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Development of Bioactive Peptide Amphiphiles for Therapeutic Cell Delivery

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Abstract

There is great clinical interest in cell-based therapies for ischemic tissue repair in cardiovascular disease. However, the regenerative potential of these therapies is limited due to poor cell viability and minimal retention following application. We report here the development of bioactive peptide amphiphile nanofibers displaying the fibronectin-derived RGDS cell adhesion epitope as a scaffold for therapeutic delivery of bone marrow derived stem and progenitor cells. When grown on flat substrates, a binary peptide amphiphile system consisting of 10% by weight RGDS-containing molecules and 90% negatively charged diluent molecules was found to promote optimal cell adhesion. This binary system enhanced adhesion 1.4 fold relative to substrates composed of only the non-bioactive diluent. Additionally, no enhancement was found upon scrambling the epitope and adhesion was no longer enhanced upon adding soluble RGDS to the cell media, indicating RGDS-specific adhesion. When encapsulated within self-assembled scaffolds of the binary RGDS nanofibers *in vitro*, cells were found to be viable and proliferative, increasing in number by 5.5 times after only 5 days, an effect again lost upon adding soluble RGDS. Cells encapsulated within a non-

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bioactive scaffold and those within a binary scaffold with scrambled epitope showed minimal viability and no proliferation. Cells encapsulated within this RGDS nanofiber gel also increase in endothelial character, evident by a decrease in the expression of CD34 paired with an increase in the expression of endothelial-specific markers VE-Cadherin, VEGFR2, and eNOS after 5 days. In an *in vivo* study, nanofibers and luciferase-expressing cells were co-injected subcutaneously in a mouse model. The binary RGDS material supported these cells *in vivo*, evident by a 3.2 fold increase in bioluminescent signal attributable to viable cells; this suggests the material has an anti-apoptotic and/or proliferative effect on the transplanted bone marrow cells. We conclude that the binary RGDS-presenting nanofibers developed here demonstrate enhanced viability, proliferation, and adhesion of associated bone marrow derived stem and progenitor cells. This study suggests potential for this material as a scaffold to overcome current limitations of stem cell therapies for ischemic diseases.

Keywords

regenerative medicine; ischemic tissue disease; RGDS; peptide amphiphile; therapeutic cell delivery

1. Introduction

Despite advances in modern therapy, ischemic tissue disease remains one of the foremost causes of morbidity and mortality [1]. Interest in the regenerative potential of cell-based therapies for ischemic tissue gained momentum through the description of endothelial progenitor cells (EPCs), lineage-committed precursors of mature endothelial cells that combine endothelial and stem cell characteristics [2]. It has been shown that EPCs are mobilized from the bone marrow in response to an ischemic trigger and home to the ischemic zone where they participate in repair of ischemic tissue through paracrine effects and *de novo* blood vessel formation [3,4]. Subsequent studies have attempted to leverage the endogenous stem/progenitor cell mechanism through therapeutic applications of various cell types to the region of interest in cardiovascular diseases. In the clinical setting, the application of unselected bone-marrow mononuclear cells (BMNCs) is most advanced, showing promise in the treatment of acute [5] and chronic [6] myocardial infarction and peripheral arterial disease [7]. It remains uncertain, however, which subtypes of BMNC-derived cells elicit this regenerative effect and comparative studies of different cell lineage are pending. The excitement surrounding these cell-based therapies has been tempered, however, by several practical limitations including limited local retention and poor viability of transplanted cells within the ischemic tissue [8,9]. In order to exploit the full regenerative potency of these cell therapies, overcoming these limitations is crucial; especially when considering the observed dose-response relationship found in preclinical and clinical studies coupled with the known dysfunctionality and pro-apoptotic state of cells isolated from older multimorbid cardiovascular patients in autologous strategies [6,10,11]. Thus, promoting retention and viability of transplanted cells within the target region is of particular clinical interest to improve the efficacy of cell-based therapies.

Synthetic cell delivery scaffolds, often polymer-based systems, have been developed for cell-based therapies in order to enhance their retention at the treatment site [12]. Attempts to incorporate a signaling capacity to these otherwise non-bioactive materials has led to the incorporation of epitopes for cellular interaction, particularly motifs that foster cell adhesion [13]. Biological adhesion to native ECM occurs, in part, through binding of integrin proteins on the cell surface to specific epitopes present on proteins of the ECM, creating a focal adhesion, anchoring the cell and allowing for communication with the surrounding environment [14–16]. One such ECM protein responsible for biological adhesion, fibronectin, binds to integrins through a domain containing Arg-Gly-Asp-Ser (RGDS) [17,18]. Previous studies have shown that RGDS epitope-spacing is a crucial factor for cell recognition and response [19–22]. The RGDS sequence, or sometimes the abbreviated RGD sequence, has

been incorporated into a variety of synthetic materials to promote cell interaction and adhesion. Recently, alginate scaffolds presenting an RGD epitope were used as a depot for therapeutic applications of vascular progenitor cells to a hind-limb ischemia model, with results showing enhanced efficacy when using this biomaterial delivery vehicle [23].

The use of supramolecular self-assembly to create biomaterials offers the possibility of controlling the architecture, shape and dimensions of bioactive nanostructures, as well as the spatial display and density of bioactive signals. This is made possible by the local order in the assembled one-dimensional structures [24,25]. Previously, our laboratory developed several classes of self-assembling biomaterials [26–30] including a class of synthetic peptide amphiphiles (PA). PAs contain a hydrophobic alkyl segment covalently grafted to an amino acid sequence composed of a domain controlling self-assembly of the molecules into nanofibers through hydrogen bonding and a domain allowing for presentation of cell signaling sequences or protein binding sequences. The assembly of molecules into nanofibers emulates ECM architecture, and by design allows the bioactive domain to be presented on the surface of the nanostructures as the alkyl tail is buried in the core of the fiber through hydrophobic collapse. Electrostatic screening of charged amino acids on these molecules by electrolytes in physiologic media triggers the self-assembly into high aspect-ratio nanofibers that form gel networks at relatively low concentrations, on the order of 1% by weight [31,32]. PA nanofibers have been used previously for a host of biological applications. When presented on a PA, the laminin-derived IKVAV epitope showed differentiation of neural progenitor cells [33] and inhibition of glial scar formation while promoting axon elongation in a spinal cord injury model [34]. Another PA was designed to bind heparin for the delivery of angiogenic growth factors [35,36], while still others have been used for applications as MRI contrast agents [37,38]. RGDS has been previously incorporated into PAs using various covalent architectures including linear, branched and cyclic epitope presentations [22,39,40]. Different PA molecules are capable of co-assembly, allowing for a specific bioactive molecule to be mixed with a different bioactive molecule or a non-bioactive diluent molecule to vary the epitope density on the assembled nanostructure for optimized cell signaling [41,42]. The optimal composition of an RGDS-presenting PA co-assembled with a diluent molecule was previously determined to be between 2.5 and 10% for 3T3-fibroblasts, depending on the covalent architecture used [22].

In this work, we investigate RGDS-presenting PA nanostructures as a potential bioactive vehicle for BMNC delivery. We first explore optimization of BMNC biological adhesion *in vitro* and then assess the feasibility of this RGDS nanofiber gel to support these cells *in vivo*. Since the limitations of BMNC-based therapies for ischemic cardiovascular diseases center around cell viability and retention following targeted application, our goal is to develop a bioactive RGDS-presenting nanofiber matrix that could serve as a cell delivery system for ischemic tissue therapies.

2. Materials and Methods

2.1 Synthesis and Purification of Peptide Amphiphiles

We synthesized five different PAs for this study having the following amino acid sequences covalently linked to a 16-carbon alkyl segment: C₁₆-V₃A₃K₃RGDS (RGDS), C₁₆-V₃A₃K₃DGSR (scrambled), C₁₆-V₃A₃K₃ (K₃ diluent), C₁₆-V₃A₃R₃ (R₃ diluent), and C₁₆-V₃A₃E₃ (E₃ diluent). Structures for the primary PAs used in this study are shown in Figure 1. All PAs were synthesized by standard solid phase Fmoc chemistry on a CS Bio automated peptide synthesizer. Fmoc-protected amino acids, MBHA rink amide resin, and HBTU were purchased from NovaBiochem and all reagents were purchased from Mallinckrodt. The resulting product was purified using standard reversed-phase HPLC. TFA counter-ions were exchanged by sublimation from 0.1 M hydrochloric acid. All PAs were dialyzed against

deionized water using 500 MWCO dialysis tubing and isolated by lyophilization. The purity and accurate mass for each PA was verified using LC/MS on an electrospray ionization quadrupole time-of-flight mass spectrometer (Agilent).

2.2 BMNC Isolation and Culture

Total bone marrow was obtained from eight week old male FVB/N wild-type mice (Charles-River). The mononuclear cell fraction was isolated by density gradient centrifugation using Histopaque (Sigma). Isolated BMNCs were plated on rat fibronectin-coated (Sigma-Aldrich) culture dishes (Nunc) and maintained in endothelial basal media medium-2 supplemented with EGM-2 SingleQuots (Lonza) containing FBS and VEGF-1, FGF-2, EGF, IGF-1, and ascorbic acid, in accordance with previously established culture methods [43]. Cells were cultured in this way to enrich for subpopulations of endothelial character while preventing differentiation into other lineage. After 4 days of culture, non-adherent cells were removed by exchanging the culture medium. Cells were used for experiments after 7 days in culture. This isolation protocol was approved by the Northwestern University Animal Care and Use Committee.

2.3 Diluent Charge Preference Screening

Solutions of E₃, K₃, and R₃ diluent molecules were prepared at 0.01wt% in water and 100 μ l was added to wells of a 96-well tissue culture plate. To coat the surfaces, the PA solution was evaporated overnight in a sterile tissue culture hood. Cells were seeded onto the surfaces in serum-free endothelial basal media-2 (EBM-2) at a density of 5000 cells per surface. Samples were analyzed for cell viability after 1, 3, and 6 days using a live/dead two color assay (Invitrogen) where Calcein AM indicates live cells with green fluorescence and ethidium homodimer shows dead cells with red fluorescence. The surfaces were imaged using an inverted fluorescent microscope (Nikon). To quantify the percentage of viable cells in each sample, two images from each well were captured and the number of live and dead cells counted, with 4 wells analyzed for each condition at each time-point. Viability is expressed as the fraction of viable cells.

2.4 Cell Adhesion Assay

The RGDS PA and E₃ diluent were mixed to prepare 0.01wt% solutions with an RGDS composition of 0%, 1%, 2%, 5%, 7%, 10%, 15%, 25%, 50%, 75% and 100% by weight. These solutions were added to wells of a 96-well plate (n=8 wells per condition) and the solutions were left to evaporate overnight in a sterile tissue culture hood. BMNCs were incubated in serum-free EBM-2 supplemented with 4 mg/ml bovine serum albumin and 50 μ g/ml cyclohexamide at 37°C for 1 hour prior to use to inhibit ECM production. After washing with PBS, the cells were isolated and resuspended in serum-free EBM-2 at 25,000 cells per ml and 100 μ l was added to each coated well. The surfaces were incubated at 37°C for 4 hours, at which point the media was aspirated and the plates were rinsed once with PBS. The number of cells per well was quantified using the Cyquant NF cell proliferation assay (Invitrogen), measuring fluorescence (Ex/Em=485/530 nm) with a conventional microplate reader (Molecular Devices). Background fluorescence was accounted for by processing an additional four surfaces for each condition without cells added. Cell adhesion was quantified by subtracting the background signal and results were expressed as the signal intensity relative to that of the no-epitope control (0% RGDS case). In order to assess viability, surfaces were similarly prepared and a live/dead two color assay (Invitrogen) was performed following the 4 hour incubation, with viability quantified as previously described for the diluent charge screening.

To control for epitope bioactivity, the RGDS PA and the scrambled PA were mixed with the E₃ diluent to make 0.01wt% solutions containing 10% RGDS PA, 10% scrambled PA, or 100% diluent PA. Additionally, the adhesion assay outlined previously was performed in the presence

and absence of soluble Ac-RGDS peptide (0.1mg/ml). Once again, results were expressed as the signal intensity relative to the diluent control

2.5 Visualization of Cell-PA Interaction

The RGDS PA was mixed with the E₃ diluent to make a 0.01wt% solution containing 10% RGDS PA. Glass coverslips (12 mm) were placed into 24 well tissue culture plates, and 250 μ l of each solution was added to the well and left to evaporate overnight as before. 20,000 cells were plated onto each coated coverslip and cultured for one day in serum-free EBM-2. The samples were processed for scanning electron microscopy (SEM) by fixation in 2% glutaraldehyde and 3% sucrose in PBS for 1 hour at 4°C followed by sequential dehydration in ethanol. They were then dried at the critical point and coated with 7.3 nm gold/palladium. Samples were imaged using a Hitachi S4800 SEM (Ontario, Canada) with a 3 kV accelerating voltage.

2.6 Cell Viability and Proliferation Within PA Scaffolds

PA solutions were prepared consisting of either 100% E₃ diluent PA, 10% RGDS PA, or 10% scrambled PA, at gelation concentrations of 1.33wt%. Cells were resuspended at 40,000 cells per 15 μ l in serum-containing EGM-2 supplemented with CaCl₂ to a final concentration of 0.1 M to serve as the gelation media. 15 μ l of each PA solution was added to wells of a 96-well round bottom tissue culture plate. An equal volume of the calcium supplemented cell suspension was mixed with each aliquot of PA and the samples were placed at 37°C for 30 minutes, at which point 200 μ L of complete EGM-2 was added on top of the gels. Viability was examined after 4 days using the same live/dead two-color assay and an inverted fluorescent microscope.

For proliferation studies, gels were prepared as above and harvested at days 0 and 5, along with background control gels that did not contain cells. We performed our proliferation studies in the presence and absence of soluble Ac-RGDS (0.1 mg/ml). The gels were lyophilized and resuspended in phosphate buffered EDTA (pH=6.5) supplemented with 0.01 M L-cysteine and 0.5% papain (Sigma). Digestion was performed at 60°C for 24 hours. Following this, a PicoGreen DNA quantification assay (Invitrogen) was performed, collecting PicoGreen fluorescence (Ex/Em=485/535 nm) using a microplate reader. The background signal was subtracted, and the signal intensity was expressed relative to the day 0 intensity.

2.7 Gene Expression of BMNCs in PA Scaffolds

PA gels were prepared using the same methodology as that used for three-dimensional viability experiments, testing the same material combinations. Gels formed with 100,000 cells were harvested at days 0 and 5, and RNA was isolated by standard Trizol extraction. RNA quality was assessed spectroscopically to ensure the 260/280 was within the range of 1.7–2.0. Total RNA was then reverse transcribed with a Taqman cDNA Synthesis Kit (Applied Biosystems) and amplification was performed using a Taqman 7500 thermocycler (Applied Biosystems) with real-time analysis. The relative mRNA expression for each gene was calculated by the comparative threshold cycle (CT) method and normalized to the 18s housekeeping expression. Results here are expressed as the 18s normalized values relative to the day 0 expression for each gene.

2.8 In Vivo Cell Delivery and Bioluminescent Imaging

Bone marrow mononuclear cells were harvested from hemizygous FVB/N-Tg(β -Actin-luc)-Xen mice (Xenogen). These transgenic mice have a modified firefly luciferase gene driven under the murine β -Actin promoter that is constitutively expressed throughout ubiquitous tissues and is not inducible. BMNCs from these animals were resuspended at 30 million cells

per ml in serum-free EBM-2 supplemented with calcium to a final concentration of 0.1 M. Solutions of E3 diluent PA and 10% RGDS PA were prepared at 1.33wt%. Cells were combined with the various materials and injected subcutaneously into eight-week old male FVB/N wildtype mice (Charles River). Animals were imaged at baseline, day 1, and day 4 using longitudinal bioluminescent imaging (BLI). Mice were anesthetized by inhaled anesthesia (2.0% isoflurane in air) and D-luciferin potassium salt (Regis Technologies) dissolved in PBS was administered by intraperitoneal injection (100 mg/kg body weight). Mice were placed supine on the heated shelf of a light-tight, low-background imaging chamber. In order to minimize electronic background and maximize sensitivity, this imaging system (IVIS 100 Imaging System, Xenogen) features a 25 mm² back-thinned, back-illuminated, charge-coupled camera sensor, which is cryogenically cooled via a closed cycle refrigeration system. Photon transmission emitted from intracellular luciferase of viable BMNCs was measured every 2 minutes from the time of injection until determination of the peak signals (Living Image 2.50.1 software, Xenogen). All images were captured with an acquisition time of 1 minute, a 20 cm field of view, constant binning, and excitation and emission filters. To localize the spatial distribution of the detected photons, a grayscale body image was overlaid with the pseudo-color luminescent image. Luminescence was quantified as the sum of all detected photons per second within a constant region of interest in all mice (Igor Pro 4.09A image analysis software, Wavemetrics) and background signal was subtracted in each mouse. To control for baseline signal variability between mice, results are presented as relative change in signal intensity from the baseline level [44,45]. These studies were approved by the Northwestern University Animal Care and Use Committee.

2.9 Statistics and Data Analysis

All error bars represent the standard error of the mean. Differences between groups were determined using a one-way analysis of variance (ANOVA) with a Bonferonni multiple comparisons post-hoc test. Significance between groups was established for $p < 0.05$, $p < 0.01$, and $p < 0.001$.

3. Results and Discussion

3.1 Diluent PA Screening

As demonstrated in previous studies, peptide amphiphiles displaying an RGDS epitope benefit from the presence of a diluent molecule to co-assemble with the epitope-presenting molecule and space the RGDS epitope for optimal cell recognition and adhesion [22]. In order to determine which molecule to use, BMNCs were screened for viability on surfaces coated with three different diluent molecules; one molecule bearing three negative charges (E₃ diluent) and two molecules bearing three positive charges (K₃ and R₃ diluent). BMNCs grown on surfaces coated with the negatively charged E₃ diluent were significantly ($p < 0.001$) more viable than on either of the positively charged PA-coated surfaces (Figure 2). Viability on these E₃ surfaces was greater than 60% for up to six days in serum-free media, while the viability on surfaces coated with K₃ and R₃ diluents was approximately 20% after a single day of culture, maintaining this viability through the six-day study. Viable cells cultured on the E₃ diluent substrate have numerous process extensions and appear phenotypically healthy, while the few viable cells found on the K₃ and R₃ diluent substrates were primarily rounded suggesting a less cell amiable substrate. Our objective here was to assess whether PAs bearing different charges affected the viability of BMNCs directly in contact with our nanofibers. Thus, we used serum-free media to limit any confounding effects associated with serum coating the PA surface. In spite of the lack of serum in the media, cells remained viable for at least six days on the E₃ coated substrates.

The results of this initial screening indicate that BMNCs have a preference for negatively charged PA nanofibers, whereas substrates coated in positively charged PA nanofibers were detrimental to viability. This is consistent with evidence from the literature, where several studies have found polycationic substrates to induce cell death [46–48]. Poor cell viability was observed for materials with both arginine and lysine as the charged group, allowing us to conclude that the observed cytotoxicity is most likely a feature of the overall positive charge and less dependent on the specific amino acid sequence. The viability exhibited by BMNCs to the negatively charged E₃ diluent was ideal, and therefore this molecule was used as the diluent for the remainder of the studies.

3.2 RGDS Signal Density Optimization

Previous studies have shown that the presentation density of the RGDS epitope is important for cell recognition and biological adhesion [21,22]. To specifically determine the optimal RGDS density for BMNCs, a two-dimensional adhesion assay was performed using surfaces with varying RGDS composition. The RGDS-presenting molecule was specifically designed to co-assemble with the E₃ diluent by incorporating positively charged amino acids in the positions corresponding to the negative charges on the E₃, with the aim of producing mixed binary nanofibers [41,42]. Various ratios of RGDS to E₃ were sampled, and it was found that significantly ($p < 0.001$) more BMNCs adhered to surfaces composed of a binary mixture of 10% RGDS than to surfaces of any other composition (Figure 3A). The only exception was the 15% RGDS case, which exhibited less adhesion than the 10% case, though the difference was not significant. When compared to a non-bioactive surface coated with E₃ alone, the 10% RGDS surface showed a 1.4 fold increase in the number of cells adhered.

Adhesion is enhanced as the RGDS composition is increased from 0% to 10%. Beyond this point, BMNC adhesion begins to decrease rapidly. Most likely, this can be attributed to a combination of factors. The primary effect is likely attributable to epitope crowding and saturation; increasing the epitope density beyond that which is optimal for cell adhesion. However, this alone does not explain the extent to which the observed decrease occurs at very high RGDS densities, since if this were the only factor at play, the value at 100% RGDS should not vary considerably from that for the diluent alone. The dramatic decrease in cell adhesion observed when the RGDS content is in excess of 50% is likely also attributable to the positive charge of the RGDS PA molecule negatively affecting cell viability. Cells cultured under the same conditions as those used for evaluation of adhesion showed limited viability when RGDS was mixed at 50% or more (Figure 3B). Since this molecule has a net charge of +3, the surface becomes increasingly positively charged as the RGDS content is increased relative to the negatively charged diluent. This supports our findings of charge-dependent effects on cell viability when screening diluent molecules alone. The point at which this positively charged character of the surface begins to interfere with adhesion studies appears to be around 50% RGDS. Before this point, likely epitope density is the major factor affecting cell adhesion differences.

In order to further verify that the cells were in fact responding to the presence of RGDS and not to changes in the overall charge or composition of the substrate, a PA molecule bearing a scrambled epitope was prepared. The adhesion assay was repeated, testing the binary 10% epitope system with either the RGDS PA or scrambled PA mixed with the E₃ diluent. Significantly ($p < 0.001$) more cells adhered to surfaces coated with 10% RGDS than to surfaces coated with 10% scrambled PA (Figure 4). Also, significantly ($p < 0.001$) more cells adhered to the 10% RGDS surface than to a surface coated with E₃ diluent alone. However, adhesion to surfaces coated with 10% scrambled PA did not vary significantly from surfaces coated only with diluent PA. When soluble RGDS was added, there was no significant difference in cell adhesion to the 10% RGDS surfaces relative to surfaces consisting of E₃ diluent or 10%

scrambled PA. These findings upon scrambling the epitope or adding a soluble RGDS indicate that the increase in adhesion observed for the 10% RGDS-coated surface is likely a result of specific cellular recognition of the RGDS signal on the nanostructures. In order to verify that BMNCs are able to interact with RGDS PA coated surfaces, cell adhesion and morphology on these surfaces was observed using SEM (Figure 5). These cells cultured on the binary RGDS PA surface show an adherent morphology with extensive process formation. At high magnification, these cells are shown to be in contact with PA nanofibers coating the substrate surface. This alleviates any concern that the cells are not in direct contact with a PA coated surface, and shows apparent cell health when interacting with these bioactive surfaces.

3.3 Viability and Proliferation of Encapsulated BMNCs

In order to evaluate the effects of encapsulation within nanofiber networks of these peptide amphiphiles, we examined BMNC viability and proliferation in this geometry (Figure 6). As shown, cells were minimally viable when encapsulated within scaffolds comprised of only E₃ diluent. Cells were also minimally viable when encapsulated within binary scrambled epitope gels. However, a high number of viable cells were found within the binary 10% RGDS nanofiber scaffolds. To quantify these effects, proliferation of BMNCs within these materials was evaluated. Cells encapsulated within the binary 10% RGDS scaffolds showed a 5.5 fold increase in cell number over 5 days. This was significantly greater ($p < 0.001$) than for the corresponding E₃ diluent scaffolds and the binary scrambled epitope scaffolds, where the cell number after 5 days in culture did not differ significantly from the initial cell number, suggesting no proliferation in these cases. This is presumably due to the poor viability of BMNCs within these materials. When soluble RGDS was added, this highly proliferative response of BMNCs encapsulated within the RGDS-presenting networks was completely lost (Figure 6C).

The preserved viability and proliferation demonstrated by BMNCs when encapsulated within the binary RGDS scaffold points to epitope-dependent bioactivity. Others have shown that systems functionalized with RGDS have the potential to enhance the proliferation of cells [49]. The proliferation-inducing effect of the RGDS peptide sequence has been linked to the activation of mitogen-activated protein kinase signaling cascades [50,51]. In conjunction with the pro-adhesive effect of the binary RGDS system, the induction of proliferation in encapsulated BMNCs makes this scaffold even more appealing for applications to cell-based therapies, especially in the context of the previously observed dose-response relationship for therapeutic use of these cells.

For our preliminary studies, coated surfaces were used for evaluating viability, optimizing epitope density and assessing epitope recognition. To apply PA systems for cell therapies, however, dispersion of the cells within a three-dimensional PA scaffold is more relevant as a matrix for *in vivo* cell delivery. Certainly, there is the potential for differences in optimizing epitope presentation in a two- versus a three-dimensional geometry. Others have emphasized this and have done extensive studies optimizing epitope presentation in three-dimensions [52,53]. With our systems, optimizing cell adhesion in a three-dimensional geometry is challenging, however the 10% RGDS that was determined to be optimal in our two-dimensional studies produced good results when cells were encapsulated *in vitro* in nanofiber gels of this composition, evident by the viability and proliferation observed.

3.4 Gene Expression of Encapsulated BMNCs

It is of interest to determine whether BMNCs, when exposed to these materials, differentiate or lose potency through interactions with the binary RGDS PA system. Prior evidence suggests the differentiation of BMNC-derived hematopoietic stem/progenitor cells into endothelial cells (EC), differentiation we desired to promote through selection of the soluble factors used to

culture BMNCs in these studies. Using RT-PCR, cells encapsulated within the binary RGDS system were examined for their expression of certain target genes (Figure 7). The expression of CD34, a hematopoietic stem cell marker, decreased 0.53 fold after 5 days encapsulated within the binary RGDS scaffold ($p<0.01$). Over the same time, increases in expression were seen for EC markers VE-Cadherin (1.73 fold, $p<0.05$), Vascular Endothelial Growth Factor Receptor 2 (VEGFR2, 4.48 fold, $p<0.001$) and Endothelial Nitric Oxide Synthase (eNOS, 2.49 fold, $p<0.01$) after 5 days encapsulated within the 10% RGDS PA networks.

This decrease in CD34 expression, paired with increases in VE-Cadherin, VEGFR2, and eNOS expression suggests an endothelial-lineage maturation and loss of stem-like character of BMNCs when encapsulated within the binary RGDS PA scaffold. This is likely due to the culture conditions that were used to enrich for subpopulations of endothelial character. We cannot conclude if this expression pattern when encapsulated is related to the presence of the RGDS epitopes on the nanofibers due to poor viability when encapsulated in the E3 and scrambled controls. This observation, however, demonstrates that encapsulation of BMNCs within the binary RGDS system is feasible and that cells in this environment maintain their endothelial-lineage phenotype and increase in EC character.

3.5 BMNCs Supported Within PA Scaffolds In Vivo

Finally, an animal study was performed to determine if the binary RGDS PA system optimized *in vitro* proved advantageous when translated to an *in vivo* setting (Figure 8). When luciferase-expressing cells were encapsulated within the binary RGDS scaffold and injected subcutaneously, the resulting luminescence after one day did not differ significantly from cases where the E₃ diluent was used without the bioactive epitope. Additionally, the RGDS group did not differ significantly from a saline control. Moreover, none of these conditions at day 1 varied significantly from their day 0 baseline levels. However, at 4 days post-transplantation, the cells transplanted within the binary RGDS scaffold showed a significant ($p<0.001$) increase in signal of 315% when compared to the day 0 baseline. The bioluminescence after 4 days for cells transplanted within the binary RGDS scaffold was also significantly greater ($p<0.01$) than the corresponding signal for cells transplanted with the E₃ diluent (127%) and saline control (147%) at the corresponding time.

This experiment was designed to assess whether our developed binary RGDS material was able to support cells *in vivo*. Encapsulation within the binary RGDS scaffold imparts a beneficial effect on the transplanted cells, seen by a substantial increase in the relative bioluminescent signal intensity from viable cells after 4 days. The E₃ group and the saline group did not show the same signal increase. This points to bioactivity of the RGDS-displaying nanofibers, indicating possible cell proliferation in this *in vivo* model, which would corroborate results obtained from *in vitro* experiments, where cell proliferation was enhanced by encapsulation in the binary RGDS scaffold. In this animal model, the binary RGDS system once again indicates bioactivity, and the support of cells *in vivo* holds promise to enhance regenerative potential of cell therapies using these bioactive, injectible self-assembling biomaterials. Another factor that would make this material amenable for use in the delivery of BMNCs is its presumed biocompatibility. Additional studies showed the subcutaneously implanted material to have only a mild tissue reaction, and suggested the material is degraded over an applicable time course (see supplemental information).

4. Conclusions

Self-assembling nanofibers, formed by co-assembly of RGDS-displaying peptide amphiphile molecules and a diluent, led to enhanced biological adhesion of cultured bone marrow mononuclear cells relative to nanostructures comprised of only diluent molecules. Moreover, this binary RGDS nanofibrous material enhanced viability and proliferation of encapsulated

bone marrow mononuclear cells *in vitro* while allowing endothelial cell maturation. When applied *in vivo*, our system showed the ability to act as a supportive matrix for transplanted bone marrow mononuclear cells. We conclude that the system developed here has the potential to overcome current limitations of stem and progenitor cell therapies through enhancing cell retention, viability, and proliferation, all desirable to assist in bone marrow mononuclear transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Chemical structure of the E₃ diluent PA (A), RGDS PA (B), and DGSR scrambled PA (C) along with molecular graphics representations of binary PA fibers assembled from 90% E₃ diluent and 10% RGDS PA accented in yellow (D) or 10% scrambled PA accented in green (E).

Figure 2.

Viability of BMNCs when cultured for 1 day on surfaces coated with E₃ diluent PA (A), K₃ diluent PA (B), and R₃ diluent PA (C) with red indicating dead cells and green indicating live cells. Viability was quantified after 1, 3, and 6 days (E). n=5 for each group. The scale bar for all images is 500 μm. *** p<0.001

Figure 3.

(A) Optimization of BMNC adhesion to PA coated surfaces by altering the content of RGDS PA using E₃ PA. n=8 for each group. *** p<0.001, displayed for 10% RGDS relative to other RGDS compositions. (B) Quantitative viability of BMNCs cultured on PA coated surfaces of the same RGDS content as those used in (A).

Figure 4. BMNC adhesion to surfaces coated with 10% RGDS PA compared to surfaces coated with E₃ diluent only and 10% scrambled PA only, in the presence and absence of soluble RGDS. n=12 for each group. *** p<0.001

Figure 5. BMNCs cultured on surfaces coated with 10% RGDS PA, exhibiting extensive process formation (A, B, C) in contact with the PA coated surface at higher magnification. (D) Scale bars are 20 µm (A), 5 µm (B), 2 µm (C), and 500nm (D).

Figure 6.

Viability BMNCs encapsulated within E₃ PA (A) and 10% RGDS PA (B). Viable cells are noted by green fluorescence. The red channel is not shown due to background fluorescence from interaction of the PA with EthD-1. The scale bar for all images is 500 μm. Also, proliferation after 5 days for BMNCs encapsulated within E₃ diluent, binary mixtures of 10% RGDS or 10% scrambled PAs, expressed as a percent of the day 0 cell signal, in the presence and absence of soluble RGDS (C). *** p<0.001. n=5 for each group.

Figure 7.

Gene expression of BMNCs encapsulated within the binary RGDS scaffold for 0 and 5 days, examining hematopoietic stem cell (CD34) and endothelial cell (VE-Cadherin, VEGFR2, and eNOS) marker expression by RT-PCR. Data are normalized to 18s expression and displayed relative to day 0 values. n=5 for each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 8.

Quantification from in vivo bioluminescent imaging of transplanted luciferase-expressing BMNCs (A) injected subcutaneously, encapsulated within the binary RGDS system (B, n=15) and E₃ diluent PA (C, n=11), along with a saline control (D, n=13). ** p<0.01