compared to PA-S. PA-VAPG also showed significantly greater spreading of AoSMCs (63 ±11%) when compared with other PAs. Also, all the PAs showed significantly reduced platelet adhesion when compared with collagen I (control). These findings would facilitate the development of novel vascular grafts, heart valves, and cell based therapies for cardiovascular diseases.

Keywords

Self-assembly; Vascular grafts; Peptide amphiphiles; Tissue engineering

Introduction

Tissue engineering solutions to cardiovascular diseases have become highly attractive due to paucity in conventional methods of treatments and the enormous burden on medical budgets in the western world. For perspective, over 500,000 coronary bypass grafts are performed per annum in the world [1]. In some cases, these procedures are performed with autologous mammary arteries or saphenous veins. However, due to shortage of supplies, synthetic vascular

* Corresponding author: Dr. Ho-Wook Jun Assistant Professor, Department of Biomedical Engineering University of Alabama at Birmingham 1825 University Blvd 806 Shelby Building Birmingham, AL 35294, USA Phone: 205-996-6938 hwjun@uab.edu.
[#]Equally contributing first authors

grafts have been widely used. There are several synthetic vascular grafts available in the market, but limitations exist for these grafts as well. The usage of synthetic materials commonly results in graft failure, especially in small diameter blood vessels, due to thrombosis and the lack of re-endothelialization, followed by restenosis and intimal hyperplasia. Thus, it is imperative to develop a tissue engineered vascular graft by mimicking the native endothelium to provide the best possible environment, thereby enhancing clinical patency.

The success of a tissue engineered graft depends largely on its ability to replicate the microenvironments in the native tissue. Despite recent advances, the generation of a functional microvasculature remains elusive [2,3]. Several types of scaffolds have been used, ranging from synthetic materials, such as Dacron [4], to natural ECM proteins, such as collagen [5]. Most scaffolds, however, result in failure due to the early onset of thrombosis, especially at the small diameter level [6].

To develop tissue engineered vessels, the critical components needed include a functional endothelium, a collagenous network for mechanical strength, and an elastin network to recreate the mechanical flexibility of native blood vessels [7]. Endothelial cells (ECs) and smooth muscle cells (SMCs) form the cellular components of a blood vessel. Therefore, to adequately aid the functional development of engineered vessels, the scaffolds should interact with the cells and improve their adhesion and survival, thus promoting integration into the surrounding tissue. The scaffold should provide the cells with their native environment by mimicking the natural ECM. This homing of cells leads to their secretion of essential proteins, such as collagen and elastin. The formation of a confluent endothelial cell layer is essential for developing an artificial vessel, as it provides an antithrombogenic surface [7]. However, ECs are susceptible to shear forces and scaffold adaptations are needed, such as entities that provide the cells with an adhesive moiety. Various techniques have been attempted to induce endothelial cell attachment and retention, including plasma treatment of scaffolds [8] and inclusion of peptides for cell adhesion [9]. Several scaffolds have been modified by the addition of Arg-Gly-Asp (RGD), a general cell adhesive ligand derived from fibronectin. However, RGD also promotes the attachment of platelets, which can lead to thrombosis [10]. Thus, the incorporation of a general cell adhesive moiety, such as RGD, proves counterproductive. Tyr-Ile-Gly-Ser-Arg (YIGSR) is a laminin derived peptide that has shown affinity for endothelial cells [11]. Laminin is a major component of the basement membrane that comprises the native vasculature for endothelial cells. YIGSR has been found to selectively enhance the endothelialization of polyurethaneurea scaffolds, without platelet adhesion [12]. Thus, the incorporation of YIGSR into scaffolds for vascular tissue engineering offers a promising step in the right direction.

Similarly, to aid the adhesion, growth, and proliferation of SMCs, scaffolds should provide the same type of natural environment. In vivo, SMCs are found in the medial layer of the blood vessel. An elastin derived peptide sequence, Val-Ala-Pro-Gly (VAPG) [13], which is a quantitative marker for elastins, has been shown to specifically enhance the adhesion and growth of vascular SMCs [14].

Altogether, the goal of this study is to evaluate the effect of novel endothelium mimicking self-assembled peptide amphiphile (PA) nanomatrices. PAs typically consist of alkyl chains attached to short peptides [15], and they can assemble into a variety of shapes, depending on dimensions, charge, and environment [16]. Conical shaped PAs with a bulky head group self assemble into cylindrical micelles with the PAs stacked in form of β-sheets parallel to the long axes, packed radially within the nanofibers, as the hydrophilic peptide segments extend outward towards the surface [15,17]. The PAs developed for this study consist of a hydrophobic alkyl (palmityl) tail coupled to a matrix metalloprotease-2 (MMP-2) enzyme degradable site, Gly-Thr-Ala-Gly-Leu-Ile-Gly-Gln (GTAGLIGQ), and a cell adhesive ligand. The alkyl tail constitutes the hydrophobic component of the molecule and is responsible for driving the self-

assembly of the PAs into a nanofibrous matrix with the cell adhesive ligands presented on the outer surface. The presence of the MMP-2 sensitive site within the PA molecules is designed to allow matrix remodeling, as cells grow and constitutively secrete MMP-2. MMP-2 mediated degradation of PAs and their remodeling has been studied in 3D networks [17]. The cell adhesive ligands used in this study are YIGSR, which is a known endothelial cell adhesive moiety, and VAPG, which is known to elicit a favorable response from SMCs. Therefore, the two PAs synthesized are C16-GTAGLIGQYIGSR (PA-YIGSR) and C_{16}-GTAGLIGQVAPG (PA-VAPG). A third PA was synthesized without any cell adhesive ligands, C_{16}-GTAGLIGQS (PA-S), to serve as a control. Thus, the incorporation of these cell adhesive sequences and enzyme degradable sites into the nanomatrices potentially provide cells with native endothelium mimicking environments, which are tailored to meet their needs for survival, growth, and integration into host tissue.

Materials and methods

Synthesis of peptide amphiphiles

The peptides were synthesized in solid phase using Fmoc chemistry in an Aapptech Apex 396 Peptide synthesizer as previously described [17]. Three PAs were developed for this study: (1) PA-YIGSR - $CH_3(CH_2)_{14}$CONH-GTAGLIGQ-YIGSR, (2) PA-VAPG - $CH_3(CH_2)_{14}$CONH-GTAGLIGQVAPG, and (3) PA-S - $CH_3(CH_2)_{14}$CONH-GTAGLIGQ-S. The monomers, which were Fmoc-protected amino acids, were added as triple equivalent solutions in dimethyl formamide to a resin that was linked to the carboxyl terminal amino acid of the desired sequence. Piperidine (three equivalents) was used to deprotect the monomers. Three equivalents of o-benzatriazole- N,N,N′,N′- tetramethyluronium hexafluorophosphate (HBTU) and six equivalents diisopropylethylamine (DIEA) were used to promote the sequential coupling of deprotected amino acids. Dimethyl formamide (DMF) and Dichloromethane (DCM) were used as system solvents. Alkylation was performed by reacting two equivalents of palmitic acid with the N-terminal of the peptide in the presence of HBTU and DIEA for 12 hours at room temperature. The peptide was then be cleaved from the resin using 20 ml of trifluoroacetic acid (TFA). The acidic solution was dried in a rotary evaporator, and the PA was precipitated in cold ether. The PA precipitates were centrifuged, and the pellet was washed two times with cold ether. The pellet was then dried under vacuum overnight and re-dissolved in deionized water adjusted to pH 7 by the addition of NaOH. Precipitation was induced by lowering the pH to approximately three. The precipitates were centrifuged and collected. This purification procedure was repeated several times and the final solution was frozen and lyophilized. The PAs were characterized using HPLC and MALDI-TOF analysis, as previously described [17].

Self-assembled coating of PA nanomatrices

The self assembly of PAs into nanofibrous nanomatrices was characterized by Transmission electron microscopy (TEM). For TEM, 5 μl of 0.1 wt% PA were coated onto a 400-mesh carbon coated copper grid and dried overnight in a chemical fume hood. 10 μl of a negatively charged stain, 20% phosphotungstic acid (PTA), buffered to pH 7, were added to the grid and allowed to stain for 30 seconds. The grid was then imaged using a FEI Technai T12 TE microscope at 60 kV accelerating voltage.

For cell studies, self-assembled coatings of PA nanomatrices were prepared on 48-well tissue culture plates. 200 μl of 0.1 wt% PA solution in DI water were added to each well in the tissue culture plate. The solvent was evaporated by drying in a chemical fume hood for 24 hours and then transferred to an incubator for drying under 37°C for 48 hours. As the solvent evaporated, the PAs self assembled into nanofibrous matrices with the cell adhesive ligands exposed. These coatings were used for further cell studies.

Cell culture

Human umbilical vein endothelial cells (HUVECs) and human aortic smooth muscle cells (AoSMCs) were purchased from Lonza (Walkersville, MD). HUVECs were cultured in EBM media supplemented with EGM singlequot kit (2% FBS, 0.1% hEGF, 0.1% hydrocortisone, 0.1% gentamycin A, 0.4% bovine brain extract), and AoSMCs were cultured in SmBM supplemented with SmGM-2 singlequot kit (5% FBS, 0.1% Insulin, 0.2% hFGF-B, 0.1% gentamycin A, 0.1% hEGF). The cells were cultured in T-75 cell culture flasks (Corning, NY) at standard culture conditions of 37°C, 95% humidity, and 5% carbon dioxide. Cells were passaged by trypsinization with 0.05 wt % trypsin/EDTA and subcultured at a density of 2000-3000 cells per square centimeter.

Evaluation of cell behavior

Cell adhesion and spreading were studied on self-assembled nanomatrices coated onto 48- well tissue culture plates. Self-assembled coating of PA-YIGSR, PA-VAPG, and PA-S were prepared as described above by the addition of 200 μl of 0.1 % PA to each well and subsequent evaporation in a chemical hood for 24 hours. The wells then were incubated at 37°C for 48 hours and sterilized by UV exposure for 4 hours. After trypsinization with 0.05 wt% trypsin/EDTA, the cells were resuspended in culture media at a density of approximately 180,000 cells/ml. The cells were seeded in the wells at a concentration of 15,000 cells/cm^2 for AoSMCs and 30,000 cells/cm^2 for HUVECs. These cultures were maintained at 37°C, 5% CO_2, and 95% humidity.

After 4 hours, cell adhesion was evaluated using the fluorometric PicoGreen DNA assay kit (Molecular Probes, OR). Cells were collected by trypsinization and permeabilized by repeated freeze-thaw cycles. The PicoGreen dye was added to the cell sample. The PicoGreen dye binds to double stranded DNA in cells and allows for quantification. Known volumes of calf thymus dsDNA were used as standards. The fluorescence was measured in a microplate fluorescence reader (Synergy HT, Bio-Tek Instruments, VT). The amount of DNA was correlated to cell number by an empirical value of 8 pg DNA per cell. Three independent experiments were conducted for cell studies. For all cell quantifications, eight wells were tested for each condition making up the experiments (n=8). Thus, more than 76 samples were analyzed in the 3 independent experiments.

To examine cell spreading, cells were stained with LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR). Calcein AM is converted to a green fluorescent product within live cells due to enzymatic activity, while ethidium homodimer-1, a red fluorescent compound, accumulates in dead cells due to increased membrane permeability. The cells were exposed to the dye for 30 minutes and then were imaged using a Nikon TE2000-S fluorescence microscope. Three independent studies were conducted. For all cell spreading quantifications, five random fields were imaged and averaged for each well. Four samples were tested for each condition making up the experiment. Thus, more than 120 images were analyzed in 3 independent experiments. The results shown in the graph are representative of over 120 images from 3 independent experiments (n=12).

Also, the cells were treated with rhodamine-phalloidin (Molecular Probes, OR) to stain the actin filaments in the cells. The cells were fixed with 4% paraformaldehyde in PBS for 10 minutes and permeabilized with 0.1% Triton-X100 in PBS for 20 minutes. The cells were then treated with rhodamine-phalloidin (Molecular Probes, OR) for 30 minutes in a dark, moist environment. DAPI (Molecular Probes, OR) was used to counterstain the nuclei of the cells. They were then imaged using a Nikon TE2000-S fluorescent microscope.

Long term cell viability was assessed by performing a Live/Dead viability assay. Cells were seeded on PA-YIGSR, PA-VAPG and PA-S at concentrations of 15,000 cells/cm^2 for AoSMCs and 30,000 cells/cm^2 for HUVECs. Tissue culture treated plates were used as controls. After 24 hours, one set of controls were treated with media containing 10% ethanol, while a second set of controls were treated with 70% ethanol for 5 minutes. These served as negative controls, while a third set of controls, which were unexposed to ethanol served as positive controls. The cells were then stained using Live/Dead viability/cytotoxicity kit (Molecular Probes, Eugene, OR). Each condition consisted of 4 samples. Three independent experiments were conducted. After 30 minute incubation, the cells were imaged. 5 images were taken for each sample and averaged.

Cell proliferation on the substrates was studied using proliferating cell nuclear antigen (PCNA) cell proliferation kit (Molecular Probes, Eugene, OR). HUVECs and AoSMCs were seeded, at 30,000 cells/cm^2 and 15,000 cells/cm^2, respectively, on PA-YIGSR, PA-VAPG and PA-S coatings. After 48 hour incubation at 37 °C, 95% humidity, 5% CO_2, the cells were fixed with 10% neutral buffered formalin (Sigma Chemicals, St. Louis, MO) and permeabilized using methanol. The cells were then incubated in 3% hydrogen peroxide for 5 minutes to block endogenous peroxidases. The cells were then incubated with mouse IgG anti-PCNA antibody (Dako, Carpinteria, CA), diluted 1:100 in 10%FBS in PBS, for 60 minutes in a humid chamber. After washing, the cells were incubated with anti-mouse IgG HRP (Dako, Carpinteria, CA) diluted 1:100 in 10%FBS in PBS. This was followed by incubation with aminoethylcarbazole chromogen (Dako, Carpinteria, CA), which precipitates a red color in proliferating cells. After washing with PBS, the cells were counterstained with Mayer's hematoxylin. Excess hematoxylin was washed away with 37 mM ammonium hydroxide. The percentage of proliferating cells per field of view (20X) was determined by counting the red proliferating cells and the hematoxylin stained non-proliferating cells using phase contrast microscopy (Nikon Eclipse TE2000-S) after averaging 5 fields per sample.

For studying platelet adhesion, similar coatings of PA-YIGSR, PA-VAPG and PA-S were made. Tissue culture treated plastic and collagen I films were used as controls. Collagen I films were prepared by making a 2.5 mg/ml collagen I dilution in 3% glacial acetic acid. This solution was cast into films in a similar manner to PAs. Platelet adhesion study was approved by the Institutional Review Board at the University of Alabama at Birmingham, and the informed consent was obtained from volunteers prior to collection of blood. Whole blood from a healthy volunteer was collected in BD vacutainer® heparin tubes (BD, NJ) and the cells were fluorescently labeled with 10 μM mepacrine. The coatings were then separately incubated in mepacrine labeled whole blood for 20 minutes at 37°C. The number of platelets per field of view was determined by using a fluorescent microscope (Nikon Eclipse TE2000-S) after averaging five fields per sample.

Statistical analysis

All experiments were performed at least three independent times. All values are expressed as mean ± standard deviation. One way ANOVA was used to determine statistical significance and $p<0.05$ was considered significant. Within the ANOVA analysis, Tukey multiple comparisons test was performed to find significant differences between pairs. All statistical tests were performed with SPSS 15.0 software (SPSS Inc., IL).

Results

The PAs were successfully synthesized and contained enzyme-mediated degradable sites coupled to laminin derived, YIGSR (PA-YIGSR), elastic derived VAPG (PA-VAPG), or no cell adhesive ligand (PA-S). Self-assembly of PAs into nanofibrous nanomatrices was achieved by solvent evaporation and characterized by transmission electron microscopy. Evaporation

increases the concentration of PAs in solution, and thereby, causes two dimensional self-assembly to form a coating. As shown in figure 2, all PAs self-assembled into uniform cylindrical nanofibers by micelle formation. In aqueous media, the hydrophobic alkyl chains group together with the hydrophilic peptide chains on the exterior. This forms a structure akin to a wheel. Since each PA is approximately 4-5 nm in length, the wheel structure is about 8-10 nm in diameter. Several wheels then stack up in three dimensions to form a nanofiber. Each individual nanofiber consistently exhibited a fiber diameter of approximately 8-10 nm and a length of several microns. This self assembly occurs such that the cell adhesive ligands are exposed on the exterior of the nanofiber, presenting surface receptors for cell adhesion. It is evident from the images that all three PA nanofibers stacked up in a consistent manner, forming a multilayered coating that covers the entire area. Thus, a uniform, smooth surface is presented at the nanoscale. The multilayered network of nanofibers also ensures that the functional cell adhesive ligands present in the PAs are continually available to the cells.

The behavior of cells on these PAs was evaluated by seeding HUVECs and AoSMCs on the nanomatrix coated surfaces. Cell adhesion was evaluated by using PicoGreen DNA assay Kit (Molecular Probes, Oregon). PicoGreen dye, which is fluorometric, attaches specifically to dsDNA. As shown in the figure 3A, HUVECs showed significantly greater ($p < 0.05$) adhesion on PA-YIGSR (26704±2708) when compared to PA-VAPG (17198±4809) and PA-S (19651 ±5373). This can be attributed to the recognition of laminin derived YIGSR ligand, which has been shown to promote endothelial cell adhesion in previous literature [9, 12]. HUVEC spreading was also more pronounced on PA-YIGSR, as shown by rhodamine-phalloidin staining in figure 5. Again, this is attributed to the YIGSR ligand, as their spreading on PA-VAPG and PA-S was significantly lower ($p< 0.05$). Also, almost all the cells were spread on PA-YIGSR (84±8%) by the 4 hour time point (Figure 4A). This was significantly greater than the spreading seen on PA-VAPG (68±8%) and PA-S (67±7%). This is also consistent with previous studies using the YIGSR peptide to evaluate endothelial cell behavior. From the 24 hour Live/dead viability assay, it is evident that all three PAs show cell viability that is comparable to tissue culture treated plates. As shown in figure 6A, PA-YIGSR (97±2%), PA-VAPG (95±2%), PA-S (91±3%) showed viability that was similar to tissue culture treated polystyrene (97±2%), while the negative controls, where cells were exposed to 10% ethanol and 70%ethanol showed 80±8% and 0% viability respectively. This shows that these scaffolds support long term viability and proliferation of HUVECs. This was confirmed by performing PCNA proliferation assay. PCNA is a 36 kDa non-histone protein that is found in the nucleus, and plays an important role in the initiation of cell proliferation. Its presence in the nucleolus in the late S phase of the cell cycle makes it an ideal marker for cell proliferation. From figure 7A, HUVECs showed significantly higher proliferation ($p < 0.05$) on PA-YIGSR (50±2%) when compared to PA-VAPG (47±1%) and PA-S (27±2%). These results further indicate that PA-YIGSR is an ideal material for endothelialization, as they promote initial attachment, viability, and proliferation of HUVECs.

Studies for cell behavior of AoSMCs were carried out in a similar manner. As shown in figure 3B, adhesion of AoSMCs on PA-YIGSR and PA-VAPG was similar. Elastin derived VAPG sequence has been shown to promote smooth muscle cell adhesion and spreading previously [14]. However, smooth muscle cells are more robust and showed similar adhesion on two uniform scaffolds (15090±2958 for PA-YIGSR and 13557±1073 on PA-VAPG). However, adhesion on PA-S (11028±2506) was significantly lower than adhesion on PA-VAPG and PA-YIGSR. These observations infer that AoSMCs adhesion was improved by the presence of a cell adhesive ligand in the PA, but there is no difference between YIGSR and VAPG adhesive ligands. Interestingly, it is also noted that AoSMCs do not show the same proclivity to spreading on PA-YIGSR as endothelial cells. The actin filaments and stress fibers of AoSMCs (figure 5) were positively stained on PA-YIGSR, but the extent of spreading was greater on PA-VAPG. From figure 4B, AoSMC spreading was significantly greater ($p < 0.05$) on PA-VAPG (63

±11%) when compared to PA-YIGSR (49±15) and PA-S (45±6%). This shows that the two PAs interact with the two cell types in different ways. PA-YIGSR clearly promotes endothelial cell adhesion and spreading, while smooth muscle cells, being more robust, attach well on either surface but exhibit greater spreading on PA-VAPG. As was with HUVECs, long term viability of AoSMCs on the PAs was assessed by a 24 hour Live/dead viability assay. As shown in figure 6B, PA-YIGSR (94±4%), PA-VAPG (95±3%) and PA-S (93±5%) showed similar viability as tissue culture treated polystyrene (positive control, 96±2%), while negative controls, where cells were treated with 10% ethanol and 70% ethanol showed 66±10% and 0% viability, respectively. This shows that AoSMCs are able to retain their viability on the PAs over longer time points. Proliferation of AoSMCs was also studied by performing the PCNA assay. From figure 7B, PA-YIGSR (47±6%) and PA-VAPG (51±4%) showed significantly greater AoSMC proliferation when compared to PA-S (30±6%). AoSMCs, being more robust, seem to proliferate well on both PA-YIGSR and PA-VAPG.

Platelet adhesion study was performed to evaluate the ability of PAs to prevent thrombosis. As shown in figure 8, PA-YIGSR (70±7), PA-VAPG (93±22), PA-S (52±16) showed similar platelet adhesion as tissue culture treated polystyrene (81±25), which was significantly lower ($p < 0.05$) that the positive control, collagen I (5978±74). These results indicate that the PAs may not cause, or result in thrombosis, which is a major limitation in most vascular grafts.

Discussion

A great emphasis has been placed on mechanical stability in constructing vascular grafts. There have been limited successes due to lack of re-endothelialization, restenosis, and thrombosis. Thus, to construct the ideal vascular graft, it is essential to replicate the native blood vessel as closely as possible. The structure of an artery, as shown in figure 1(a), provides us with a direction to proceed in order to engineer its regeneration. Blood vessels have three layers; an intimal layer comprising of endothelial cells on a basement membrane, a medial layer comprising of smooth muscle cells, and an adventitious layer comprising of fibroblasts and collagens. The layer of endothelial cells provides a continuous, selectively permeable, thrombosis-resistant barrier that supports laminar blood flow [7]. It also controls vascular tone, platelet activation, adhesion and aggregation, leukocyte adhesion, and smooth muscle cell migration and proliferation.

Thus, an effective vascular graft may be constructed by duplicating the native environment of vascular cells. This can be achieved by the incorporation of cell adhesive ligands into the grafts. Along these lines, considerable effort has been devoted to the design and controlled fabrication of structured materials with various bioactive sequences. RGD, an integrin ligand, has been the most extensively studied cell adhesion peptide and has been covalently bound to surfaces and scaffolds and shown to support growth of various cell types including fibroblasts, osteoblasts, and endothelial cells [18-20]. Fibronectin derived peptide REDV has also been shown to allow specific adhesion of endothelial cells but not fibroblasts, smooth muscle cells, or platelets [21]. However, laminin derived YIGSR has emerged as the most popular choice for endothelial cell adhesive ligand [22,23]. While YIGSR has been shown to selectively promote endothelial cell adhesion and growth [9,11], elastin derived sequence VAPG has been shown to favor smooth muscle cell adhesion and growth [14], as depicted in figure 1(b) and (c). YIGSR is expected to improve the adhesion and spreading of endothelial cells, while VAPG is believed to improve SMC adhesion and spreading. These cell adhesive ligands were incorporated into the PAs to produce PA-YIGSR and PA-VAPG. The PAs comprise a 16 carbon hydrophobic palmityl chain that is attached to the amino end of a functional peptide sequence. These peptide sequences also contained a matrix metalloprotease-2 (MMP-2) degradable sequence (GTAGLIGQ). MMP-2 is constitutively produced by proliferating cells as a means of remodeling the extracellular matrix. This allows for cellular migration,

accompanied by the local cellular secretion of ECM. The incorporated cell adhesive ligands are individually attached to the carboxyl ends of the enzyme degradable sequence.

It is clear from this study that enhanced adhesion, spreading and proliferation of HUVECs on PA-YIGSR is due to the presence of the YIGSR peptide sequence. It is also interesting to note that YIGSR can be functionally incorporated into the PA to endow endothelium mimicking environments. The PA can be self-assembled into nanomatrix coatings by simple solvent evaporation method. Thus, it can be applied to various cardiovascular implants, including vascular grafts. This is a significant finding, as the importance of endothelial cells for treatment of multiple cardiovascular pathologies cannot be overstated. When viewed from a vascular graft perspective, PA-YIGSR can be used to develop a biomaterial that potentially aids the formation of a healthy thrombosis resistant endothelialized layer, and thereby, restores blood vessel homeostasis. The development of a functional endothelium is the primary requisite for a vascular graft [7], as it can prevent thrombosis and restenosis. These are the main culprits in most vascular graft failures. Additionally, a healthy endothelium is self-regulating, which means that it produces factors that maintain vascular cell homeostasis. This is essential for long term patency of a vascular graft. Apart from these implications for vascular grafts and potentially, heart valves, this finding can provide the basis for development of novel cell based therapies for treatment of several cardiovascular diseases. Also, the effect of VAPG peptide on endothelial cells has never been documented and compared with a favorable peptide, such as YIGSR. Likewise, the effect of VAPG peptide on AoSMCs has been researched, but relative cell behavior when exposed to YIGSR peptide is unknown. VAPG is known to be smooth muscle specific and to promote smooth muscle cell spreading in a concentration dependent manner [14]. The ability of PA-VAPG to self-assemble by solvent evaporation under physiological conditions could provide the foundation for its utilization in vascular grafts. In this study, PA-VAPG has been shown to assist AoSMCs adhesion and greatly improve spreading. Thus, PA-VAPG has the potential to be an important component of a vascular graft for developing a medial layer, which is essential for mechanical properties. It is also an important step in recreating the blood vessel trilayer. In a native blood vessel, a healthy endothelium is required to prevent thrombosis and intimal hyperplasia, while simultaneously, a strong medial layer is essential for elasticity and mechanical integrity. These novel PAs with incorporated cell adhesive ligands can manipulate cell behavior, do not support platelet adhesion and have great potential, thus making an excellent case for further use in an ideal vascular graft.

In conclusion, endothelium mimicking self-assembled PAs have been developed. Functional peptide sequences were incorporated, such as enzyme degradable sequences and cell adhesive ligands. The self assembly of PAs into biomimetic nanomatrices was achieved by simple solvent evaporation method. In this study, PA-YIGSR has shown great potential for vascular graft applications. It greatly enhances endothelial cell adhesion, spreading and proliferation, which is essential for developing a functional endothelium. PA-VAPG has also shown to assist smooth muscle cell adhesion, and it greatly increases smooth muscle cell spreading. These findings are of significance for vascular grafts, as PA-YIGSR can enhance endothelialization leading to the formation of a functional endothelium, which is essential for the success of the graft. Also, PA-VAPG could play an important role in developing a smooth muscle cell layer, imparting mechanical integrity to the graft, and aiding in the recreation of the vascular trilayer. Also, these PAs do not support platelet adhesion, and therefore, may not cause thrombosis. These PAs could facilitate the development of novel vascular grafts, heart valves, and potentially, novel cell based therapies for cardiovascular diseases.

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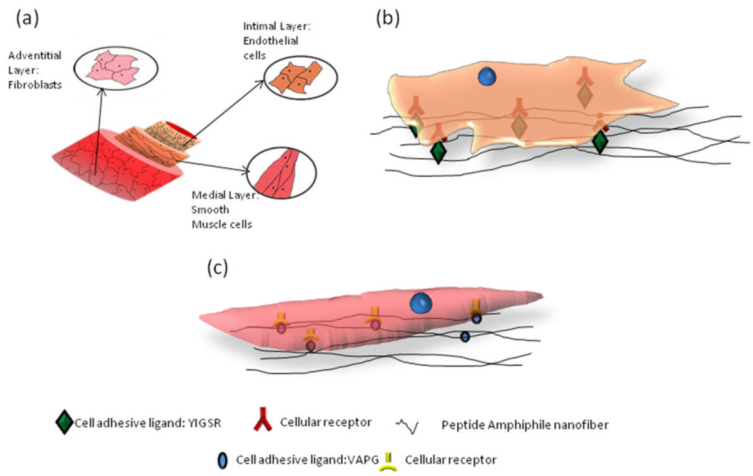

Figure 1.

The cellular hierarchy in a typical blood vessel provides us with a direction for constructing ideal vascular grafts. As shown in (a) It comprises three layers; the innermost intima, composed of endothelial cells and the outermost adventitia, composed of fibroblasts sandwiching the media, composed of smooth muscle cells. The intimal endothelium is thrombosis-resistant while the smooth muscle cells in the medial layer provide mechanical integrity. This condition may be achieved by using scaffolds that mimic the natural extracellular environment experienced by the respective cells. PA-YIGSR and PA-VAPG provide endothelial and smooth muscle cells with their respective native environments. The PA self assembles into nanofibers, exposing cell adhesive ligands that dictate cellular behavior. Image (b) shows an endothelial cell on PA-YIGSR, which contains the laminin derived YIGSR peptide that improves endothelial cell adhesion and spreading. The endothelial cell attaches to the PAs through specific receptors via the YIGSR sequence, followed by cell spreading. In image (c), a smooth muscle cell attaches to PA-VAPG through cell receptor via the elastin derived VAPG sequence, and shows characteristic spreading.

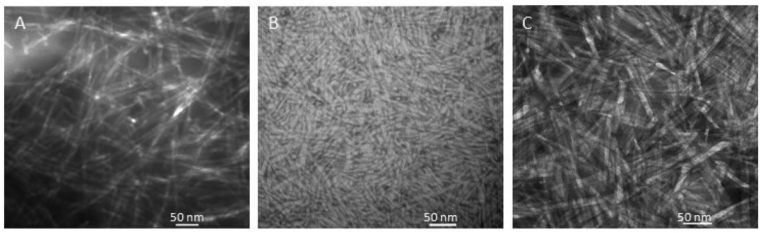

Figure 2.
TEM images of (A) PA-YIGSR; (B) PA-VAPG; (C) PA-S

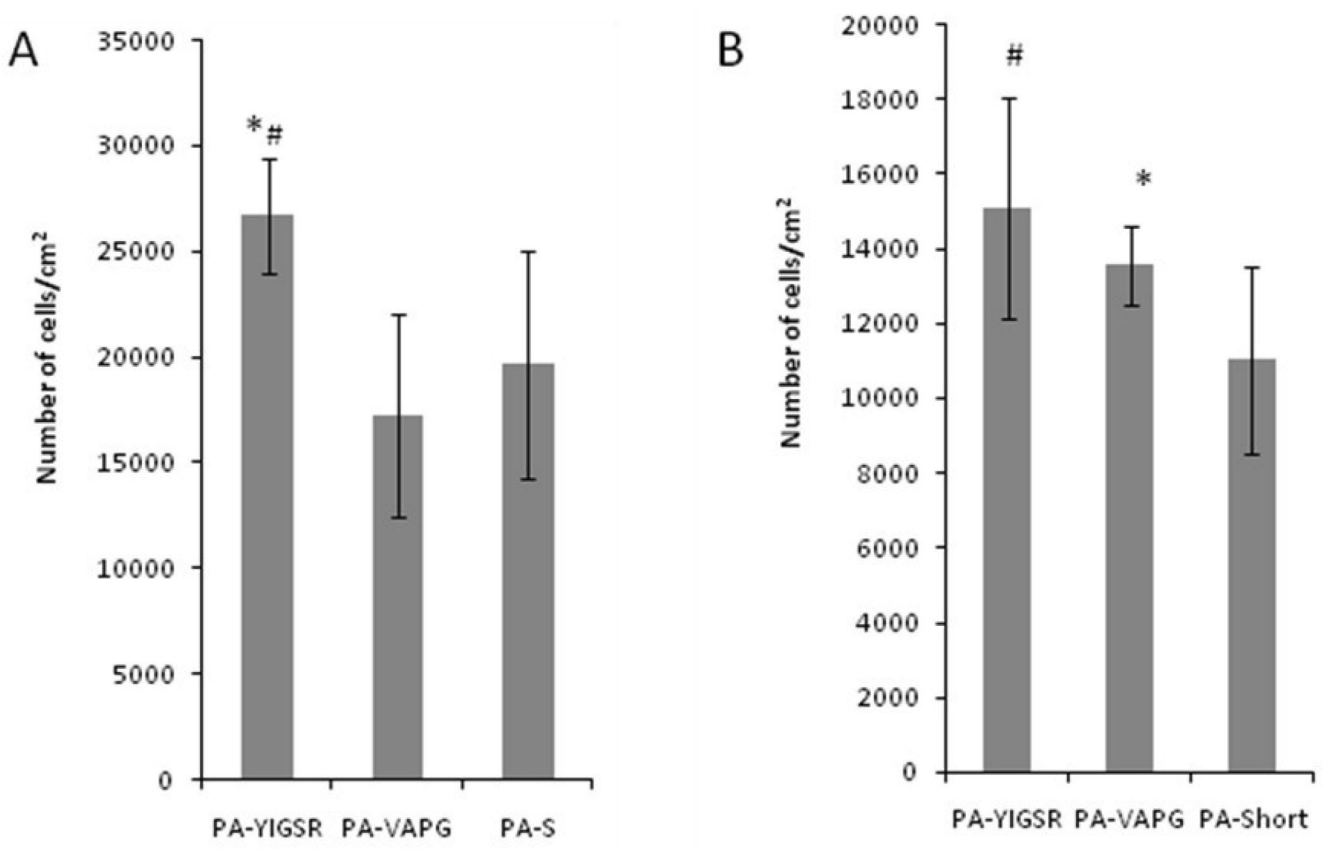

Figure 3.

Cell adhesion on PA-YIGSR, PA-VAPG and PA-S. Error bars represent mean ± standard deviation; (A) HUVECs at 4hrs. PA-YIGSR showed significantly greater HUVEC adhesion when compared with (*)PA-VAPG and (#)PA-S ($p<0.05$); (B) AoSMCs at 4hrs. PA-YIGSR showed significantly greater AoSMC adhesion when compared to (#)PA-S ($p<0.05$). *PA-VAPG showed significantly greater cell adhesion when compared with (*)PA-S ($p<0.05$).

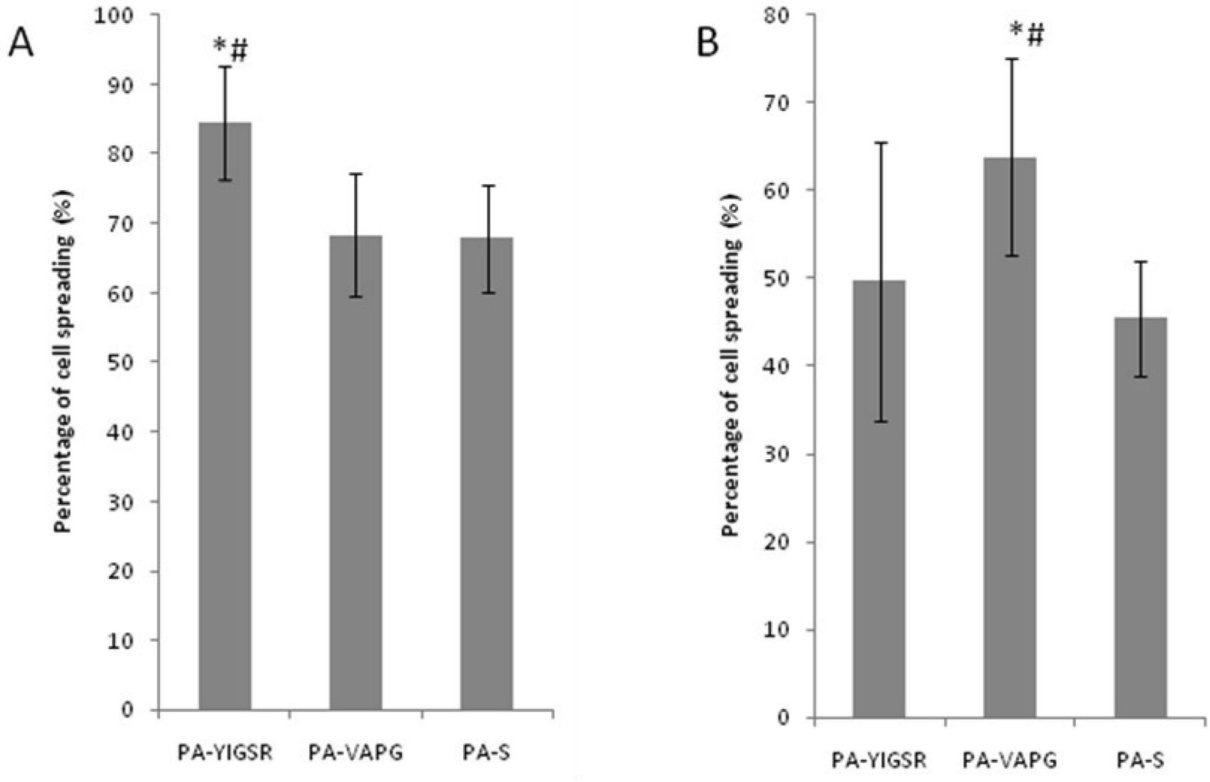

Figure 4.

Cell Spreading on PA-YIGSR, PA-VAPG, PA-S. Error bars represent mean ± standard deviation; (A) HUVECs at 4hrs. PA-YIGSR showed significantly greater HUVEC spreading when compared to (*)PA-VAPG and (#)PA-S ($p<0.05$); (B) AoSMCs at 4hrs. PA-VAPG showed significantly greater AoSMC spreading when compared with (*)PA-YIGSR and (#) PA-S ($p<0.05$).

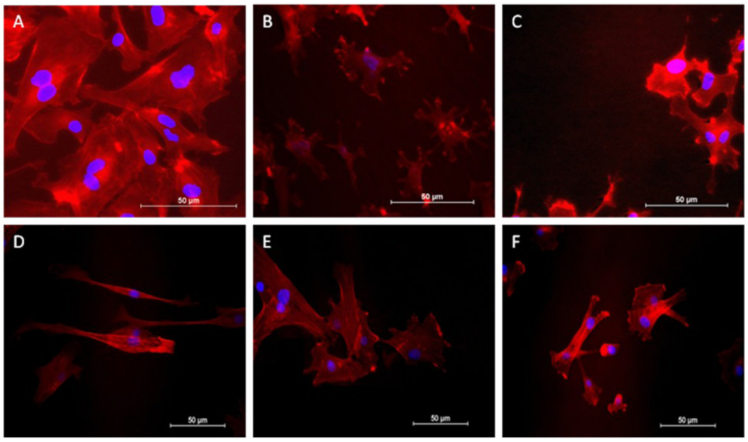

Figure 5.

F-actin staining of cells on the nanomatrices. Cells were stained with rhodamine phalloidin at 4hrs. (A), (B) and (C) represent HUVECs on PA-YIGSR, PA-VAPG and PA-S respectively. Endothelial cells clearly showed greater spread morphology and stress fiber formation on PA-YIGSR; (D), (E) and (F) represent AoSMCs on PA-YIGSR, PA-VAPG and PA-S respectively. AoSMCs show spreading and stress fiber formation on PA-YIGSR and PA-VAPG, and clearly, show lesser spreading on PA-S.

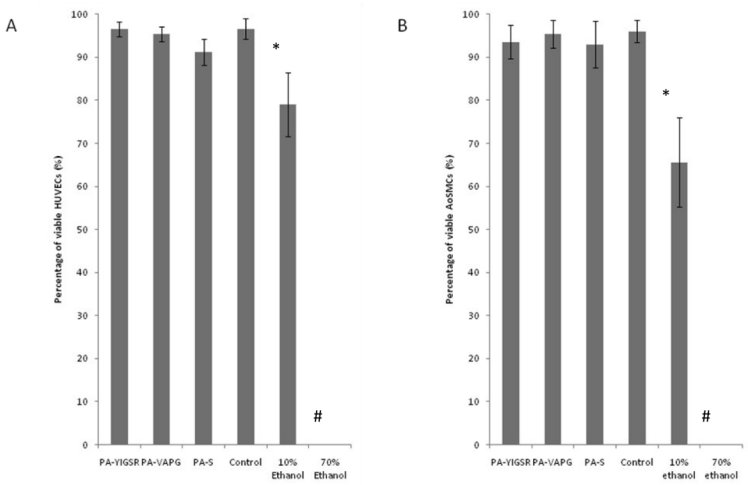

Figure 6.

Cell viability at 24 hours on the nanomatrices. (A) HUVEC viability at 24 hours. PA-YIGSR, PA-VAPG and PA-S showed similar viability as tissue culture treated polystyrene (positive control), which was significantly higher than the negative controls (10% ethanol (*)and 70% ethanol(#) respectively). (B) AoSMC viability at 24 hours. PA-YIGSR, PA-VAPG and PA-S showed similar viability as tissue culture treated polystyrene (positive control), which was significantly higher than then negative controls (10% ethanol (*) and 70% ethanol (#) respectively).

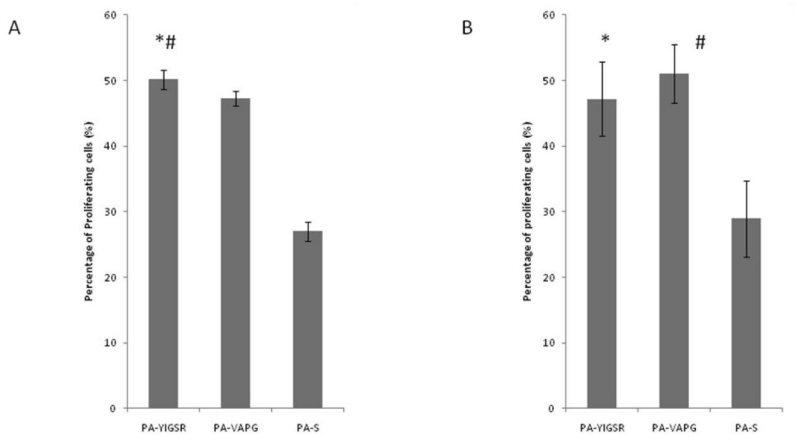

Figure 7.

Percentage of proliferating cells on the nanomatrices at 48 hrs. (A) HUVEC proliferation. PA-YIGSR shows significantly greater percentage of proliferating cells ($p<0.05$) when compared to (*) PA-VAPG and (#) PA-S. (B) AoSMC proliferation. PA-YIGSR (*) and PA-VAPG (#) show significantly greater percentage of proliferating cells when compared to PA-S ($p<0.05$).

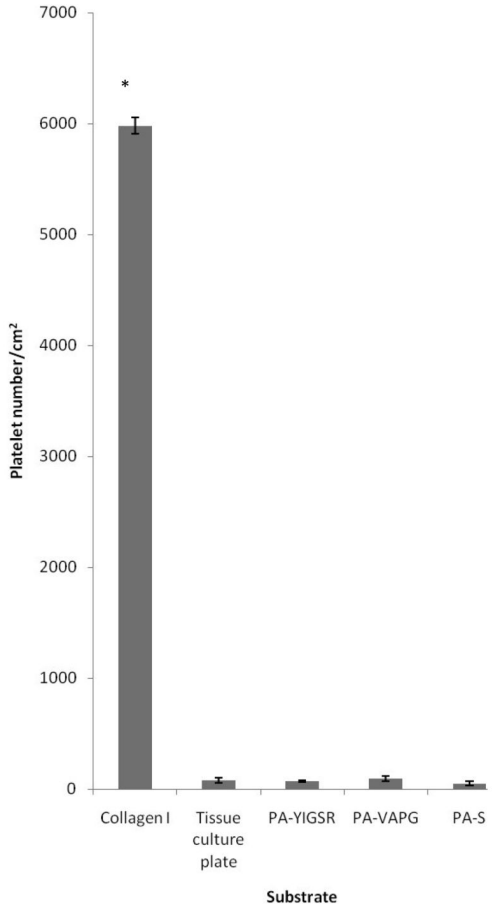

Figure 8.

Platelet adhesion on the nanomatrices and collagen I. PA-YIGSR, PA-VAPG and PA-S showed platelet adhesion that was similar to tissue culture treated polystyrene. All three PAs showed significantly lower platelet adhesion ($p<0.05$) when compared to the (*) positive control (Collagen I).