

# Installing Multifunctionality on Titanium with RGD-Decorated Polyurethane-Polyurea Roxithromycin Loaded Nanoparticles: Toward New Osseointegrative Therapies

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Metallic biomaterials such as titanium (Ti) and its alloys have long been used for applications in bone fixation and regeneration owing to their excellent biocompatibility and mechanical properties.<sup>[1]</sup> However, their use is not exempt from limitations, which still today may compromise the long-term performance of these materials. In the first place, the high degree of “bioinertness” displayed by such metals may, in some clinical scenarios, translate into poor rates of osseointegration (i.e., insufficient implant-bone interactions) and inadequate mechanical fixation.<sup>[1]</sup> Moreover, bacterial infection and subsequent inflammation (i.e., peri-implantitis) has been described as another major

cause of implant failure.<sup>[2]</sup> To address these drawbacks surface biofunctionalization with bioactive motifs has extensively been investigated.<sup>[1,3]</sup> Thus, cell adhesive peptides and proteins from the extracellular matrix (ECM) have been immobilized aiming at increasing the material's capacity to improve the adhesion of osteoblast cells.<sup>[4]</sup> Alternatively, antimicrobial coatings including antifouling polymers, drug releasing matrices or bactericidal peptide sequences, have also been developed.<sup>[5]</sup>

As evidenced by recent research and debate in the literature, it is expected that the combination of distinct biological functions on the surface may improve the biological performance of classical biomaterials.<sup>[6]</sup> However, the majority of current strategies only focus on either improving cell adhesion or reducing bacterial adherence. Thus, the aim of the present study was to exploit the novel concept of multifunctionality by simultaneously installing cell adhesive and antimicrobial properties on the surface of Ti.

Herein, we present a new class of shell-stratified amphiphilic polyurethane-polyurea nanoparticles (PUUa NPs, **Figure 1A**).<sup>[7]</sup> These innovative nanopolymeric systems display the following unique features:

- (i) NPs shell decorated with an  $\alpha\beta3$ -selective cyclic RGD peptide, which provides cell specificity.
- (ii) High capacity to encapsulate hydrophobic drugs in the NPs oily core.

The RGD peptide motif, an integrin-binding sequence present in natural ECM proteins, such as fibronectin and vitronectin,<sup>[8]</sup> is a common cell adhesive motif used to enhance biointegrative effects on biomaterials.<sup>[9]</sup> In particular, the cyclic RGD peptide cRGDfK, designed and developed by Kessler and co-workers,<sup>[10]</sup> has gained increasing interest over the last years due to its much higher specificity toward integrins  $\alpha\beta3$  and  $\alpha\beta5$ , which are widely expressed by osteoblasts and bone precursors, and capacity to effectively promote osseointegrative events.<sup>[11]</sup> Moreover, encapsulation of an antibiotic drug in the PUUa NPs would tackle another recurrent problem: bacterial infection associated with orthopedic and dental implants surgery. In this regard, the drug roxithromycin (Rx) was considered a promising candidate due to its potent antibacterial and anti-inflammatory effects.<sup>[12]</sup> Moreover, the lipophilicity of this drug (log P 2.9) ensured its effective encapsulation into PUUa NPs via hydrophobic interactions.<sup>[13]</sup> Thus, PUUa NPs represent an unprecedented dual approach to improve the osseointegration of implant materials: accelerated osteoblast adhesion and inhibition of bacterial infection (**Figure 1B**). To functionalize Ti materials, in this work we will focus on a novel approach we

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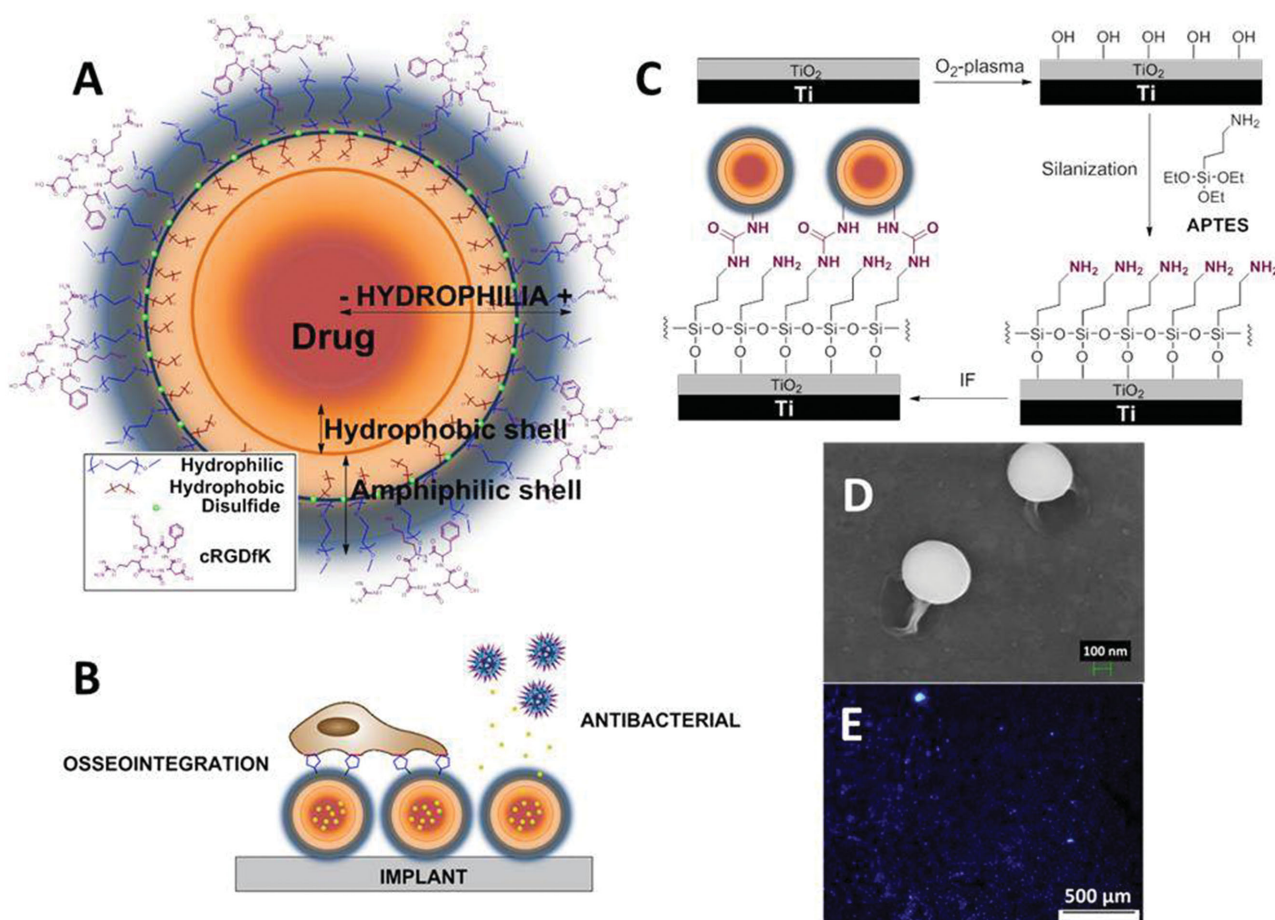
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DOI: 10.1002/adhm.201500245





**Figure 1.** Installing multifunctionality on Ti. A) Schematic representation of the stratified structure of PUUA NPs. B) A dual biological activity on Ti implants. C) Methodology to immobilize PUUA NPs on Ti by means of interfacial functionalization. D) Morphology of EDA-crosslinked PUUA NPs by scanning electron microscopy. E) Ti surfaces coated with fluorescently labeled EDA-crosslinked PUUA NPs.

described as interfacial functionalization (IF), where PUUA NPs bearing isocyanate reactivity and Ti surfaces containing superficial amino groups react to create highly stable urea bonds in the metal-nanocoating interface (Figure 1C). Significantly, one of the major advantages of this technique relies on the efficient and well established polyurethane and polyurea chemistry in the field of industrial coatings and biomedical devices.<sup>[14]</sup> Another key point of this methodology with respect to other approaches is the wide range of molecules that can be either hydrophobically encapsulated or covalently conjugated via quantitative urethane and/or urea bonds in the polymer backbone.

The production of PUUA NPs was based on the synthesis and self-assembly of a reactive amphiphilic prepolymer designated as Amphil (Figure S1, Supporting Information). This prepolymer bears two terminal isocyanate groups and multiple pendant hydrophilic and hydrophobic chains, which represent key structural features to mediate the stratification of the PUUA shell in the oil–water interface. Given the fact that the hydrophilic dangling chains of Amphil self-orient toward the water phase, while hydrophobic chains orient into the oily core, an increasing gradient of hydrophilicity is created from the hydrophobic core to the outer shell. This process stabilizes the structure of PUUA NPs in water and encapsulates the hydrophobic

shell with the drug in the inner core. Hence, such emulsification process yields a hydrophobically stratified PUUA NP with high encapsulation efficiency and capacity (see the Supporting Information for details).

Emulsification in water of the reactive prepolymers was followed by a precrosslinking step to fix the amphiphilic structure of the PUUA NPs. To this end, a diamino molecule was added to crosslink 50% of the total amount of free isocyanate functional groups of the shell (Figure S3, Supporting Information). This methodology ensured that PUUA NPs still contained accessible reactive isocyanate groups for further functionalization.

As schematized in Figure 1C, Ti surfaces were activated by oxygen plasma treatment followed by amino-silanization with (3-aminopropyl)triethoxysilane (APTES) to obtain an accessible monolayer of amino groups. Next, amino-activated Ti disks were immersed in reactive PUUA NPs and left to react during either 1 h or overnight (ON) both at 5 °C to prevent reaction of isocyanate groups with water. After such time, the remaining unreacted isocyanate groups in the NP shell were totally crosslinked adding the 100% of the crosslinking diamine.

To characterize the presence and distribution of PUUA NPs on Ti as well as to optimize the process of surface biofunctionalization, the fluorophore Clear Blue DFSB-C0 was encapsulated

into the PUUA NPs and the fluorescence intensity on the NP-coated surfaces quantified by microscopy. Two crosslinking diamines of different hydrophilicity were used, L-lysine (Lys) or ethylenediamine (EDA). Moreover, two surface immobilization times were studied, 1 h and ON. As shown in Figure S4, Supporting Information, statistically significant higher densities of PUUA NPs were achieved at longer incubation times and when using EDA as crosslinker. Based on these data, the use of EDA and 1 h of incubation seemed to be optimal conditions for surface biofunctionalization.

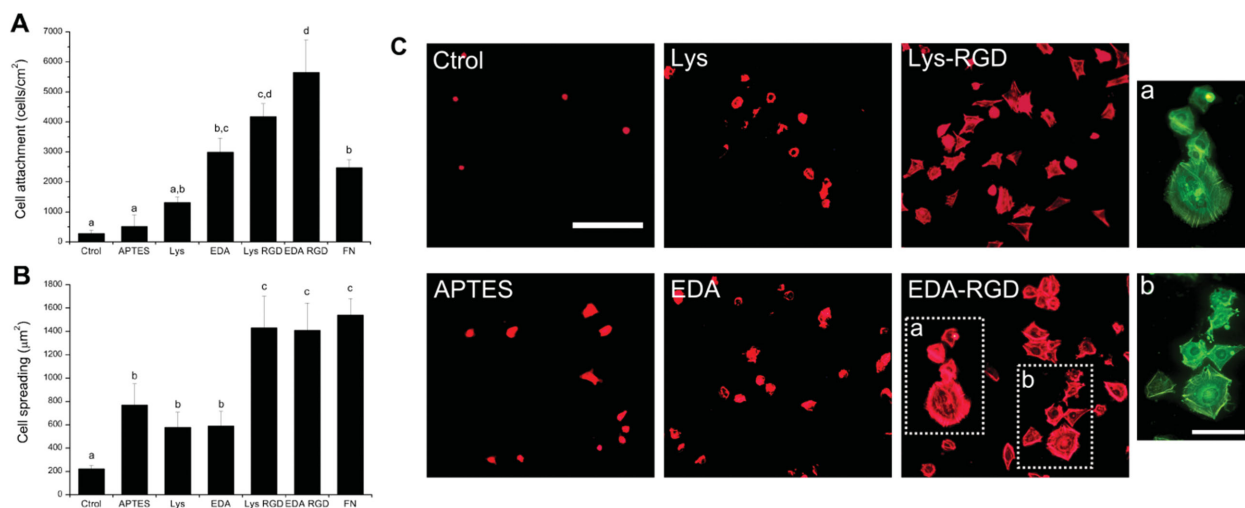
Fluorescence analysis also revealed a very homogenous distribution of the PUUA NPs under all conditions (Figure 1E and Figure S5, Supporting Information). Moreover, scanning electron microscopy corroborated the presence of the PUUA NPs, which showed a well-defined spherical shape and suggested the formation of “root-like” polymeric linkages with the metallic surface (Figure 1D and Figure S6, Supporting Information).

The stability of the coatings was also analyzed with PUUA NPs that contained the fluorophore cadaverine-Oregon Green 488 covalently anchored via urea bonds. These NPs were immobilized on Ti surfaces and their stability analyzed after one and two weeks of incubation in phosphate-buffered saline (PBS) under orbital agitation. The binding of PUUA NPs onto Ti was proven stable under these conditions (Figure S7, Supporting Information).

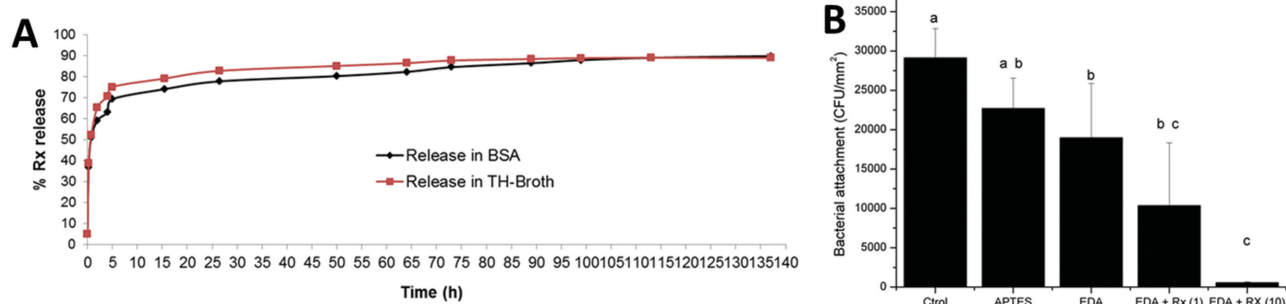
As previously introduced, PUUA NPs were decorated with the integrin selective cRGDfK peptide to convey osseointegrative properties to the surfaces. For such purpose, the free primary amine of the Lys residue of cRGDfK was the cornerstone of our experimental design because represented the functionalization point between the polymeric shell and the peptide. Thus, the hydrophilic polyisocyanate linker Bayhydur 3100 (B3100)<sup>[15]</sup> was bounded to cRGDfK without affecting its bioactivity and capacity to mediate the specific interaction with  $\alpha\beta3$  integrin receptors<sup>[10b]</sup> (Figure S1B, Supporting Information).

As monitored by MALDI-TOF MS and HPLC techniques (Figure S10 and Table S2, Supporting Information), B3100 reacted in a quantitative manner via urea linkage with cRGDfK peptide in PBS. At this point, the resulting hydrophilic B3100-cRGDfK reactive conjugate was added to the prepolymer mixture, homogenized and coemulsified leading to PUUA NPs encompassing the cRGDfK functionality in the outer shell (Figure S2, Supporting Information).

To determine the capacity of these functionalized biomaterials to trigger osteoblast-like cell adhesion, PUUA NPs-coated materials, and their controls, were incubated with sarcoma osteogenic (Saos-2) cells for 4 h and the number and area of adherent cells analyzed (Figure 2). Noteworthy, the presence of RGD-decorated PUUA NPs (Lys RGD or EDA RGD) yielded an outstanding increase in both cell attachment and spreading compared to uncoated (Ctrl) or aminosilanized (APTES) Ti ( $p < 0.05$ ) (Figure 2A,B). In particular, cells seeded on surfaces coated with RGD-containing NPs developed clear actin filaments, and a well-defined cytoskeleton with some nascent focal adhesions (see Figure 2C). PUUA NPs not expressing the RGD motif (Lys or EDA) also enhanced cell binding compared to plain Ti, however these values were statistically lower ( $p < 0.05$ ) than those achieved for RGD-decorated NPs. Moreover, PUUA NPs without the RGD motif failed to stimulate an adequate cell spreading and cytoskeleton (Figure 2), thus indicating that integrin binding and activation is required to successfully support cell adhesion.<sup>[16]</sup> Interestingly, the highest efficiency in cell adhesion was observed when EDA was used as crosslinking diamine, which is in agreement with previous surface characterization data (see Figure S4, Supporting Information). To demonstrate the long-term biological functionality of these coatings, cell proliferation studies were also performed for one and two weeks of incubation (Figure S11, Supporting Information). Of note, surfaces biofunctionalized with EDA RGD supported and promoted the growth of Saos-2 cells with



**Figure 2.** Adhesion of Saos-2 cells on biofunctionalized Ti surfaces after 4 h of incubation in serum free medium. A) Cell attachment (cells cm<sup>-2</sup>). B) Cell spreading (averaged cell area, μm<sup>2</sup>). Cell numbers and spreading were analyzed by immunostaining and fluorescence microscopy. Distinct letters denote statistically significant differences ( $p < 0.05$ ) between groups. C) Visualization of actin filaments with phalloidin-rhodamine staining (scale bars: 200 μm). Visualization of vinculin on Ti surfaces functionalized with EDA-RGD was done with mouse anti-vinculin and anti-mouse Alexa 488 at a higher magnification (images a and b, scale bar: 100 μm).



**Figure 3.** A) Cumulative release profiles of Rx in Todd-Hewitt (TH) broth (red) and in BSA-liposomes (black). B) Bacterial adhesion of *S. sanguinis* on biofunctionalized Ti surfaces after 4 h of incubation in TH broth. Distinct letters denote statistically significant differences ( $p < 0.05$ ) between groups.

proliferation rates statistically comparable to those obtained with the ECM protein fibronectin.

Incorporation of antibacterial properties to the Ti surface was the final step of our studies. In this regard, the antibiotic drug Rx was selected and encapsulated in our system. The encapsulation efficiency (EE) and drug loading (DL) of Rx into PUUA NPs was of  $95\% \pm 1\%$  and  $9.5\% \pm 0.1\%$ , respectively, as proved by HPLC analysis. Moreover, its in vitro drug release was monitored by mixing Rx-loaded PUUA NPs with either bacterial growth medium or a protein and lipid-rich buffer, which aims to mimic physiological conditions (Figure 3A).

Interestingly, crosslinked PUUA NPs released 60%–70% of the encapsulated drug during the first 4–6 h of incubation. Such initial burst release would respond to the elevated risk of bacterial infection described for implant materials during the 6 h post-implantation period,<sup>[17]</sup> followed by a prolonged and sustained antibacterial effect.

Thus, as a final proof of concept study, Ti surfaces were functionalized with PUUA NPs loaded with Rx, and bacterial adhesion to these surfaces investigated after 4 h of incubation. *Streptococcus sanguinis* was chosen as model bacteria because this oral strain is commonly involved in peri-implantitis and other implant-related pathologies.<sup>[18]</sup> The susceptibility of this bacterial strain to Rx was demonstrated in preliminary assays (Figures S12 and S13, Supporting Information). As illustrated in Figure 3B, PUUA NPs containing Rx were able to strongly suppress *S. sanguinis* adhesion in a concentration-dependent manner. The efficacy of our coating system after the initial burst release of the drug was also investigated. To this end, biofunctionalized samples were first incubated in TH broth for 20 h (i.e., a time point where more than the 70% of the drug is released, see Figure 3A) and bacterial adhesion checked afterward. Noteworthy, biofunctionalized surfaces were still able to significantly inhibit bacterial adhesion on Ti substrates (inhibition rate of 76%, see Figure S14, Supporting Information). Therefore, PUUA NPs are not only effective in preventing initial bacterial colonization but also reducing the risk of repetitive or late bacterial infection. Furthermore, the presence of Rx did not affect the positive effects previously observed on Saos-2 cell adhesion (Figure S15, Supporting Information), thereby proving the feasibility of our strategy to confer multifunctionality to Ti-based materials.

To conclude, we have described a novel methodology to produce PUUA NPs and their immobilization on Ti surfaces. These

systems combine outstanding cell-adhesive and antibacterial properties and thus represent excellent candidates to install multifunctionality on metallic surfaces in order to develop new generation biomaterials for bone regeneration.

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

## Acknowledgements

The authors thank the Spanish Government for financial support through Project No. MAT2012–30706, cofunded by the European Union through European Regional Development Funds, and the Government of Catalonia (SGR2009 1039). C.M.-M. thanks the support of the Secretary for Universities and Research of the Ministry of Economy and Knowledge of the Government of Catalonia (2011-BP-B-00042) and the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme (FP7-PEOPLE-2012-CIG, REA Grant Agreement No. 321985). P.R., J.R., and F.A. thank the Spanish Government (MICINN) for financial support into the INNPACTO Project No. IPT-090000–2010–1 (Polysfera).

Received: April 7, 2015

Revised: July 1, 2015

Published online: August 14, 2015

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