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Tuning Cell Adhesion on Titanium with Osteogenic Rosette Nanotubes

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Abstract

Self-assembled rosette nanotubes (RNTs), obtained from a twin GAC base functionalized with lysine-arginine-serine-arginine (KRSR-(GAC)₂), were designed and investigated as bioactive coatings on titanium. These results were compared to RNTs derived from Lysine GAC (K GAC), Arg-Gly-Asp GAC (RGD GAC) and aminobutane-(GAC)₂ (AB-(GAC)₂). The results from this study revealed that these materials had excellent cytocompatibility properties as they enhanced osteoblast (bone forming cell) adhesion when coated on titanium. In particular, KRSR and RGD functionalized RNTs coated on titanium promoted the greatest osteoblast densities relative to untreated titanium. Furthermore, KRSR functionalized RNTs selectively improved osteoblast adhesion relative to fibroblast (soft-tissue forming cell) and endothelial cell adhesion. In contrast with these results, RNTs obtained from an unfunctionalized twin base (AB-(GAC)₂), RGD GAC co-assembled with K GAC and K-GAC significantly enhanced endothelial cell attachment, which may find applications in the vascularization of newly formed bone tissue. In summary, these studies suggest that the surface of orthopedic implant materials (such as titanium) could be tailored to promote selective cell adhesion using biologically-inspired nanotubular structures functionalized with osteogenic compounds.

Keywords

rosette nanotubes; nanomaterials; biomimetic; osteogenic peptides; coating; orthopedic implant

INTRODUCTION

Each year, millions of Americans endure intense pain caused by various bone diseases often resulting in costly orthopedic implant surgeries. Specifically, for hip and knee replacements, surgical procedures drastically increased from 258,000 and 299,000 in 2000, respectively, to 482,000 and 542,000 replacements in 2006, respectively [1]. However, due to frequently reported complications (such as inflammation and implant loosening), revision surgeries are required, which add to patient pain as well as health insurance costs. A significant paradigm

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shift in this field is the design of biologically-inspired coatings that can turn conventional inert metal implant surfaces (such as titanium) into biomimetic nanostructured surfaces that enhance cell adhesion and osseointegration [2].

For this purpose, we investigated rosette nanotubes (RNTs) not only as a biomimetic nanostructured material but also as a delivery system for osteogenic peptides. The RNTs are obtained through the self-assembly of low molecular weight synthetic DNA base analogues (GAC) in aqueous solutions. The GAC moiety [3] undergoes a self-assembly process, fueled by an array of hydrogen bonds, to produce a six-membered supermacrocycle (rosette). Stacking of these rosettes gives rise to a nanotube with a very high aspect ratio. While in the case of the mono base (e.g. K-GAC), the rosette is maintained by 18 H-bonds, for the twin base (e.g. KRSR-(GAC)₂), the hexameric twin rosette is held by 36 H-bonds (Figure 1) [4]. As such, the resulting RNTs are more stable due to increased H-bonding interactions and reduced steric repulsion and charge density as opposed to RNTs from the mono base system. Unlike carbon nanotubes, the RNTs possess the advantages of being water soluble, less toxic, metal-free, and readily obtained in analytically pure form [3–9].

Previous studies have highlighted the exceptional cytocompatibility properties of RNTs assembled from single GAC motifs for various tissue engineering applications [5–9]. For example, a composite of these RNTs and nanocrystalline hydroxyapatite in poly(2-hydroxyethyl methacrylate) hydrogels enhanced osteoblast adhesion more than two times compared to hydrogel controls [8]. Journeay et al. have also demonstrated that the same RNTs have low acute toxicity in vivo [10]. In summary, RNTs are a promising nano-biomaterial for orthopedic applications because they are biocompatible, architecturally and nano-structurally similar to the extra-cellular matrix of bone [6–8]; they offer a versatile surface chemistry to tailor cell adhesion and subsequent functions [7]; and are an excellent calcification template [8].

Since greater osteoblast activity is crucial at the bone-implant interface where numerous cells are activated after implantation, an implant surface that selectively promotes osteoblast adhesion over other cells holds great potential for orthopedic implants. Osteoblast adhesion on implants can be mediated by the use of various amino acids or peptide sequences (such as RGD), which activate osteoblast membrane integrin receptors. Osteoblast adhesion may also be regulated via cell-membrane heparin sulphate proteoglycans and heparin-binding sites on proteins (i.e., fibronectin and collagen) [11–17]. For example, Dee and co-workers demonstrated for the first time that KRSR peptides immobilized on glass selectively enhanced osteoblast adhesion via heparin sulphate proteoglycan mediated mechanisms, but not endothelial cells or fibroblasts [17]. The latter, in particular, are undesirable cells for orthopedic applications because of their tendency to synthesize fibrous soft tissue around implants.

While these earlier reports highlight the separate capabilities of RNTs, RGD, and KRSR, the present study addresses the question as to whether osteoblast, fibroblast and endothelial cell adhesion on Ti can be controlled using a combination of the above to create specific osteogenic RNTs. To this end, we investigated the effect of RNTs derived from twin GAC base [4,18] functionalized with the osteogenic peptide KRSR (KRSR-(GAC)₂) on cell adhesion and compared them to other RNTs derived from lysine functionalized mono GAC base (K-GAC), RGD-GAC co-assembled with K-GAC [6,7], and unfunctionalized aminobutane (AB) twin GAC (AB-(GAC)₂) (Figure 1).

MATERIALS AND METHODS

Materials

Synthesis of twin GAC bases—All the reagents and solvents used in the synthesis of RNTs, were obtained from Aldrich, Novabiochem, BaChem, Fluka, Fisher Scientific or Advanced ChemTech, and were used without further purification. Reagent grade dichloromethane (CH_2Cl_2), methanol (MeOH) and ether (Et_2O) were purified on an MBraun solvent purification system. The KRSR-(GAC)₂ and AB-(GAC)₂ modules were synthesized according to the procedures illustrated in Figure 2.

Standard Fmoc solid-phase peptide synthesis was used to prepare the Wang resin-supported KRSR peptide. To anchor the first amino acid to the resin, Fmoc-amino acid (4 eq) and *p*-dimethylaminopyridine (DMAP) (1 eq) in *N,N*-dimethylformamide (DMF, 8 mL) were poured into a disposable plastic syringe containing the Wang resin (1 eq). After activating the resin for 20 min, *N,N'*-diisopropylcarbodiimide (DIC, 4 eq) was added to the vessel and the reaction mixture was shaken for 6 h. The resin was then filtered under a vacuum, washed with 10 mL each of CH_2Cl_2 , MeOH, DMF and then treated with 50:50 acetic anhydride/pyridine (5 mL, 1 × 10 min and 2 × 20 min) to cap the unreactive hydroxyl groups. The resin was then filtered and washed with (3 × 10 mL) DMF, CH_2Cl_2 and MeOH and dried under vacuum. The substitution degree (0.52 mmol/g) was determined by spectroscopic quantification of the fulvene-piperidine adduct at 301 nm on a resin sample.

Subsequent amino acids were coupled as follows: the Fmoc protecting group was removed by incubation of the resin in 20% piperidine/DMF (5 mL, 1 × 5 min, 1 × 30 min). The resulting peptidyl resin was washed with 10 mL each of CH_2Cl_2 , MeOH, and DMF. *N*-ethyl-*N*-isopropylpropan-2-amine (DIEA, 8 eq) was added to the mixture of amino acid (4 eq relative to resin loading) and 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 4 eq) in DMF solution, and the mixture was activated by shaking for 3 min. The resulting mixture was then added to the peptidyl resin and was shaken for 3 h. The peptidyl resin was then drained and washed with 10 mL each of CH_2Cl_2 , MeOH and DMF. The absence of free amino groups was confirmed with the Kaiser test [19]. The Fmoc protecting group was removed by incubation of the resin in 20% piperidine/DMF (5 mL, 1 × 5 min, 1 × 30 min). The resulting peptidyl resin was washed with 10 mL each of CH_2Cl_2 , MeOH, and DMF.

To prepare KRSR-(GAC)₂, the Wang resin-supported KRSR peptide was coupled to the GAC aldehyde **1** (Figure 2A). The GAC aldehyde **1** (4 eq relative to resin loading) was added to the peptidyl resin in 1,2-dichloroethane (1,2-DCE, 5 mL), and the mixture was shaken for 4 h. $\text{NaBH}(\text{OAc})_3$ (2 eq) and DIEA (4 eq) were then added and the mixture was shaken for 36 h, after which another 2 eq $\text{NaBH}(\text{OAc})_3$ and 4 eq of DIEA were added and shaken for an additional 36 h. The resin was drained and the resulting peptidyl-resin was washed with CH_2Cl_2 , MeOH and DMF (4 × 10 mL each), and dried under vacuum. Cleavage from the resin and deprotection was achieved by treating the resin with 95% TFA/water for 2 h. The beads were filtered over celite and the resulting filtrate was concentrated to a viscous liquid (rotavap). Cold Et_2O was then added to precipitate crude KRSR-(GAC)₂, which was isolated by centrifugation. The supernatant liquid was removed by decantation. The residual solid was resuspended in Et_2O (2 × 15 mL), sonicated, and centrifuged. The precipitate was dried to yield the desired KRSR-(GAC)₂ as an off-white powder.

To prepare AB-(GAC)₂, commercially available amine **2** (1.00 g, 1.57 mmol) was added to a solution of GAC aldehyde **1** (0.148 g, 0.784 mmol) in 1, 2 DCE (10 mL) at room temperature under N_2 and stirred for 30 min. $\text{NaBH}(\text{OAc})_3$ (0.395 g, 1.88 mmol) was added and the resulting mixture was stirred for an additional 15 h. The reaction mixture was

diluted with CH_2Cl_2 (50 mL) and then washed with water (10 mL), brine (15 mL), dried over Na_2SO_4 and concentrated (rotavap). Compound **3** (1.36 g, 93%) was obtained as a white foam after silica gel flash chromatography (0–10% MeOH/EtOAc). G $\wedge$ C aldehyde **1** (0.100 g, 0.155 mmol) was then added to a solution of monomer **3** (0.126 g, 0.155 mmol) in 1,2 DCE (10 mL) at room temperature under N_2 and stirred for 30 min. $\text{NaBH}(\text{OAc})_3$ (0.039 g, 0.186 mmol) was added and the resulting mixture was stirred for an additional 15 h. The reaction mixture was diluted with CH_2Cl_2 (50 mL) and washed with water (10 mL), brine (15 mL), dried over Na_2SO_4 and concentrated. Compound **4** (0.204 g, 91%) was obtained as a white foam after silica gel flash chromatography (0–50% EtOAc/Hexanes). Compound **4** (0.106 g, 0.074 mmol) was stirred in 95% TFA/thioanisole (10 mL) for 72 h. Et_2O (60 mL) was then added to the reaction mixture and the precipitate formed, was centrifuged. The residual solid was resuspended in Et_2O , sonicated and centrifuged. This process was repeated until no UV-active product could be detected in the Et_2O wash (by spotting on a silica plate). The resulting TFA salt of AB–(G $\wedge$ C) $_2$ was dried and then dissolved in 1M hydrochloric acid (10 mL), followed by removal of the solvent under reduced pressure. This process was repeated twice before the solid was dried under vacuum for 72 h to give the HCl salt of AB–(G $\wedge$ C) $_2$ as an off-white powder in quantitative yield.

Self-assembly of RNTs in water—Stock solutions (1 mg/mL) of RNTs assembled from functionalized twin bases AB–(G $\wedge$ C) $_2$ and KRSR–(G $\wedge$ C) $_2$ (referred to as AB RNT^t and KRSR–RNT^t, respectively) were prepared by dissolving the corresponding motifs (AB–(G $\wedge$ C) $_2$ isolated either as a TFA or HCl salt) in deionized water (dH_2O). The stock solutions were then diluted to 0.1 mg/mL and 0.01 mg/mL solutions for comparison purposes as required for this study. RGD–G $\wedge$ C and K–G $\wedge$ C were prepared as previously reported [3,7]. K–RNT^m refer to RNTs assembled from functionalized mono base K–G $\wedge$ C. K⁹⁹/RGD¹–RNT^m and K⁹⁵/RGD⁵–RNT^m refer to RNTs co-assembled from mono bases K–G $\wedge$ C and RGD–G $\wedge$ C in a molar ratio of 99% and 95%, respectively [7]. All the RNT solutions were filtered through a 0.22 μm syringe filter.

Characterization of KRSR–(G $\wedge$ C) $_2$ and AB–(G $\wedge$ C) $_2$ —KRSR–(G $\wedge$ C) $_2$, AB–(G $\wedge$ C) $_2$ and all intermediate molecules were characterized by $^1\text{H}/^{13}\text{C}$ NMR spectroscopy, high resolution electrospray ionization mass spectrometry (HR EI-MS), and elemental analysis. $^1\text{H}/^{13}\text{C}$ NMR spectra were recorded with the solvent as an internal reference on Varian Inova NMR spectrometers (500 or 600 MHz) at the National Institute for Nanotechnology or Department of Chemistry, University of Alberta. The NMR data are presented as follows: chemical shift, peak assignment, multiplicity, coupling constant, and integration. The mass spectra were obtained from the Mass Spectrometry Laboratory at the Department of Chemistry, University of Alberta. These data are summarized in Tables 1 and 2.

Transmission electron microscopy (TEM) imaging—TEM was used to characterize the various single and twin base RNT morphologies. As previously described [20], carbon-coated 400-mesh copper grids (EM Sciences, PA) were floated on a dH_2O droplet of each RNT (0.1 mg/mL or 0.01 mg/mL) for 2 min to adsorb the RNTs. The grids were then placed onto a droplet of dH_2O for 20 s to remove excess non-adherent RNTs before they were placed on a second droplet of 2% aqueous uranyl acetate for 20 s to negatively stain the RNTs. The grids were then dried with filter paper and imaged on a Philips EM410 under an acceleration voltage of 120 kV. The KRSR peptide (not coupled to G $\wedge$ C base) was also imaged as a control experiment.

Scanning electron microscopy (SEM) imaging and atomic force microscopy (AFM) imaging—For SEM imaging, twin bases (0.5 mg/mL) were dissolved in dH_2O by

sonication at room temperature for ~2 min. The solutions were filtered on 0.25 μm Whatmann filter membranes, heated to boiling (to promote self-assembly), and aged for 1 day. The solutions were diluted to 0.025 mg/mL with dH_2O prior to imaging. SEM samples were prepared by floating a carbon-coated 400-mesh copper grid on a droplet of the diluted RNT solution for 10 s. The grid was blotted and floated onto a drop of 2% uranyl acetate for 10 s. The RNT-coated grid was then air-dried and heated on a hot-plate (100 $^{\circ}\text{C}$) for 15 min before imaging on a high resolution Hitachi S-4800 SEM.

For AFM imaging, one drop of the diluted RNT solution (0.05 mg/mL) was deposited onto a freshly cleaved mica substrate (1 cm^2) for 10 s and excess solution was blotted using filter paper. The sample surface was imaged using a Digital Instruments/Veeco Instruments MultiMode Nanoscope IV AFM equipped with an E scanner in tapping mode. Silicon cantilevers (MikroMasch USA, Inc.) with low spring constants of 4.5 N/m, a scan rate of 0.5-1 Hz and amplitude setpoint of 1 V were used.

Cytocompatibility assays of RNT coatings on Ti

Preparation of RNT coatings on Ti—Titanium (1 cm $\times$ 1 cm $\times$ 0.05 cm) (Alfa Aesar Ti foil) and glass coverslips were soaked in acetone for 15 min, sonicated for 15 min in acetone, and rinsed with dH_2O . They were then soaked and sonicated in 70% ethanol and rinsed with dH_2O . Lastly, they were soaked and sonicated in dH_2O for another 15 min and rinsed. The glass was then etched in 1 M NaOH for 1 h and thoroughly rinsed in dH_2O . All of the Ti and glass coverslips were oven-dried overnight and autoclaved for sterilization. The day before cell seeding, the cleaned Ti substrates were coated with the various RNTs (0.01 mg/mL) and KRSR peptide (0.01 mg/mL) solutions for 45 min at room temperature. They were then removed from the solutions and air-dried overnight.

Osteoblast, fibroblast and endothelial cell culture—A human fetal osteoblast cell line (ATCC, CRL-11372, VA) was cultured in Dulbecco's modified eagle's medium (DMEM, Invitrogen Corporation) supplemented with 10% fetal bovine serum (FBS, Hyclone, UT) and 1% penicillin/streptomycin (P/S, Hyclone, UT) under standard cell culture conditions (37 $^{\circ}\text{C}$, humidified, 5% CO_2 in air). Cells were used up to passage numbers of 8–11 in the experiments without further characterization.

A rat skin fibroblast cell line (FR, ATCC, CRL-1213) was cultured in Eagle's Minimum Essential Medium (EMEM, ATCC, 30–2003) supplemented with 10% FBS under standard cell culture conditions. Rat aortic endothelial cells (RAEC, VEC Technologies) were cultured in MCDB-131 complete medium (VEC Technologies) under standard cell culture conditions. The fibroblasts were used at passage numbers 6–9 and the endothelial cells were used at passage numbers 6–11 during culture. The cell medium was replaced every other day.

Osteoblast, fibroblast and endothelial cell adhesion—Osteoblasts, fibroblasts and endothelial cells were seeded onto the substrates at a density of 3500 cells/ cm^2 in 24-well cell culture plates and were incubated in 1 mL cell culture medium (specifically, DMEM supplemented with FBS and P/S for osteoblasts, EMEM supplemented with FBS for fibroblasts and MCDB-131 complete medium for endothelial cells) for 4 h. Then, the substrates were rinsed three times with a phosphate buffered saline (PBS) to remove non-adherent cells. The remaining cells were fixed using 10% normal buffered formaldehyde (Fisher Scientific) for 10 min and 0.1% Triton X-100 (Sigma-Aldrich, MO) for 5 min. Cells were then stained with rhodamine-phalloidin (staining F-actin filaments, Molecular Probes) to examine cell spreading and were further stained with DAPI (Invitrogen). The cells were observed using a fluorescent microscope (Axiovert 200M, Zeiss) and five different areas of

each sample were imaged. The cell density was then determined by counting cells using Image Pro Analyzer. All cellular experiments were run in triplicate and repeated three times for each substrate.

Statistics

Data are presented as the mean value $\pm$ the standard error of the mean and were analyzed with a student's *t*-test to make pair-wise comparisons. Statistical significance was considered at $p < 0.1$.

RESULTS AND DISCUSSION

Synthesis

Two new self-assembling modules KRSR-(GAC)₂ and AB-(GAC)₂ were synthesized according to the synthetic schemes in Figures 2A and 2B, respectively. Tables 1 and 2 summarize the NMR and MS data of KRSR-(GAC)₂ and AB-(GAC)₂ compounds. Standard Fmoc [21, 22] solid-phase peptide synthesis (SPPS) was used to prepare KRSR-(GAC)₂. SPPS [23] is a simple procedure, which allows rapid synthesis of peptides in good yields. This method eliminates solubility, purification and racemization issues, common with solution phase peptide synthesis. The carboxyl groups of the protected amino acid Fmoc-Lys(Boc)-OH was first coupled to the hydroxyl groups on the Wang resin. The Fmoc group of the lysine anchored to the resin was removed under basic conditions, after which it was reacted with the second amino acid Fmoc-Arg(PBf)-OH. The same procedure was repeated for subsequent amino acid couplings with Fmoc-Ser(^tBu)-OH, Fmoc-Arg(PBf)-OH and Fmoc- γ -Abu-OH. The terminal Fmoc group on the Wang resin-supported peptide was removed and the resulting free amine was reductively coupled to GAC aldehyde. The desired motif KRSR-(GAC)₂ was obtained upon deprotection and cleavage from the resin under strongly acidic conditions.

The synthetic scheme (Figure 2B) was carried out for preparation of module AB-(GAC)₂ from the GAC aldehyde and *t*-butyl 4-aminobutylcarbamate via two consecutive reductive amination reactions followed by removal all protecting groups.

Microscopy studies

Irrespective of the side chains on the GAC motif, all the resulting RNTs showed nanostructures with high aspect ratios (Figure 3). TEM images revealed a larger diameter (4.4 ± 0.2 nm) for KRSR-RNT^t (Figures 3A–B) as compared to AB-RNT^t (3.5 ± 0.2 nm) (Figure 3C–D) due to the bulkier KRSR moiety attached on the periphery of the twin base. K-RNT^m and K⁹⁵/RGD⁵-RNT^m (Figure 3E–F) featured a diameter of 3.4 ± 0.3 nm. As expected, the control sample with KRSR peptides only did not show similar morphologies to the RNT molecules (Figure 3G).

SEM and AFM images revealed dense nanotubular networks for both KRSR-RNT^t and AB-RNT^t (Figure 4). It is interesting to note that the average length of KRSR-RNT^t was less than that of AB-RNT^t, suggesting that the bulkiness of the peptide influences the degree of stacking and hence the length of the RNTs.

Osteoblast responses to RNT-coated Ti

All of the RNTs coated on Ti significantly enhanced osteoblast adhesion compared to uncoated Ti after 4 h ($p < 0.01$) (Figure 5). Impressively, compared to uncoated Ti, the 0.01 mg/mL KRSR-RNT^t and K⁹⁵/RGD⁵-RNT^m coated Ti improved osteoblast adhesion by 122% and 124%, respectively. In fact, KRSR-RNT^t and K⁹⁵/RGD⁵-RNT^m promoted the greatest osteoblast densities on Ti. In addition, the KRSR peptide alone on Ti promoted

more osteoblast cell adhesion relative to uncoated Ti. There was no statistically significant difference among AB-RNT^t, K⁹⁹/RGD^l-RNT^m, and K-RNT^m.

It is well-known that surface chemistry of implant materials plays an important role in mediating cell adhesion, proliferation and differentiation [2,24]. Thus, one of the objectives of this study was to investigate the influence of different surface chemistry (particularly RNTs functionalized with KRSR peptides) on osteoblast adhesion. Balasundaram et al. immobilized KRSR peptides on commercially available nanophase and conventional Ti surfaces and measured improved osteoblast adhesion relative to bare Ti [25]. They found that not only did the KRSR-functionalized Ti have improved osteoblast adhesion relative to uncoated Ti, but that there were more osteoblasts on uncoated nanophase Ti relative to conventional KRSR functionalized Ti. The latter observation underscores the importance of nanostructured biomaterials in improving osteoblast adhesion. In this report, the osteoblast-adhesive KRSR peptide was conjugated with RNTs to provide an improved orthopedic material. The resulting osteogenic RNTs offered a biomimetic nanostructured biomaterial displaying osteogenic peptides that could be readily distributed on the Ti surface to potentially enhance the efficiency of traditional implants. In effect, our results demonstrated that the novel nanotube KRSR-RNT^t greatly improved osteoblast adhesion relative to K-RNT^m, AB-RNT^t, and uncoated Ti, thus, confirming the important role of the osteogenic peptide display function of the RNTs on osteoblast adhesion.

It is also important to note that more osteoblasts adhered to Ti when the latter was coated with KRSR-RNT^t versus KRSR. Specifically, osteoblast adhesion on Ti coated with KRSR-RNT^t was 84.4% higher than Ti coated with the peptide KRSR alone, which is consistent with earlier results obtained with hydrogels coated with K-RNT^m versus poly L-lysine [7]. Additionally, osteoblasts were better spread with more extended filopodia on RNT-coated Ti than on uncoated Ti (Figure 6). Such trends highlight the importance of the unique nano-biomimetic features of RNTs for promoting osteoblast functions.

Fibroblast responses to RNT coated Ti

Compared to uncoated Ti, results from the present study provided evidence that KRSR-RNT^t, K⁹⁹/RGD^l-RNT^m, K-RNT^m, and AB-RNT^t did not alter fibroblast adhesion after 4 h (Figure 7). Fibroblast attachment on Ti coated with 0.01 mg/mL KRSR-RNT^t was slightly greater than the KRSR-coated Ti. Considering a similar surface chemistry but a greatly different supramolecular organization between KRSR-RNT^t and the KRSR peptide, this result suggests that the nanostructure of RNTs plays a role in augmenting fibroblast adhesion.

AB-RNT^t was assembled from the HCl and TFA salts of AB-(G⁺C)₂. The differences in fibroblast attachment with these two types of RNTs may be associated with their different counter ions. In contrast with its effect on osteoblast adhesion, the KRSR peptide did not enhance fibroblast adhesion on Ti, thus providing for cell selectivity and a potential for orthopedic applications. Finally, many small filopodia extensions from rounded fibroblasts were visible on all substrates, indicating that fibroblasts attached similarly regardless of the Ti coatings (Figure 8).

Endothelial cell responses to RNT-coated Ti

Results from this study showed a significantly higher endothelial density on the Ti coated with RNTs (except for KRSR-RNT^t) compared to uncoated Ti after 4 h (Figure 9). This result is consistent with a previous report wherein different K-RNT concentrations enhanced endothelial cell adhesion and proliferation on Ti compared to uncoated Ti [26]. In addition, AB-RNT^t (HCl) coated Ti promoted the greatest endothelial cell adhesion compared to all

other substrates. Furthermore, more endothelial cells attached on Ti coated with K-RNT^m and AB-RNT^t (HCl) than on Ti coated with KRSR-RNT^t. KRSR-RNT^t and KRSR peptide coated Ti did not enhance endothelial cell adhesion here, confirming that KRSR selectively promoted osteoblast adhesion as previously described [17]. Endothelial cell morphologies are shown in Figure 10. These results suggested excellent cytocompatibility properties of K-RNT^m and RGD-RNT^m for endothelial cell adhesion and the selectivity of KRSR-RNT^t only for osteoblast adhesion.

Enhanced endothelial cell adhesion on RNT coated Ti may be closely related to the nanotubular structure and surface chemistry of RNTs. In fact, other nanomaterials such as polymers and metals improved vascular cell (endothelial and smooth muscle cells) functions compared to conventional (nano-smooth) materials [27]. For example, Choudhary et al. reported greatly improved vascular cell adhesion and proliferation on nanostructured commercially pure Ti particle compacts compared to conventional micron Ti particle compacts [28]. It was speculated in that study that the increased nanoscale roughness and particle boundaries on nanostructured Ti favored endothelial cell functions.

From the surface chemistry point of view, it was reported that poly L-lysine coated vascular stents significantly improved endothelial cell adhesion compared to uncoated controls, perhaps due to the electrostatic or optimal initial protein interactions between poly L-lysine and endothelial cells [29]. In addition, it is well-known that RGD is a cell adhesive domain in many proteins which interacts specifically with integrin receptors of cell membrane receptors to improve the adhesion of a variety of cells [30,32]. Thus, as supported in this study, a thin film of nanostructured RNTs with numerous K or RGD side chains may alter the surface chemistry and surface roughness of conventional Ti to provide a favorable environment for enhancing endothelial cell adhesion. Due to the advantages of growing new blood vessels in bone formed around an orthopedic implant, the increased endothelial cell functions on RNTs may be an added value for orthopedic applications.

CONCLUSIONS

In this study, a KRSR peptide was successfully coupled onto RNTs to serve as a potentially improved orthopedic implant material. Improved cytocompatibility properties of unfunctionalized or functionalized RNTs with KRSR, K, or RGD on conventional Ti were observed. It was found that osteoblast, fibroblast and endothelial cells responded differently on RNTs with different amino acid or peptide side chains. Specifically, greater osteoblast adhesion occurred on KRSR-RNT^t and K⁹⁵/RGD⁵-RNT^m coated Ti than on uncoated Ti, K-RNT^m and AB-RNT^t coated Ti. In contrast, fibroblast adhesion was similar on the various RNTs compared to uncoated Ti. Furthermore, the various RNTs (except KRSR-RNT^t) coated on Ti greatly enhanced endothelial cell densities after 4 h. KRSR-RNT^t coated on Ti selectively enhanced the adhesion of bone cells after 4 h. RGD RNT^m, K-RNT^m and AB-RNT^t enhanced both osteoblast and endothelial cell adhesion. In summary, results of this study demonstrated that nanostructured RNTs with diverse amino acid and peptide side chains possess excellent cytocompatibility properties and favorable surface chemistry for selectively enhancing bone cell attachment. While further studies are needed, the ease of chemical modification and presence of a biologically-inspired nanostructured geometry, make RNTs promising materials for improved orthopedic implant applications.

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Figure 1.

Self-assembly of rosette nanotubes from twin and single DNA bases. The models shown are not drawn to scale. (A) Self-assembly of six KRSR-(GAC)₂ twin modules to give a twin rosette, which then stack to give the KRSR RNT^l nanotube. (B) Self-assembly of six AB-(GAC)₂ twin modules to give a twin rosette, which then stack to give the AB-RNT^l nanotube. (C) Co-assembly of K-GAC and RGD-GAC modules to give a rosette, which then stack to give the K⁹⁵/RGD⁵-RNT^m nanotube. (D) Self-assembly of six K-GAC modules to give a rosette, which then stack to give the K-RNT^m nanotube.

Figure 2.
A synthetic scheme of (A) Wang resin protected KRSR peptide coupling onto the twin GAC bases (KRSR-(GAC)₂) and (B) AB-(GAC)₂ twin modules.

Figure 3. TEM images of RNTs investigated: (A) and (B) 0.1 mg/mL and 0.01 mg/mL KRSR-RNT^t; (C) and (D) 0.1 mg/mL AB-RNT^t (HCl and TFA salts, respectively); (E) 0.1 mg/mL K-RNT^m; (F) 0.1 mg/mL K⁹⁵/RGD⁵-RNT^m; and (G) 0.1 mg/mL KRSR peptide only. Arrows point at nanotubes.

Figure 4. SEM (A, C) and AFM (B, D) images of KRSR-RNT^t (A, B); and AB-RNT^t (C, D).

Figure 5.

Osteoblast adhesion on Ti coated with RNTs. Data are mean values $\pm$ SEM, N=3. * $p < 0.01$ and $\&p < 0.1$ compared to uncoated Ti; ** $p < 0.05$ compared to 0.01 mg/mL KRSR coated on Ti; *** $p < 0.1$ compared to 0.01 mg/mL K-RNT^m, K⁹⁹/RGD¹-RNT^m, AB-RNT^t (HCl) coated on Ti; # $p < 0.05$ compared to 0.01 mg/mL K-RNT^m coated on Ti; and § $p < 0.1$ compared to K⁹⁹/RGD¹-RNT^m, AB-RNT^t (HCl or TFA salts) coated on Ti.

Figure 6.

Fluorescence microscopy images of osteoblast adhesion on 0.01mg/mL RNT-coated Ti at low magnification (50 $\times$, DAPI stained nuclei, A–F) and high magnifications (400 $\times$, rhodamine-phalloidin stained F-actin filaments, A'–F'). K–RNT^m (A, A'); AB-RNT^t (HCl) (B, B'); AB-RNT^t (TFA) (C, C'); KRSR–RNT^t (D, D'); K⁹⁹/RGD¹–RNT^m (E, E'); K⁹⁵/RGD⁵–RNT^m (F, F'); and KRSR (G, G'). Uncoated Ti at low (H) and high (H') magnifications. Arrows point to long filopodia.

Figure 7.

Fibroblast adhesion on RNT-coated Ti. Data are mean values $\pm$ SEM, N=3. * $p < 0.05$ compared to 0.01 mg/mL KRSR coated on Ti; # $p < 0.05$ compared to 0.01 mg/mL AB-RNT^t (HCl) coated on Ti; and & $p < 0.05$ compared to uncoated Ti.

Figure 8.

Fluorescence microscopy images of fibroblast spreading. (A) K-RNT^m coated on Ti; (B) AB-RNT^t (HCl) coated on Ti; (C) K⁹⁹/RGD¹-RNT^m coated on Ti; (D) KRSR-RNT^t coated on Ti; (E) KRSR coated Ti; and (F) uncoated Ti. F-actin filaments were stained by rhodamine-phalloidin.

Figure 9. Endothelial cell adhesion on RNT coated on Ti. Data are mean values $\pm$ SEM, N=3. * $p < 0.05$ and ** $p < 0.1$ compared to uncoated Ti. # $p < 0.05$ compared to all other substrates. & $p < 0.1$ compared to 0.01 mg/mL KRSR-RNT^t coated on Ti.

Figure 10.

Fluorescence microscopy images of endothelial cell spreading on various coatings after 4 h. (A) K-RNT^m coated on Ti; (B) AB-RNT^t (HCl) coated on Ti; (C) K⁹⁹/RGD¹-RNT^m coated on Ti; (D) KRSR-RNT^t coated on Ti; (E) KRSR coated Ti; and (F) uncoated Ti. F-actin filaments were stained by rhodamine-phalloidin (green color) and cell nuclei were stained by DAPI (blue color).

Table 1¹H NMR, ¹³C NMR, HR EI-MS and elemental analysis data of KRSR-(GAC)₂

	KRSR-(GAC) ₂ Characterization Data
¹ H NMR (DMSO; 600 MHz)	12.34 (bs, 2H), 9.24 (bs, 2H), 9.06 (bs, 2H), 8.97 (m, 1H), 8.26 (m, 2H), 8.13 (m, 2H), 8.08–7.98 (m, 2H), 7.65 (bs, 3H), 7.54–7.50 (m, 2H), 7.39–7.16 (bs, 4H), 7.10–6.65 (bs, 4H), 4.43 (m, 4H), 4.35–4.27 (m, 3H), 4.12 (m, 1H), 3.62 (m, 1H), 3.59–3.47 (m, 4H), 3.33 (m, 1H), 3.08 (bs, 5H), 2.91 (d, 6H, <i>J</i> = 4.2 Hz), 2.74 (m, 4H), 2.34–2.23 (m, 2H), 1.90–1.86 (m, 2H), 1.77–1.64 (m, 2H), 1.60–1.43 (m, 8H), 1.34–1.27 (m, 2H)
¹³ C NMR (DMSO; 150 MHz)	174.9, 174.1, 173.7, 172.0, 171.9, 170.4, 169.8, 162.6, 161.7, 160.3, 157.2, 156.7, 156.0, 148.6, 128.9, 128.7, 83.0, 82.2, 62.2, 55.4, 52.6, 52.5, 52.2, 52.0, 49.5, 40.9, 39.2, 39.1, 31.0, 30.7, 29.8, 29.5, 28.3, 27.1, 27.0, 25.4, 25.3, 22.9
HR EI-MS	Calculated mass for C ₄₃ H ₇₁ N ₂₄ O ₁₁ [M+H] ⁺ 1099.5729; found 1099.5727
Elemental analysis	Calculated for (C ₄₃ H ₇₀ N ₂₄ O ₁₁)(TFA) ₅ (H ₂ O) ₃ (H ₂ SO ₄) _{1.5} (Et ₂ O) = C (35.22), H (4.82), N (17.30), S (2.47); found: C (35.20), H (4.71), N (17.25), S (2.49)

Table 2

¹H NMR, ¹³C NMR, HR EI-MS and elemental analysis data of AB (GAC)₂ and intermediates

	AB-(GAC) ₂ and intermediate Characterization Data
Compound 3	R _f = 0.25 (10% MeOH in EtOAc); ¹ H NMR (CDCl ₃ , 500 MHz) (ppm) 7.44–7.32 (m, 5H), 5.56 (s, 2H), 4.75 (bs, 1H), 4.50 (t, 2H, J = 6.3 Hz), 3.46 (s, 3H), 3.07 (app. t, 4H, J = 6.0 Hz), 2.73 (t, 2H, J = 6.9 Hz), 1.58 (s, 9H), 1.54–1.44 (m, 4H), 1.42 (s, 9H), 1.33 (s, 18H); ¹³ C NMR (CDCl ₃ , 125 MHz) (ppm) 165.8, 161.2, 160.9, 160.5, 156.0, 155.9, 152.6, 149.3, 134.9, 128.6, 128.5, 128.3, 93.1, 83.8, 83.3, 70.1, 50.0, 47.0, 42.6, 40.2, 34.9, 28.4, 28.1, 27.8, 27.6, 26.3; HR EI-MS ⁺ calculated for C ₄₀ H ₆₁ N ₈ O ₁₀ [M] ⁺ 813.4505, found 813.4507.
Compound 4	R _f = 0.26 (50% EtOAc in hexanes); ¹ H NMR (CDCl ₃ , 500 MHz) (ppm) 7.46–.33 (m, 10H), 5.57 (s, 4H), 4.87 (bs, 1H), 4.39 (t, 4H, J = 7.2 Hz), 3.48 (s, 6H), 3.06 (m, 2H), 2.90 (t, 4H, J = 7.3 Hz), 2.67 (m, 2H), 1.56 (s, 18H), 1.41 (s, 14H), 1.31 (s, 36H); ¹³ C NMR (CDCl ₃ , 125 MHz) (ppm) 165.7, 161.2, 161.1, 160.3, 156.1, 155.6, 152.6, 149.3, 135.0, 128.6, 128.5, 127.8, 114.0, 92.9, 83.7, 82.9, 78.8, 70.1, 53.9, 50.9, 41.3, 40.5, 35.0, 29.7, 28.5, 28.1, 27.9, 25.1; HR EI-MS ⁺ calculated for C ₇₁ H ₁₀₀ N ₁₄ O ₁₈ Na[M+Na] 1459.72377, found 1459.72376.
AB-(GAC) ₂	mp = 296–301 °C (Decomposition); ¹ H NMR (DMSO, 600 MHz) (ppm) 12.32 (s, 2H), 9.15 (s, 2H), 8.90 (s, 2H), 8.57 (app. q, 2H, J = 4.8 Hz), 8.11 (bs, 3H), 4.45 (bs, 2H), 3.46 (bs, 3H), 3.30 (bs, 4H), 2.95 (d, 6H, J = 4.7 Hz), 2.81–2.75 (m, 2H), 1.85–1.75 (m, 2H), 1.70–1.58 (m, 2H); ¹³ C NMR (DMSO, 100 MHz) (ppm) 160.3, 159.6, 155.9, 155.6, 147.6, 82.4, 51.2, 48.3, 37.8, 36.0, 27.7, 23.8, 19.7; HR EI-MS calculated for C ₂₂ H ₃₃ N ₁₄ O ₄ [M+H] ⁺ 557.2804, found 557.2803; Elemental analysis calculated for C ₂₂ H ₃₂ N ₁₄ O ₄ (HCl) ₄ (H ₂ O) _{1.5} C, 36.22, H, 5.39, N, 26.88, found C, 36.31, H, 5.35, N, 26.44.