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Covalent Attachment of P15 Peptide to Ti Alloy Surface Modified with Polymer to Enhance Osseointegration of Implants

Liye Fu^{†,‡}, Maiko Omi^{‡,§}, Mingkang Sun[†], Boyle Cheng[§], Gordon Mao[§], Tong Liu[†], Gustavo Mendonça[†], Saadyah E. Averick^{*,§}, Yuji Mishina^{*,‡}, Krzysztof Matyjaszewski^{*,†}

[†]Department of Chemistry, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213, United States

[‡]Department of Biologic and Materials Sciences & Prosthodontics, University of Michigan School of Dentistry, Ann Arbor, Michigan 48109, United States

[§]Neuroscience Disruptive Research Lab, Allegheny Health Network, 320 East North Avenue, Pittsburgh, Pennsylvania 15212, United States

Abstract

Titanium (Ti) and its alloys are used in orthopedic and dental implants due to their excellent physical properties and biocompatibility. Although Ti exhibits superior osteoconductive properties compared to those of polymer-based implants, improved bone-on growth properties are required for enhanced surgical outcomes and improved recovery surgical interventions. Herein, we demonstrate a novel surface modification strategy to enhance the osteoconductivity of Ti surfaces through the grafting-from procedure of a reactive copolymer via surface-initiated atom transfer radical polymerization (SI-ATRP). Then, postpolymerization conjugation of the P15 peptide, an osteoblast binding motif, was successfully carried out. Subsequent *in vitro* studies revealed that the surface modification promoted osteoblast attachment on the Ti discs at 6 and 24 h. Moreover, mineral matrix deposition by osteoblasts was greater for the surface-modified Ti than for plain Ti and P15 randomly absorbed onto the Ti surface. These results suggest that the strategy for postpolymerization incorporation of P15 onto a Ti surface with a polymer interface may provide improved osseointegration outcomes, leading to enhanced quality of life for patients.

Graphical Abstract

^{*}**Corresponding Authors:** saadyah.averick@ahn.org (S.E.A.), mishina@umich.edu (Y.M.), km3b@andrew.cmu.edu (K.M.).
[#]L.F. and M.O. contributed equally to this work.

Supporting Information

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Characterization of aqueous phase catalysts and polymerization results ([PDF](#))

The authors declare no competing financial interest.

P15-polymer Modified Ti Surface for Rapid Osseointegration

Keywords

titanium; implant; osseointegration; surface modification; polymer grafting; P15 peptide; SI-ATRP

INTRODUCTION

Titanium (Ti) alloys are the most commonly used materials in spine, orthopedic, and dental implants due to their low modulus, superior biocompatibility, enhanced corrosion resistance, and osteoconductive properties when compared to more conventional stainless-steel- and cobalt-based alloys.^{1–3} Osseointegration of implants is characterized as the direct structural and functional connection between living bone and the surface of a load-bearing artificial implant.⁴ Ideally, implant materials facilitate functional tissue regeneration of bones in addition to providing structural support of damaged tissue. Dental implants are a well-established treatment for replacing lost teeth and have achieved high success rates. Unfortunately, all implant materials including Ti-based implants require a several months long postimplantation period before fixation and bone growth on implanted materials in otherwise healthy individuals. In the case of heavy smokers, or patients with severe diabetes or osteoporosis, there are higher rates of implant failure due to impaired bone healing.^{5,6} The negative outcomes of poor osseointegration with insufficient bone healing need to be improved for successful implant application in challenging cases.^{7,8} Therefore, novel technologies that can enhance osteoconductive properties of Ti and thus facilitate osseointegration are of great medical importance.

The success of an implant's osseointegration is dictated by the cell–biomaterial interactions that are primarily governed by cell adhesion originating from the binding of cellular integrin receptors to the material's surface.⁹ Enhancement of binding can be achieved by attaching biomacromolecules via adsorption, tethering, or deposition onto the implants. Osteoblastic differentiation requires binding of $\alpha 2\text{B1}$ integrins with type I collagen¹⁰ which initiates activation of focal adhesion kinases (FAK) leading to signal transduction pathways involved in cell migration, survival, and differentiation.¹¹ Incorporation of $\alpha 2\text{B1}$ binding motifs onto implant-body interfaces can promote osseointegration of biomaterials.^{12,13} The P15 peptide is a synthetic short peptide (GTPGPQGIAGQRGVV) that mimics the cell-binding domain

of human type I collagen and has found applications in coating the surface of inorganic bone material to promote osteoblast differentiation and to enhance cell adhesion, migration, and survival.^{14,15} In addition to promoting cell adhesion, exposure of the P15 peptide to mesenchymal stem cells leads to express higher alkaline phosphatase activity, a marker of early osteoblast differentiation.^{16–18} It was reported that nonspecific absorption of the P15 peptide onto a Ti alloy surface leads to improvements of cell growth, but it had limited impact on *in vitro* matrix mineralization.¹⁹

An additional tool to enhance implant biocompatibility and osseointegration is the coating of biomolecule–polymer hybrids onto the metal surface. A surface coating consisting of polymers with an integrin binding domain (the RGD peptide) increases the bone–implant contact area by 75% when compared to that with an uncoated Ti surface.²⁰ Thus, combining polymers and integrin binding motifs may play a key role in the next generation implants with an increased cellular integration and potentially faster osseointegration.

A key challenge to preparing these complex hybrid materials is the grafting of a uniform polymer layer at the metal interface. Recent advances in reversible deactivation radical polymerization (RDRP) methods like atom transfer radical polymerization (ATRP) have ameliorated many challenges to preparing polymer-modified surfaces for applications in energy, self-cleaning, and superhydrophilic materials.^{21–24} Nevertheless, application of polymer-grafted metal surfaces in the biomedical space is still very limited. To prepare uniform layers of polymers from a metal surface, initiators are first immobilized onto a surface, then monomers are introduced, and polymers are grown *in situ* via a surface-initiated atom transfer radical polymerization (SI-ATRP), which is a versatile and robust approach widely used in the “graft-from” strategy.^{25–28} We hypothesized that the incorporation to the P15 peptide onto surfaces grafted with polymers would be a facile method to improve cell binding and *in vitro* mineralization of osteoblasts onto a Ti surface.

In this manuscript, we developed a novel method of attaching polymers and P15 peptide on the surface of Ti materials to enhance *in vitro* osseointegration. Ti discs were processed using an acid etch, followed by treatment of a tethering agent containing ATRP initiator 12-(2-bromoisobutyramido)dodecanoic acid (BiBADA). A monomer containing a ketone moiety, acetyethyl methacrylate (AEMA), was synthesized and copolymerized with 2-(dimethylamino) ethyl methacrylate (DMAEMA) via SI-ATRP. The ketone-containing polymer grafted from the Ti surface allowed for postpolymerization ligation of the P15 peptide with aminooxy-modified N-terminus via oxime chemistry.²⁹ The *in vitro* impact of polymer grafting was studied using short-term cell binding assays and late-stage mineralization studies using primary osteoblasts. Our results showed the improved osteoconductive cellular response including cell attachment and mineralization to the polymer-modified Ti surface compared to that on the untreated Ti surface.

EXPERIMENTAL SECTION

Modification of the Ti Surface.

Titanium discs (United Performance Metals, South Windsor, CT) (99% Ti, 1.0 mm thick, 14.7 mm in diameter) were cleaned by washing twice in 1 M NaOH with sonication for 5

min, twice in deionized water (dH₂O), and twice in MeOH/HCl (50:50) with sonication for 30 min, then etching by piranha solution (50:50, 98% H₂SO₄/30% H₂O₂, 4 h), and subsequently washing twice in dH₂O with sonication for 5 min and thrice with tetrahydrofuran (THF) for 5 min. To attach the ATRP initiator, the discs were treated with BiBADA (10 mg/mL in THF) with sonication for 2 h, followed by washing twice in THF for 5 min. Then, the Ti discs were dried under vacuum and placed in desiccator prior to use.

Polymer Grafting and P15 Attachment.

Ti discs (maximum of 5 per reaction) were placed into a 50 mL round-bottomed Schlenk flask DMAEMA (1.866 g, 11.88 mmol), AEMA (0.928 g, 5.94 mmol), and sacrificial initiator 2-hydroxyethyl α -bromoisobutyrate (OHEBiB, 25.1 mg, 0.12 mmol) were added to the flask. Next, 530 μ L stock solution of copper(II) bromide and tris(2-pyridylmethyl)amine (CuBr₂/TPMA = 1:5, 5.0 mg/mL of CuBr₂) was pipetted into the flask, and *N,N*-dimethylformamide (DMF) was added to a total of 20 mL of reaction mixture. The flask was then sealed and purged by bubbling N₂ gas. Photomediated ATRP was conducted under irradiation by a 4.9 mW/cm² UV lamp for 5 h. P15 with an aminooxy N-terminal end was purchased from Genscript. A 2 mg/mL solution of P15-ONH₂ was prepared in 1× PBS. Discs were then incubated with 1 mL of 0.2 mg/mL P15-ONH₂ at 37 °C and shaken on an orbital plate shaker for 24 h. Discs were washed with PBS, water, and 70% ethanol/water. A total of 4 groups of Ti discs were prepared for *in vitro* studies: (1) pristine Ti discs without any modification or treatment (Ti); (2) Ti discs with adsorption of P15 peptide (Ti + P15); (3) polymer-grafted Ti discs without P15 peptide (Ti-polymer); and (4) polymer-grafted Ti discs with P15 peptide (Ti-polymer + P15) (Scheme 1).

Cell Viability Assay.

Wild-type preosteoblasts were isolated from newborn mouse calvaria and cultured in α -minimum essential medium (α MEM) supplemented with 10% fetal bovine serum (Denville Scientific) and 1% penicillin/streptomycin (Invitrogen). All cells were used for experiments before passage 4. To assess cell adhesion, osteoblasts were seeded onto Ti discs (1 × 10⁵ cells/disc) and cultured for 6 h. After washing nonadherent cells, the osteoblasts attached to the Ti discs were incubated with culture media also containing 10% (v/v) alamarBlue reagent (Invitrogen) for 4 h. At the end of cultivation, 100 μ L of the culture media was transferred to a 96-well plate in triplicate for each sample, and levels of 580 nm emission were measured with 530 nm excitation using a fluorescence plate reader to quantify conversion of resazurin to resorufin as a surrogate of attached live cells on the Ti discs.

Phalloidin Stain and Cell Count.

Osteoblasts were cultured on the Ti discs (1 × 10⁵ cells/disc) for 24 h and fixed with 4% paraformaldehyde. Cells were subsequently stained with Acti-stain 488 phalloidin (Cytoskeleton) and 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) overnight at 4 °C. Fluorescence images were taken using a C1 confocal microscope (Nikon), and at least 10 fields per sample were counted.

Alizarin Red S Stain.

Osteoblasts were seeded onto the Ti discs (1×10^5 cells/disc) and incubated with osteogenic differentiation media containing 10 mM β -glycerophosphate and 50 μ g/mL L-ascorbic acid. After 21 days in culture, cells were fixed with 70% ethanol for 15 min at room temperature. Cells were stained with 40 mM alizarin red S (Sigma-Aldrich) for 10 min and then rinsed five times with water to minimize nonspecific staining. The stained area was digitally photographed and analyzed by using ImageJ (National Institutes of Health). Then, 10% cetylpyridinium chloride (Sigma-Aldrich) was used to dissolve bound alizarin red S, and optical density was measured at 562 nm.

Statistical Analysis.

All results were expressed as means $\pm$ standard deviation (means $\pm$ SD) of triplicate measurements. Statistical differences among the three groups were analyzed using one-way ANOVA, followed by a Tukey's test. Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSIONS

Polymer Grafting from Surface Roughened Ti Discs.

In this study, the surface of the Ti discs was washed by sonication under acidic and basic conditions, followed by acid etching using piranha solution, resulting in dull colored discs and indicative of surface-roughened Ti.³⁰ After surface roughening, the BiBADA ATRP initiator was immobilized via combination of ionic and hydrophobic interaction as previously reported.³¹ SI-ATRP was conducted using a photoregenerated catalysts.^{32–35} The grafted polymer had $M_n = 12\,400$ with low dispersity ($= M_w/M_n = 1.38$), as determined by gel permeation chromatography (GPC) using sacrificial initiator,³⁶ (Figure S1) with a monomer conversion reaching 54% after 5 h of photomediated ATRP. X-ray photoelectron spectroscopy (XPS) was used to evaluate the extent of surface modification at each stage of the Ti modification process including initiator immobilization and polymer grafting. At two positions, Ti signals (458 and 465 eV) were visible before the polymerization but disappeared afterward, indicating the surface of Ti discs were covered by a layer of polymer (Figure 1). In addition, results of atom mass percentage analyses also indicated a decrease of the Ti content with a progressive increase of C and N content. (Table S1). The modification was also proved by analysis of scanning electron microscopy (SEM) showing the surface of Ti plate was covered by a fine layer of polymer (Figure S2). The monomers grafted from the discs were selected to maximize interaction with the extracellular matrix (ECM)³⁷ and afford a postsynthetic reactive handle for P15 incorporation. DMAEMA was the primary constituent of the random copolymer giving the grafted surface a positive charge that can interact with the negatively charged proteoglycans in the ECM and on cell surfaces. The AEMA monomer was synthesized via a one-step reaction (Scheme S1) and provided ketone functionality on the side chains of the polymer. This group is orthogonally reactive to biological molecules and can be selectively activated and reacted with *N*-aminooxy-functionalized compounds such as P15 peptide derivative through oxime chemistry (Scheme S2). The ketoxime linking group is formed rapidly and efficiently and is stable against hydrolysis at pH 5.0–9.0.³⁸

Cell Binding to Ti-Polymer-Grafted Surfaces.

To address whether the surface modification technique can improve cell adhesion onto the Ti discs, cell viability at 6 and 48 h was measured by alamarBlue. The viability of cells on the polymer-modified Ti surface with P15 peptides (Ti-polymer + P15) was higher than that on the plain Ti surface (Figure 2a). However, the cell viability on the polymer-modified Ti surface without P15 peptides (Ti-polymer) was similar to that on the plain Ti surface. These results suggested that modification of the Ti surface by grafting polymer with P15 peptides enhances cell attachment and growth. Next, we investigated whether attaching polymers and P15 peptides on the Ti surface improves cell attachment on the Ti materials compared to that with the plain Ti and the P15-adsorbed plain Ti surface. Cell viability at 6 h and cell number at 24 h after seeding were measured by alamarBlue and nuclei staining, respectively. The viability of cells on the polymer-modified Ti surface with P15 peptides (Ti-polymer + P15) was significantly higher than that on the plain Ti or the P15-adsorbed plain Ti surface (plain Ti + P15) (Figure 2b). Fluorescent phalloidin staining showed that cells on the polymer-modified Ti with P15 peptides were more numerous and tightly packed compared to cells on the plain Ti or the P15 peptides to the plain Ti surface (Figure 2c). The number of cells adherent to the polymer-modified Ti surface with P15 peptides was significantly higher than that to the plain Ti or the P15-adsorbed Ti surface (Figure 2d). To address whether the surface modification technique can promote osteoblast differentiation, the expression of the typical osteoblast early markers *Col1a1* and *Alp* was determined by qRT-PCR. *Col1a1* expression significantly increased in cells on the polymer-modified Ti surface with P15 peptides compared to that in cells on the plain Ti or the P15-adsorbed plain Ti surface, while *Alp* expression had similar levels among all the groups (Figure S3). These results suggest that the surface modification technique using polymers and P15 peptides enhances cell attachment on the Ti surface and osteoblast differentiation, and the major reason was deemed to be a larger surface area created by grafted polymer densely attached with P15 peptides.

To determine whether the polymer-grafted Ti discs promote mineralization in osteoblasts, calcium deposits in cell culture were visualized by Alizarin red S staining at 21 days (Figure 3a). Osteoblasts cultured on the polymer-modified Ti surface with P15 peptides significantly increased calcium deposition compared to those cultured on the plain Ti or the P15-adsorbed plain Ti surface (Figure 3b,c). These results indicate that the surface modification technique using polymers and P15 peptides indeed enhances osteoblast mineralization.

CONCLUSIONS

In summary, we have successfully modified the Ti surface by grafting polymer via SI-ATRP. With the attachment of P15 peptide to the polymer via oxime chemistry, our results showed the improved cell adhesion of osteoblasts to the Ti surface. In addition, the mineralization assay showed the higher calcium deposition by osteoblasts seeded on the polymer-modified Ti surface discs. Although surface-modified Ti displays superior *in vitro* osteoconductivity compared to that of plain Ti, further *in vivo* studies are needed to fully demonstrate the efficacy of our surface modification technique for improved osseointegration. In conclusion, we developed a novel process to dramatically enhance the potential osteoconductive nature

of Ti surface, which may provide better results for osseointegration of orthopedic and dental implants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

Modification of Ti Surfaces by SI-ATRP and P15 Attachment by Oxime Chemistry

Figure 1. XPS analysis of Ti disc surfaces with anchored BiBADA; full view (a) and Ti region zoom in (b). Ti disc surfaces with grafted polymer (pDMAEMA-*co*-pAEMA); full view (c) and C region zoom in (d).

Figure 2.

Effects of the surface polymer-modified titanium surface (Ti) with P15 peptides on cell attachment. (a) The cell viability at 6 and 48 h were measured by alamarBlue. The polymer-modified Ti surface without P15 peptides (Ti-polymer) did not promote cell viability; $n = 2$. (b) The polymer-modified Ti surface with P15 peptides (Ti-polymer + P15) showed an increase in cell viability at 6 h compared to those of the plain Ti and the P15-adsorbed plain Ti surface (Plain Ti + P15); $n = 4$. (c) Fluorescent phalloidin staining of actin at 24 h displayed more cells attached to the polymer-modified Ti surface with P15 peptides. Osteoblasts were stained for phalloidin (green) and nuclei were counterstained with DAPI (blue); $n = 4$. (d) Quantitative analysis showed an increase in number of cells attached to the polymer-modified Ti surface with P15 peptides; $n = 4$. Values represent the mean $\pm$ SD. **, $p < 0.01$; one-way ANOVA using Tukey's test. Scale bar = 100 μm .

Figure 3.

Effects of surface polymer modification with P15 peptides on osteoblast mineralization. (a) Osteoblasts were incubated with osteogenic differentiation media for 21 days. Alizarin red staining was performed to visualize calcium deposition in osteoblasts. (b) The polymer-modified Ti surface with P15 peptides (Ti-polymer + P15) resulted in an increase in areas of calcium nodules in osteoblasts. (c) Alizarin red was eluted and the mineral content was measured at OD570 nm. The polymer-modified Ti surface with P15 peptide (Ti-polymer + P15) showed an increase in mineral content in culture. $n = 4$. Values represent the mean $\pm$ SD. ****, $p < 0.0001$; ***, $p < 0.001$. One-way ANOVA using Tukey's test.