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Cell attachment and spreading on metal implant materials

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Abstract

Strong bonding and coverage of organic siloxanes or phosphonates on both Ti and Ti–6Al–4V can be obtained using either of two novel metal–organic interfaces. Surface coverages are considerably higher than can be effected by direct silanization of the native oxide surfaces. Furthermore, these interfaces can be used to attach the fibronectin attachment peptide arginine–glycine–aspartic acid (RGD) to Ti–6Al–4V. Essentially, no osteoblast adhesion occurred on unmodified Ti–6Al–4V or on alkyl, ω-hydroxy, ω-carboxylic acid, or ω-carboxylate-terminated alkylphosphonate-modified surfaces. Adhesion and spreading of osteoblasts on RGD-modified surfaces was quite substantial.

Keywords: Titanium; Osteoblast; RGD

1. Introduction

Titanium and its alloys, notably Ti–6Al–4V, have high mechanical strength and are resistant to chemical attack. Thus, they enjoy widespread use as surgical implants that are often in contact with bone [1–5]. Paradoxically, this resistance to chemical attack can be problematic: osteointegration [6] is weakened by the absence of strong chemical bonding at the bone–implant interface. Consequently, implants can fail under shear stress, requiring revision surgery [1,2].

Bonding the fibronectin attachment peptide arginine–glycine–aspartic acid (RGD) [7–11] to a surface through an organic tether (Fig. 1) might enhance its osteoconductivity by providing sites for osteoblast attachment and spreading [7,12–14], but conventional methods for such surface peptide attachment to Ti alloys can be problematic. Silanization, which has long been considered as the benchmark method for attaching organics to the native oxide coatings of Ti or Ti–6Al–4V, is limited by the low surface hydroxyl (OH) group content of the Ti native oxide surface [15]; only about 15% of surface O is due to OH, as determined by X-ray photoelectron spectroscopic (XPS) analysis. Thus, low yields of direct surface silanization bonding can result, and cross-linked siloxanes can be the major reaction products [8,9,11]. Unfortunately, surface-bound and cross-linked siloxanes can be hydrolytically unstable, and this instability can limit the amounts of key organics that can survive the aqueous reaction conditions required for surface peptide attachment [8].

We have described two methods for surface modification of Ti and Ti–6Al–4V [16,17]. Both give strongly surface-bound metal–organic interfaces, and neither is limited by the native oxide surface OH group content. These interfaces can be used to accomplish strong bonding of organics to Ti–6Al–4V, and they enable surface coverages by critical organics far greater than can be achieved by direct silanization methods. Neither type of interface is cytotoxic; indeed, their RGD derivatives promote considerable attachment, spreading, and proliferation of human fetal osteoblasts (HFOB) on Ti–6Al–4V. RGD-modified surface-bound phosphonate films are stable under physiological conditions, and cell proliferation on them is extensive.

2. Results and discussion

Our first method for surface modification of Ti–6Al–4V involves increasing its surface OH group content to enhance direct surface silanization. We found [16] that heating a thin film of phosphoric acid on the surface of Ti–6Al–4V gave a strongly adhered and somewhat rough

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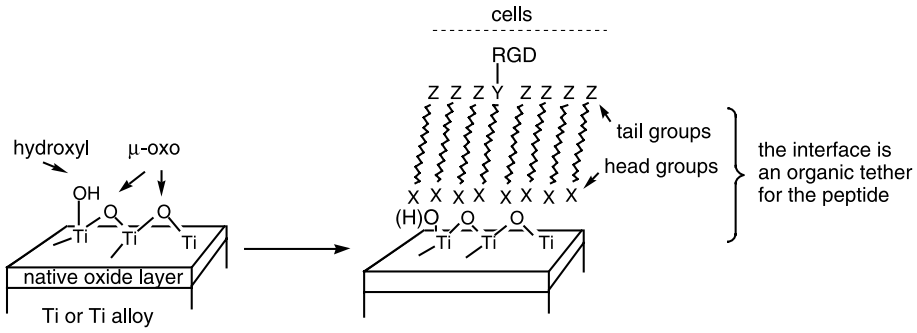

Fig. 1. An organic "tether" can be used to bind RGD to the Ti (or alloy) surface through an appropriate interface.

layer of $Ti(H_2PO_4)_3$ ("TiP") [18]. TiP has a layered structure, with OH groups above and below these layers in a titanium phosphate polymer. Thus, forming TiP on the alloy effectively converts a surface of low OH group content into one of high OH content. TiP reacts with aminopropyl(triethoxy)silane ("APTES", **1**) to give the corresponding surface-bound aminosiloxane (**2**). As expected by the increased OH content of TiP, surface coverage of TiP by **2** was much higher than could be accomplished on the native oxide, as measured by direct comparison of IR spectral intensities for derivatization of the two surfaces [16]. Following a known route [8], surface-bound **2** reacted with the typical derivatization reagent **3** to give an adduct, **4**. Comparative IR analysis showed at least an order of magnitude increase in surface coverage on TiP compared to that which was reported for the native oxide of Ti [8]. Treating surface-bound **4** with an aqueous solution of the cysteine derivative of RGD (RGDC) yielded the surface-bound peptide, **5** (Fig. 2).

Our second method for surface modification of Ti–6Al–4V involves our observation that self-assembly of organophosphonic acids occurs on this alloy surface; heating gives a strongly bound, ordered film of the alkylphosphonate [17] for which XPS analysis indicates bidentate surface coordination of phosphonate "head" groups [19]. Phosphonate self-assembly/heating also enables film formation for ω-substituted alkylphosphonates. For example, ω-hydroxy- and ω-carboxy-substituted [17,20] alkylphosphonic acid films were prepared in this way. For the carboxylic acid-derivatized material (**9**), IR shows alkyl chain order and also strongly hydrogen-bonded ω-carboxylic acid groups ($\nu_{CO} = 1694\ cm^{-1}$). Washing **9** with aqueous buffer (pH 10) caused the carbonyl peak to shift to 1575 cm^{-1}, indicating deprotonation to the ω-carboxylate-terminated film (**10**). All of these films resisted removal (as measured by maintenance of IR peak intensities) by solvent washing with sonication, or by mechanical peeling with tape. Films of **6** were used to attach RGDC to Ti–6Al–4V via maleimide adduct **7**, to give **8** (Fig. 3).

That phosphonic acid group coverage on the native oxide surface of Ti is not limited by surface OH content was demonstrated both by AFM imaging techniques and quartz

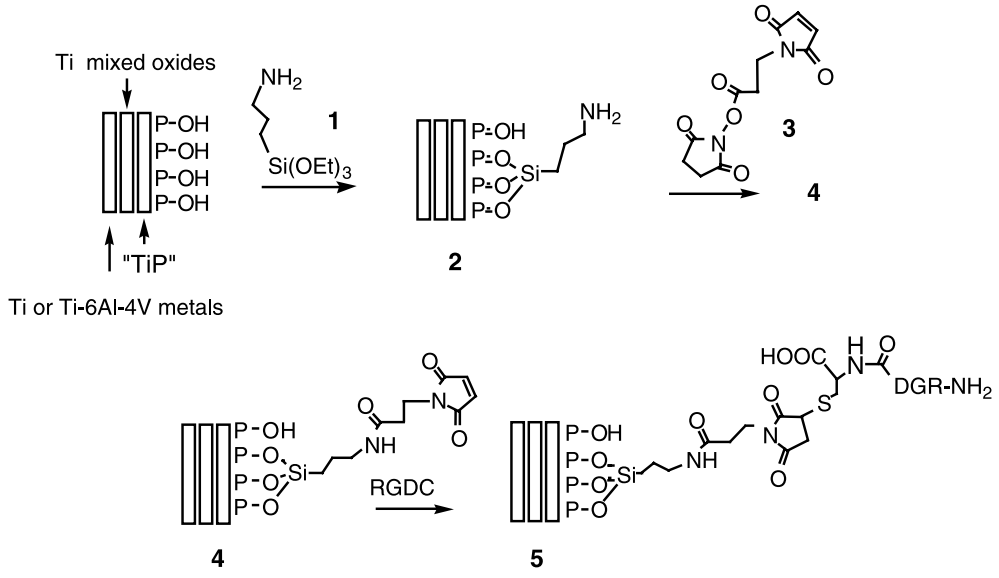

Fig. 2. Silanization of the TiP-modified surface is enhanced by its high OH content; elaboration to a surface-bound RGD product occurs with high surface coverage.

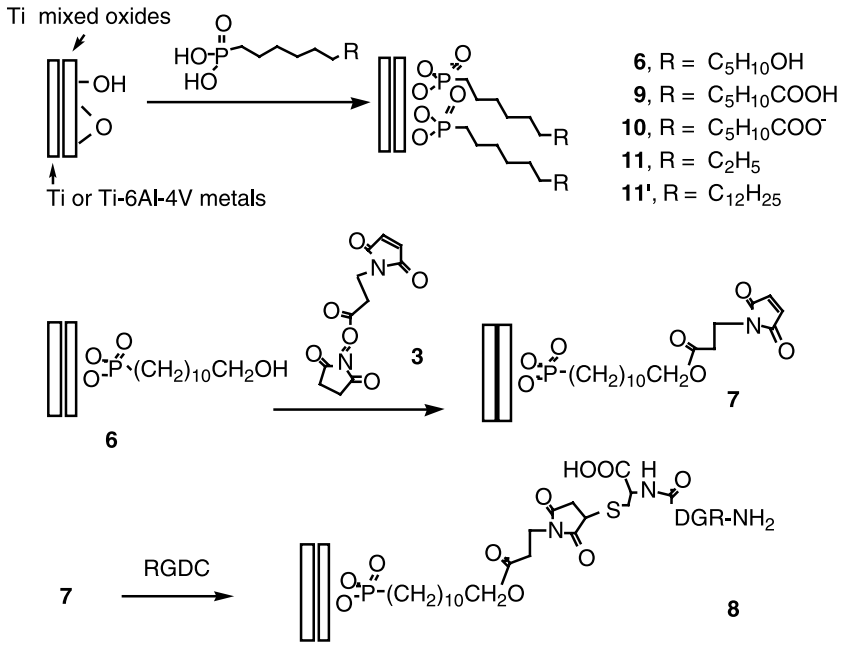

Fig. 3. Phosphonic acid films formed by self-assembly and baking on Ti–6Al–4V.

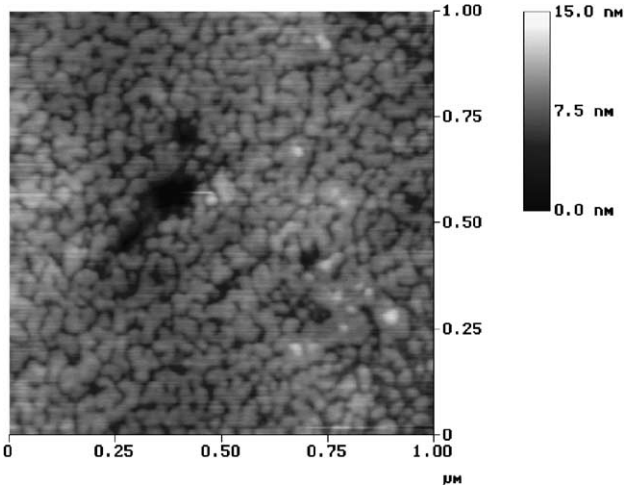

Fig. 4. AFM micrograph analysis of the polished Ti surface after reaction with octadecylphosphonic acid.

crystal microbalance (QCM) measurements. The QCM technique [21,22] allows direct gravimetric determination of mass changes on an electrode which is deposited on a piezoelectric quartz crystal. To ensure that monolayer coverage (at most) occurred on QCM crystals modified with Ti electrodes, films of **6** were subjected to copious rinsing (followed by evacuation) until a constant crystal frequency was established. QCM measurements were also made for the simple aliphatic substrate **11** in which multilayer formation through head/tail group interactions would be obviated. Direct gravimetric analyses measured surface loadings for both **6** and **11** on Ti to be on the order of 2 nmol/cm^2, slightly higher than that measured for alkanethiols on gold [23], and nearly 100 times greater than that reported for siloxane **2** (of comparable head group size) on the native oxide surface of Ti [8].

AFM imaging of a film of the octadecylphosphonate analog **11′** (Dimension 3000, Digital Instruments, operated in the "soft" tapping mode) showed comprehensive surface

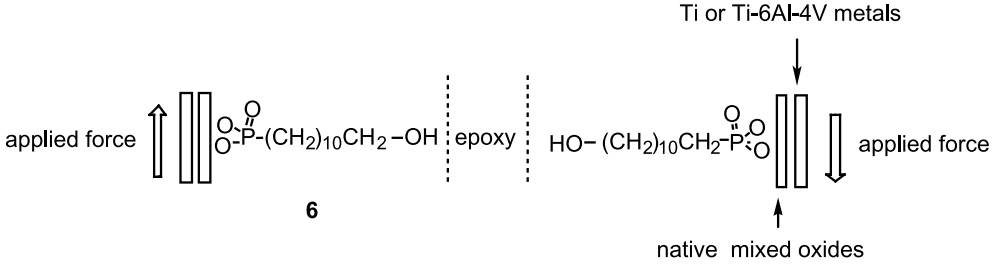

Fig. 5. Interface strength can be measured by a simple shear test involving cementing together two disks of phosphonate film-coated Ti–6Al–4V using a strong epoxy.

coverage on the Ti surface after six cycles of exposure to the phosphonic acid, baking, and rinsing with THF [17] (Fig. 4). AFM of the chain-ordered film ($\nu_{asymmCH_2} = 2914\ cm^{-1}$) showed domains of the alkylphosphonate ≈ 50 nm in diameter, of similar height (≈ 2.2 nm), and consistent with monolayer formation on the surface. Measured film height data compared with a self-assembled monolayer of the same phosphonic acid on mica [24] gave an alkyl chain tilt angle of ≈ 33° for our system.

Quantitative measure of the shear strength of the **6**/Ti–6Al–4V interface was accomplished using films grown on "lozenges" (2.7 × 1.2 × 0.3 mm) of the alloy. The lozenges were polished before films of **6** were deposited on them. Two lozenges were then glued together using epoxy cement (Cytec FM 73), and the strength of the interface was compared with that formed by epoxy bonding of native Ti–6Al–4V (Fig. 5). Since it is likely that the epoxy reacts with surface OH groups, it could be expected that films of **6**

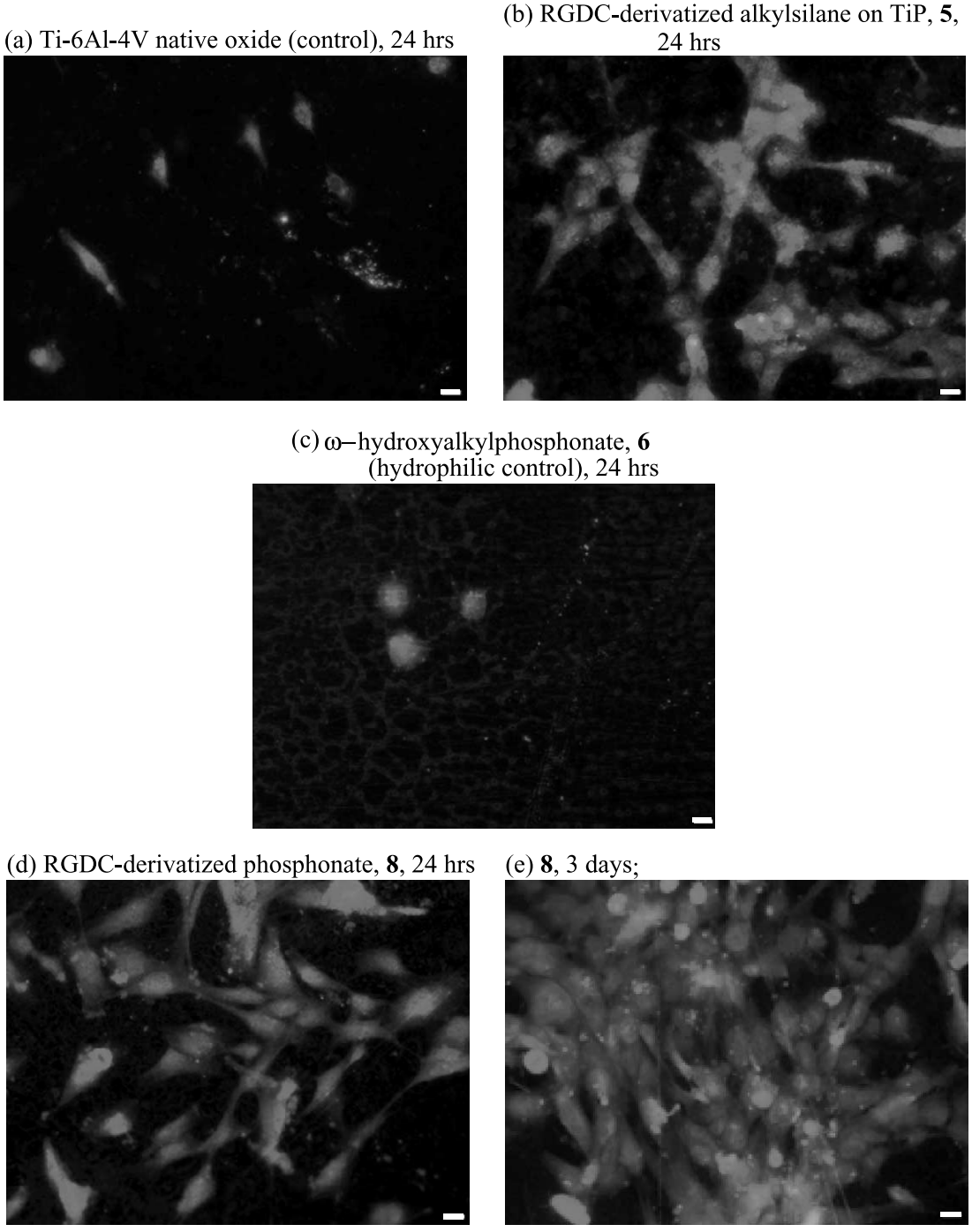

Fig. 6. Human fetal osteoblasts on (a) clean Ti (control), 24 h; (b) RGDC-derivatized alkylsilane on TiP, **5**, 24 h; (c) ω-hydroxyalkylphosphonate, **6**, 24 h; (d) RGDC-derivatized phosphonate, **8**, 24 h; and (e) RGDC-derivatized phosphonate, **8**, 3 days. Size marker bars 12 μm.

would act as adhesion "amplifiers" by supplying more surface OH as sites for epoxy bonding than exist on the native oxide coating. Compressional shear testing showed that the **6**/Ti–6Al–4V interface withstands ≥40 MPa, which far exceeds the mandated value of 20 MPa for plasma-sprayed coatings [25].

To test the stability of our interfaces under physiological conditions, demonstrate their non-cytotoxicity, and determine their efficacy for cell surface adhesion and spreading, human fetal osteoblasts (HFOB 1.19; ATTC) were incubated with unmodified or variously surface-modified Ti–6Al–4V samples for up to 3 days [26]. Cells were labeled with 10 μM fluorescent marker Cell Tracker Orange (Molecular Probes, OR) for 30 min at 34 °C. After this time, the medium was removed and replaced with fresh medium and serum for an additional 30 min at 34 °C. Essentially, no osteoblast adhesion occurred on the unmodified alloy surface or on alkyl (**11′**), ω-hydroxy (**6**), ω-carboxylic acid (**9**), or ω-carboxylate (**10**) group-terminated alkylphosphonate-modified surfaces. These observations are similar to those made for variously modified alkanethiols on gold [27]. Significantly, adhesion and spreading of osteoblasts on RGD-modified surface **8** was quite substantial and was even more striking on TiP-modified surface **5**. It may be that the presence of exposed phosphate groups of TiP in conjunction with chemically bonded RGD creates an especially attractive mixed function surface for these cells. Unfortunately, the inherent hydrolytic instability of surface siloxanes limits the long-term viability of this interface; after 3 days, loss of surface material was visually apparent. In contrast, not only were adhesion and spreading of the osteoblasts on RGD-modified surface **8** quite substantial after 24 h (Fig. 6d), but also were even more so after 3 days (Fig. 6e). Indeed, cell proliferation continued unabated throughout the test period on this surface.

3. Conclusions

The low reactivity reputed to native oxide coatings of Ti and Ti–6Al–4V and the low surface coverages achieved by direct surface silanization following aqueous treatment can be overcome by using phosphate and phosphonate reagents which can attack these oxide coatings in controllable ways. We believe that formation of our interfaces occurs in a way that is fundamentally different from silanization, which simply consumes surface OH groups. Based on observed high surface coverages, we propose that phosphoric and alkylphosphonic acids can utilize these OH groups as *catalysts* for surface attack, decoupling surface OH group content from surface film formation yields (Fig. 7). Having shown that our phosphonate interfaces bind strongly to Ti and its alloy, that they are stable to physiological conditions, and that they serve as sites to recruit human osteoblasts, we believe that a first important step in creating stable osteoconductive surfaces on Ti and Ti–6Al–4V has been taken.

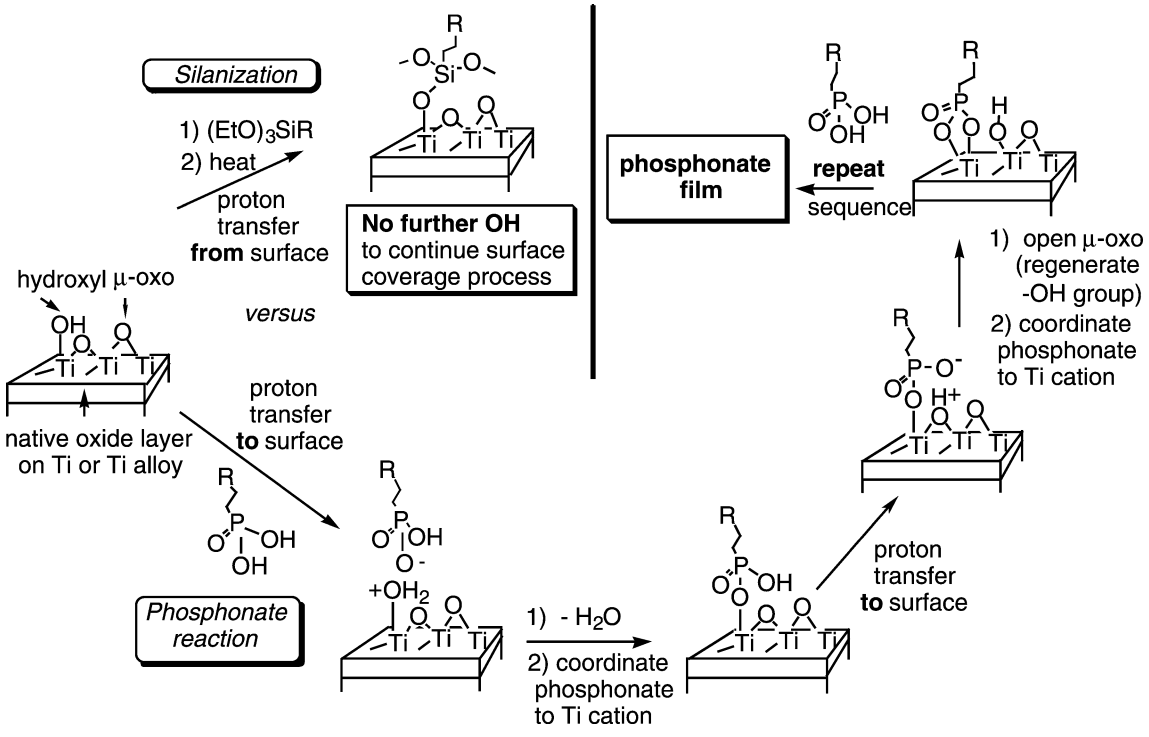

Fig. 7. In contrast to silanization, which consumes surface OH sites, our phosphonate methodology uses OH groups as catalysts for comprehensive surface coverage.

Acknowledgements

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