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Surface Chemistry of Vitamin: Pyridoxal 5′-Phosphate (Vitamin B_6) as a Multifunctional Compound for Surface Functionalization

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Vitamins are non-toxic compounds that perform a variety of biological functions and also available in a large quantity. Other than the usage as food supplements, few attempts have been made to use them as functional materials. In this study, we report that vitamin B6, pyridoxal 5′-phosphate (PLP), is a multi-functional molecule for oxide surface chemistry. PLP-immobilized surfaces exhibit superhydrophilicity and even hemophilicity, enhancing proliferation, migration, and differentiation of mammalian cells. Unlike existing molecules used so far in surface modification, PLP has an intrinsic chemical reactivity toward biomacromolecules due to the presence of the aldehyde group. In fact, RGD peptide is covalently tethered onto PLP surfaces directly in one step without any chemical activation. Furthermore, PLP-functionalized implant device showed rapid bone healing. As vitamin B6 is a FDA approved molecule for human usage, the surface chemistry of vitamin B6 potentially allows a fast route for surface functionalized medical devices into clinic.

1. Introduction

Vitamins are organic compounds that organisms require in limited amounts. They are typically nontoxic, but can display toxicity when megadoses (>500 mg d^{-1}) are ingested. Vitamins are biologically multifunctional and play a variety of important roles in cellular biochemical pathways. For example, vitamin B is in a class of water-soluble vitamins that play important roles in cell metabolism. The vitamins in this class have distinct biochemical functions including DNA production,[1] energy production in the electron transport chain,[2] and roles as coenzymes.[3] Moreover, one of the subclass of vitamin B, B_6 which is also called pyridoxal 5′-phosphate (PLP), is an important cofactor of lysyl oxidase which crosslinks collagen, the major component among extracellular matrices.[4,5] Therefore, vitamin B_6 is critically related to control collagen stiffness. It also has preventive effect on tumorigenesis which appears to be mediated through suppressing cell hyperproliferation,[6] oxidative stress,[7,8] and nitric oxide synthesis.[9]

Although research demonstrating the biological activity of vitamins is widely available, very few attempts have been made to study vitamins as functional biomaterials. One study using vitamin esters as drug nanocarriers demonstrated that amphiphilic micelles can be prepared with d-α-tocopheryl polyethylene glycol succinate[10] and vitamin E succinate.[11] The d-α-tocopheryl polyethylene glycol succinate encapsulates doxorubicin while vitamin E succinate encapsulates paclitaxel, both through hydrophobic interactions. These properties make vitamins potentially useful biomaterials. Also important is that vitamins are registered compounds in pharmacopoeia, which facilitates Food and Drug Administration (FDA) approval and commercialization. Vitamins contain a variety of chemical groups (phosphates, benzenes, phenols, hydroxyl groups, etc.) that can act as chemical moieties for surface anchorage. Vitamins are ideal for surface chemistry applications because 1) most can be obtained in large quantities without requiring time-consuming organic synthesis and 2) it is easy to introduce intrinsic bioactive functions to surfaces that originate from the immobilized vitamins. For these reasons, we hypothesized that vitamins may be useful biomaterials for functionalizing material surfaces. Subsequent commercialization of vitamin-coated devices would be easy and quick providing a fast route to the clinic.

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DOI: 10.1002/adfm.201501471

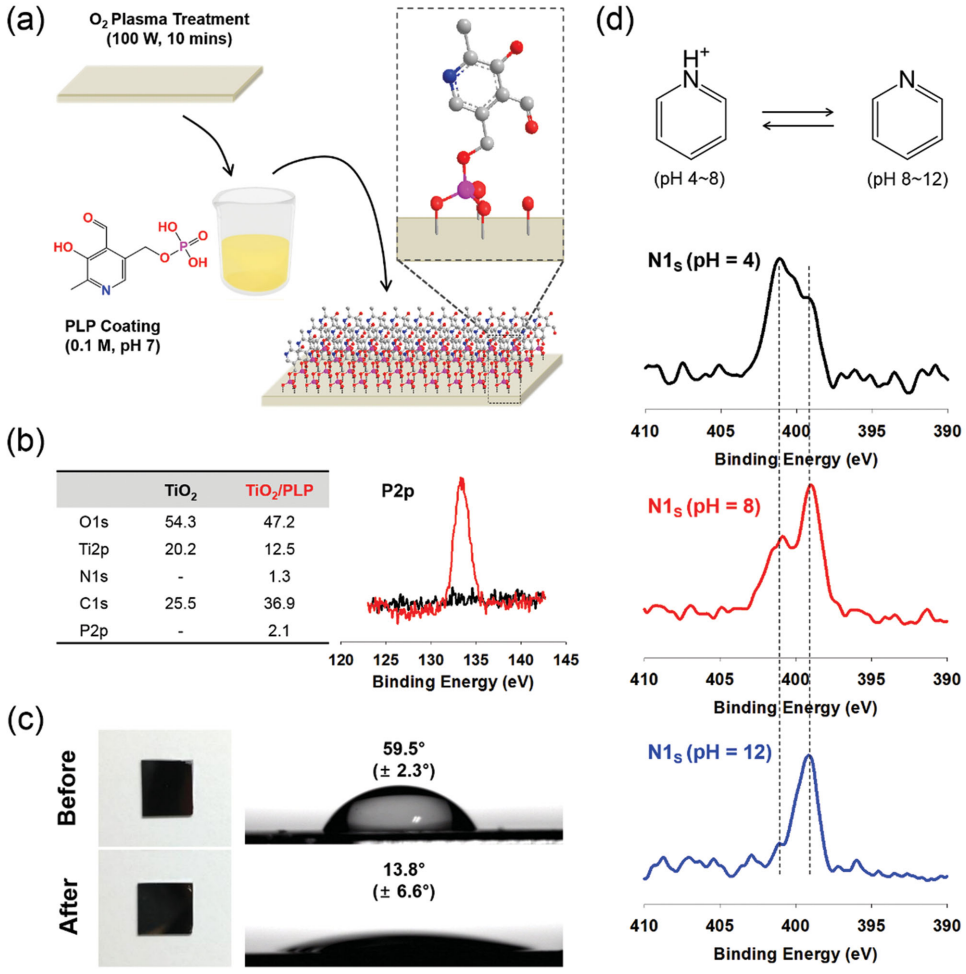

Figure 1. a) The chemical structure of PLP and schematic description of the PLP surface modification on the TiO_2 surfaces is shown. b) The surface atomic compositions of bare TiO_2 and PLP-coated TiO_2 surfaces were quantified by XPS. c) Pictures and static water contact angles of TiO_2 surfaces are shown before (top row) and after PLP coating (bottom row). d) The N1s core level photoelectron peaks are shown as a function of pH value (pH 4 [black], 8 [red], and 12 [blue]) by XPS.

2. Results and Discussion

We selected PLP, also known as vitamin B_6, as a model compound in this study. As shown in **Figure 1**a, PLP consists of phosphonic acid, a pyridine, an aldehyde, and hydroxyl groups. The phosphonic acid of PLP is able to form P-O-metal[12] coordination bonds on the metal oxide surface of titanium oxide (TiO_2). Pyridine substructure is one of the most important heterocycles found in natural products. Nitrogen in the pyridine ring acts as a hydrogen bond acceptor, providing a binding site for biomacromolecules such as tubulin.[13] The PLP aldehyde group reacts with primary amine groups of proteins and peptides via Schiff-base formation,[14] suggesting that additional layers can be formed by covalent interactions with various biological contents. We propose that the small molecule compound, vitamin B_6, provides functionality to material surfaces by facilitating immobilization of bioactive molecules.

To create PLP-functionalized surfaces, clean TiO_2 was treated with oxygen plasma for 10 min and 0.1 M of PLP solution (pH 7) was immediately applied to the surface. Oxygen plasma treatment increases hydrophilicity of the TiO_2 surfaces by enhancing surface contact of the applied PLP solution, which results in the formation of a homogeneous PLP layer (Figure 1a). The presence of a PLP layer was verified using X-ray photoelectron spectrometry (XPS) analysis. The relative composition of each peak included N1s from the pyridinium ring and P2p from phosphonic acid after PLP surface modification (Figure 1b). These compounds were not detected on the control TiO_2 surfaces (Figure 1b). PLP is a pi-electron rich molecule due to the existence of the pyridine ring and it has a methyl group (Rbond;CH_3) connected to the pyridine.Thus, from a structural point of view, PLP is not considered to be a hydrophilic molecule. However, the TiO_2 surfaces functionalized with PLP exhibited a fairly high degree of hydrophilicity displaying 10° static contact angles (Figure 1c) (13.8° ± 6.6°). We therefore hypothesized that an additional factor may contribute to the hydrophilicity of these surfaces: protonation of the pyridine ring, as shown in Figure 1d. Protonated nitrogen in pyridine resulted in formation of a quaternary amine that is known to be a hydrophilic moiety, as demonstrated previously

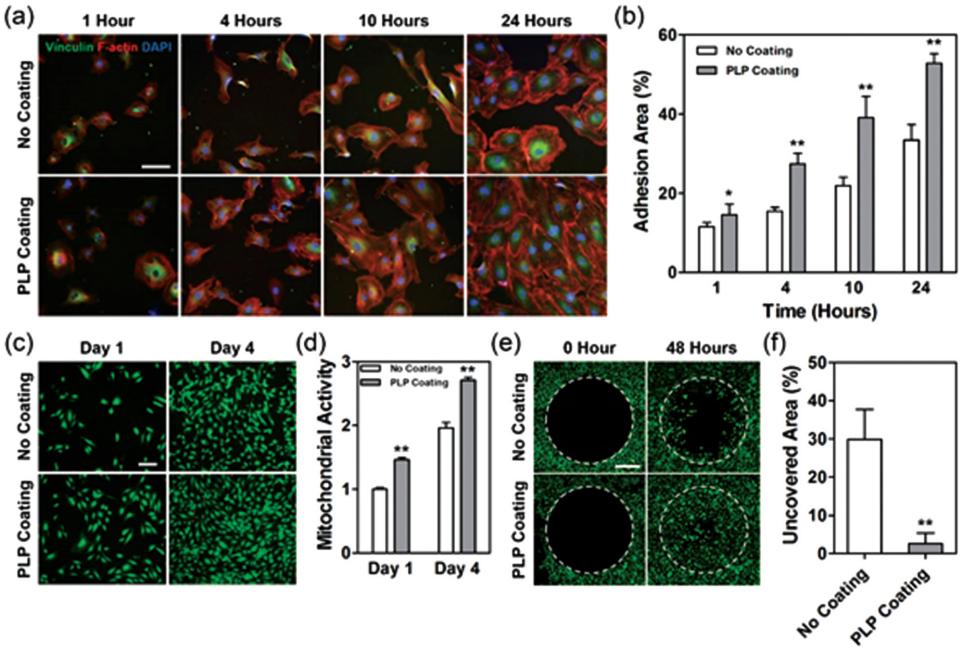

Figure 2. a) Staining of the focal adhesion vinculin and cytoskeleton protein F-actin is shown in HUVECs on TiO_2 surfaces with and without PLP coating 1, 4, 10, and 24 h after cell seeding, scale bar = 50 μm. b) HUVEC adhesion is shown on noncoated (white bars) and PLP-coated surfaces (gray bars) ($n = 6$, $*p < 0.05$ and $**p < 0.01$, compared to the no coating group). c) Live/Dead staining was performed in HUVECs cultured on each surface 1 and 4 d after seeding, scale bar = 200 μm. d) Mitochondrial metabolic activity of HUVECs was measured by MTT assay ($n = 6$, $**p < 0.01$, compared to the no coating group). e) Migration of HUVECs was visualized using Live/Dead staining, and f) the uncovered area was calculated 48 h after seeding ($n = 5$, $**p < 0.01$, compared to the no coating group), scale bar = 500 μm.

in cetrimonium bromide (known as CTAB) in which the quaternary ammonium dissolves the long carbohydrate (C16) chain in water.[15] The presence of this quaternary amine can be proven using the core N1s photoelectron. In general, binding energy of the N1s electron is upshifted when protonated.[16] The quaternary ammonium on PLP at pH 4 has an bond; NH peak at 400 eV (Figure 1d, black), which decreases at pH 8 (Figure 1d, red) and disappears at pH 12 (Figure 1d, blue). In this study, PLP was immobilized onto a TiO_2 surface in deionized water at approximately pH 7. At this pH, a large proportion of quaternary amine is still protonated making the surface hydrophilic. Generally, hydrophilic surfaces promote cellular adhesion via selective adsorption of adhesion-related proteins and subsequent cell-substrate interactions.

Enhancement of cellular adhesion has gained attention for the purpose of developing medical stents with enhanced functionality that can induce rapid endothelialization and integration into native vascular tissues. In-stent restenosis is a serious complication of stent implantation that represents a major challenge to successfully treating atherosclerosis and other vascular diseases.[17] Rapid endothelialization of the implanted stent is a critical step in inhibition of restenosis. Therefore, we first investigated whether PLP coatings on TiO_2 supports enhanced endothelialization. TiO_2 was selected for this study because it is a major metal component used in-stents due to its biocompatibility and blood compatibility.[18] Adhesion of human umbilical vein endothelial cells (HUVECs) grown on PLP-coated TiO_2 surfaces was compared to that of cells grown on noncoated TiO_2 surfaces by staining for the cytoskeletal protein F-actin and the focal adhesion protein vinculin. At early stages in cellular adhesion (1, 4, 10, and 24 h after seeding), HUVECs seeded onto PLP-coated surfaces more rapidly spread out and covered a 1.5-fold larger area than those on noncoated surfaces, indicating that the PLP coating greatly enhanced adhesion of ECs (**Figure 2**a,b). In addition, the PLP coating appeared to support significantly enhanced cellular proliferation. HUVECs cultured on PLP-coated surfaces showed a larger number of viable cells after 1 and 4 d of culture compared to cells grown on noncoated surfaces, confirmed by Live/Dead staining (Figure 2c). Significantly increased mitochondrial metabolic activity of HUVECs cultured on PLP-coated surfaces at day 1 and 4 also indicates that PLP coatings enhance cellular proliferative activity (Figure 2d). Cellular migration is another crucial factor for rapid endothelialization because ECs from surrounding vascular tissues must migrate onto the implanted stents to form endothelium.[19] Surprisingly, HUVECs cultured on PLP-coated surfaces rapidly migrated into the cell-absent region and covered most of the empty space (2.6% ± 2.8% of uncovered area) within 48 h, whereas 29.9% ± 7.8% of the total area was still uncovered on noncoated surfaces (Figure 2e,f).

Furthermore, we tested the effectiveness of PLP coatings on TiO_2 surfaces for promoting adhesion and proliferation of endothelial progenitor cells (EPCs), another important cell type involved in-stent endothelialization. Because EPCs are circulating progenitor cells that home to blood vessels with endothelial damage and differentiate into mature ECs, they are critical for re-endothelialization of injured vessels and rapid endothelialization of implanted vascular grafts or stents.[20] Adhesion and proliferation of human cord blood-derived EPCs was also significantly enhanced on PLP-coated TiO_2 surfaces compared

with noncoated TiO_2 surfaces (Figure S1, Supporting Information), as observed in the HUVEC study. Taken together, PLP coating supports promoted adhesion, proliferation, and migration of ECs and EPCs on TiO_2 surfaces. This suggests that PLP has the potential to improve the functionality of stents for treating atherosclerotic cardiovascular diseases.

The increased hydrophilicity caused by PLP coating could also improve function and biocompatibility of medical implants for bone replacement. Polar and hydrophilic medical implant surfaces typically interact better with electrolyte fluids (blood, bone marrow, and extracellular fluids). These surfaces facilitate osseointegration of implants in bone or dental applications.[21] In this study, we evaluated the potential of PLP coatings to enhance osseointegration of TiO_2-based medical implants. We examined adhesion and proliferation of primary osteoblasts on PLP-coated and noncoated TiO_2 surfaces. At early time points, osteoblasts grown on PLP-coated surfaces exhibited increased focal adhesion expression, enhanced cellular spreading, and coverage of a larger area than cells on noncoated surfaces (**Figure 3**a,b). PLP coating also supported high proliferation of osteoblasts on TiO_2 surfaces (Figure 3c,d). Live/Dead staining images in Figure 3c confirmed enhanced spreading of osteoblasts on PLP-coated surfaces at 1 and 4 d after seeding. PLP-coated surfaces significantly enhanced osteoblast proliferation, nearly twofold higher than noncoated surfaces 4 d after seeding (Figure 3d). Interestingly, adhesion and proliferation of human adipose-derived stem cells (hADSCs) that can differentiate into osteogenic lineage cells was also enhanced by PLP coating on the TiO_2 surfaces (Figure S2, Supporting Information), indicating the versatility of PLP coatings for promoting attachment and proliferation of various cell types with relevance to bone and dental applications.

PLP coatings were also tested on rough TiO_2 surfaces with microscale surface topographical features that mimic surface roughness of bone implants. Previous studies have demonstrated that micro- or nanosized surface topography can accelerate osteoblast adhesion and proliferation.[22] However, TiO_2 surfaces with roughness have low wettability, as shown in Figure 3e, and thus, we hypothesized that PLP-mediated enhanced hydrophilicity of the rough TiO_2 surface could further improve osteoblast adhesion and proliferation. PLP coating converted the rough surfaces from hydrophobic to hydrophilic without disrupting the surface topography, indicated by water contact angle measurements (Figure 3e, inner box) and field emission scanning electron microscope (FE-SEM) analysis (Figure 3e). Osteoblast proliferation was enhanced by PLP coating 1 and 4 d after seeding; however, the difference was not statistically significant (Figure 3f).

As an example of a practical application of PLP coating, we used the coating to improve hemophilicity and, in turn, tissue compatibility of TiO_2 dental implants. In the case of blood-contacting implants, such as dental implants, the hemophilic properties of implants are known to be important for healing processes due to plasma protein adsorption increase on hemophilic implant surfaces.[23] Therefore, we tested the hypothesis that the hydrophilic surface properties of PLP coatings improve hemophilicity of TiO_2 dental implants, which is critical for improved tissue compatibility. The bottom end of the TiO_2 implant fixture with and without PLP functionalization was dipped into blood. Blood adsorption along the

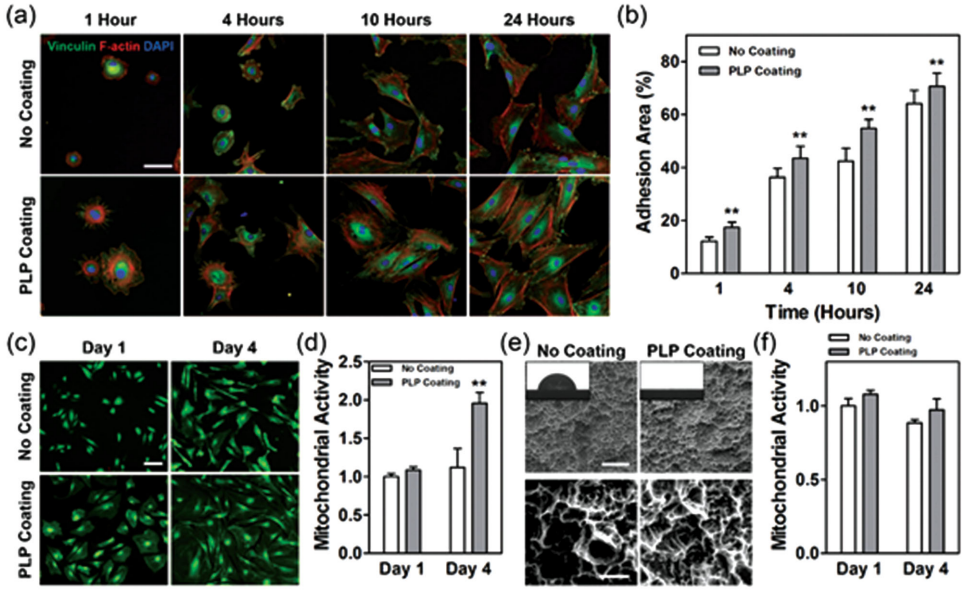

Figure 3. a) Staining of the focal adhesion vinculin and cytoskeleton protein F-actin is shown in primary osteoblasts on TiO_2 surfaces with and without PLP coating 1, 4, 10, and 24 h after cell seeding, scale bar = 50 μm. b) Osteoblast adhesion is shown on noncoated (white bars) and PLP-coated surfaces (gray bars) ($n = 10$, $^{**}p < 0.01$, compared to the no coating group). c) Live/Dead staining was performed in osteoblasts cultured on each surface 1 and 4 d after seeding, scale bar = 200 μm. d) Mitochondrial metabolic activity of osteoblasts was measured by MTT assay ($n = 3$, $^{**}p < 0.01$, compared to the no coating group). e) Surface morphology of the acid-etched rough TiO_2 surface was characterized by FE-SEM before (left) and after (right) PLP coating, upper image scale bar = 50 μm, lower image scale bar = 5 μm. The inset images show static contact angles before PLP coating (left) and after PLP coating (right). f) The mitochondrial metabolic activity of osteoblasts cultured on the rough TiO_2 surfaces was measured by MTT assay ($n = 3$).

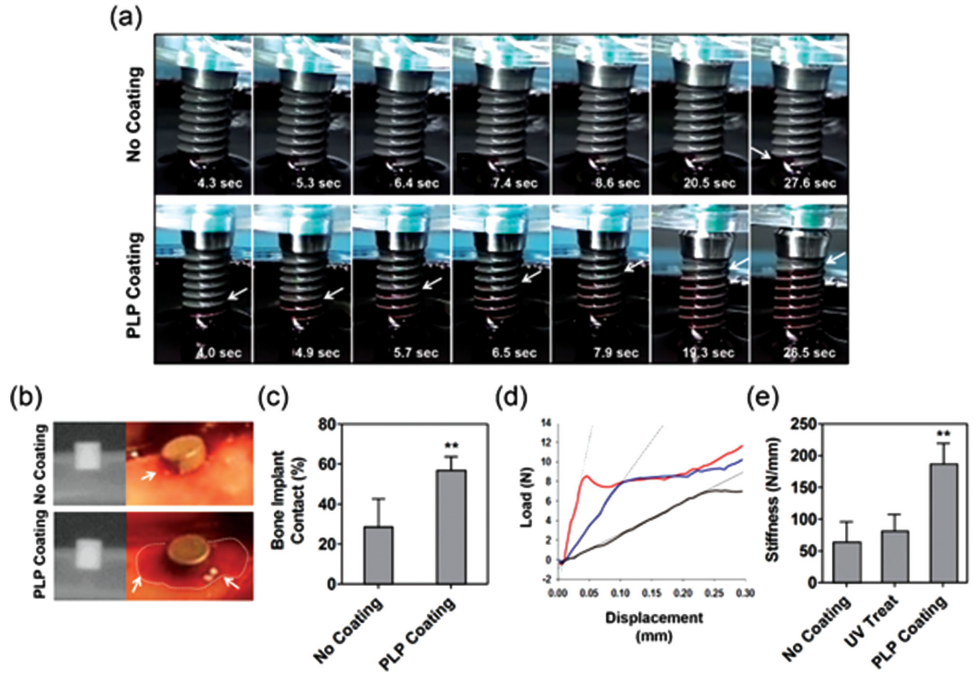

Figure 4. a) Blood adsorption was measured in the noncoated TiO_2 fixtures (top row) and the PLP-coated TiO_2 fixtures (bottom row). b) Micro-CT images of the TiO_2 fixtures 7 d after implantation into a rat femur (left) are shown. The differences in hemophilicity are shown for the noncoated fixtures (upper right) and the PLP-coated ones (lower right). The white dashed line indicates the boundary of blood overflow due to enhanced hemophilicity of the implants by PLP coating. c) The BIC of noncoated and PLP-coated TiO_2 fixtures ($n = 3$, $^{**}p < 0.01$) is shown. d) Representative load–displacement curves were obtained from push-in tests with the noncoated (black), UV-treated (blue), and PLP-treated (red) TiO_2 fixtures. e) The slope value (i.e., stiffness) was obtained from the load–displacement curves ($n = 3$ for each group, $^{**}p < 0.01$ for PLP and no coating; p value for PLP and UV is 0.014).

circumference of the PLP-coated fixture was compared to that of noncoated TiO_2 fixtures (**Figure 4**a). In the noncoated TiO_2 fixture, blood remained at the bottom of the fixture (Figure 4a, arrow in the top row), likely due to the intrinsic surface hydrophobicity of micro/nanoscale roughness created by acid etching (Figure 3e).[24] In contrast, blood was rapidly adsorbed along the circumference of the PLP-modified fixture (Figure 4a, arrows in bottom row). Surprisingly, the PLP-coated surface was hemophilic enough for blood to reach the top end of the fixture in about 26 s (Figure 4a, bottom row). This result demonstrates that PLP surface functionalization of dental implants may enhance the healing process and tissue-implant integration by increasing hemophilicity.

The effectiveness of PLP coating for in vivo integration of implants was evaluated using animal experiments. PLP-coated TiO_2 fixtures were implanted in a rat femur. Successful implantation of the fixtures was confirmed by microcomputed tomography (micro-CT) (Figure 4b, left panel). Interestingly, a significant amount of blood was found around the PLP-coated fixture implanted into the femur bone (Figure 4b, arrows in the lower image of right panel). In contrast, not much blood was found around the noncoated fixture in the femur bone (Figure 4b, arrow in the upper image of right panel). The enhanced hemophilicity of TiO_2 implants by PLP coating remarkably accelerated overall in vivo healing and implant-tissue integration. Bone-implant contact (BIC) determined by micro-CT was significantly promoted in PLP-coated TiO_2 fixtures (57.0% ± 6.4%) compared to noncoated TiO_2 fixtures (28.7% ± 14.1%) 7 d after implantation (Figure 4c). The increased BIC value suggests a greater degree of bone-to-implant integration.

We also performed push-in experiments of TiO_2 implants to further confirm the enhanced bone-to-implant integration mediated by PLP functionalization. As a positive control, we included UV light-treated TiO_2 fixtures in the experiments because enhancement of bone-to-implant integration induced by light treatment was previously reported.[25] A linear slope value in the load–displacement graph of Figure 4d indicates the force required for a unit distance movement of an implant.[26] A steep slope reveals a greater degree of bone integration of the implant. Figure 4d shows representative load–displacement curves for unmodified (black), UV-treated (blue), and PLP-coated (red) fixtures. The slope values were 63.6 ± 30.7 N mm^{-1} for unmodified fixtures, 80.6 ± 25.1 N mm^{-1} for UV-treated fixtures, and 186.4 ± 31.6 N mm^{-1} for PLP-coated fixtures (Figure 4e). Surprisingly, the degree of bone integration of the PLP-functionalized fixture observed in our study was higher than that of bone morphogenetic protein 2 (BMP2)-modified poly-L-lactide fixtures after 2 weeks (157.3 ± 39.6 N mm^{-1}).[27] Considering that the results of BMP2 fixtures were obtained after 2 weeks of fixture implantation, our result, obtained 1 week earlier than that, indicates that rapid healing and enhanced tissue integration were induced by surface PLP molecules. This might be due to the presence of an aldehyde moiety in the PLP molecule. Because aldehydes in PLP have intrinsic reactivity to proteins or peptides via Schiff-base formation,[14]

this functional group may trigger recruitment and stable immobilization of proteins onto the implant surface from blood plasma, which promotes osteoblast adhesion, proliferation, and ultimately, bone-implant integration.

Finally, with bioinspiration from the role of PLP as a cofactor for biochemical enzymatic reactions in nature, we tested whether PLP could act as a functional coating material capable of mediating bioactive peptide immobilization. It is well known that PLP can be conjugated to the amine group of a lysine residue or to the N-terminal amino group in proteins or peptides via formation of a Schiff-base intermediate.[28] Thus, PLP can function as a coenzyme for various transamination reactions in our body. Using this naturally existing biochemical reaction mediated by PLP, we conjugated two types of bioactive peptides, fibronectin-derived cell adhesion peptides (RGD) and BMP2-derived osteoinductive peptides (KIPKASSVPTELSAISTLYL),[29] onto the PLP-coated TiO_2 surfaces (**Figure 5**a). Cysteine–glycine–glycine (CGG) or lysine–glycine–glycine (KGG) sequences were added to the N-terminus or C-terminus for efficient peptide immobilization and to provide a flexible linker. Static water contact angle analysis revealed that peptide (CGGRGD) coating further increased hydrophilicity of the PLP-coated TiO_2 surfaces (Figure 5b). The immobilization efficiency of the peptides was calculated using fluorescamine assay. In most of the tested peptides, the efficiency of peptide immobilization was significantly higher in the PLP-coated groups compared to the noncoated groups (Figure 5c). Interestingly, the peptides end-modified with a lysine residue (K) showed higher immobilization efficiency than the peptides end-modified with a cysteine residue (C) (Figure 5c), reminiscent of highly efficient conjugation of PLP to the ε-amine group of lysine in transamination reactions. Although considerable amounts of peptides adhered to the noncoated surfaces via simple adsorption, it is evident that PLP-mediated peptide immobilization may further enhance conjugation and retention of bioactive peptides onto these surfaces.

Bioactivity of the immobilized peptides on the PLP-coated surfaces was confirmed by evaluating the enhancement of cellular adhesion, proliferation, and differentiation after peptide coating. HUVECs were cultured on the PLP-coated TiO_2 surfaces with immobilized RGD peptides. As a result, the surfaces with RGD peptides significantly promoted cell adhesion at early time points (within 24 h) and proliferation after 4 d in culture, compared to PLP-coated surfaces without peptide immobilization (Figure 5d,e). We also examined enhanced osteoinductivity by BMP2 peptides conjugated to the PLP-coated TiO_2 surfaces. Osteogenic differentiation of hADSCs was induced on the PLP-coated TiO_2 surfaces with BMP2 peptide immobilization at varying concentrations (1, 2, and 4 mg mL^{-1}) in osteogenic induction medium for 21 d. Immunocytochemical staining showed that higher levels of osteopontin (OPN), a glycoprotein secreted by osteoblasts, were expressed in hADSCs cultured on the BMP2-PLP-coated surfaces than in cells on surfaces with

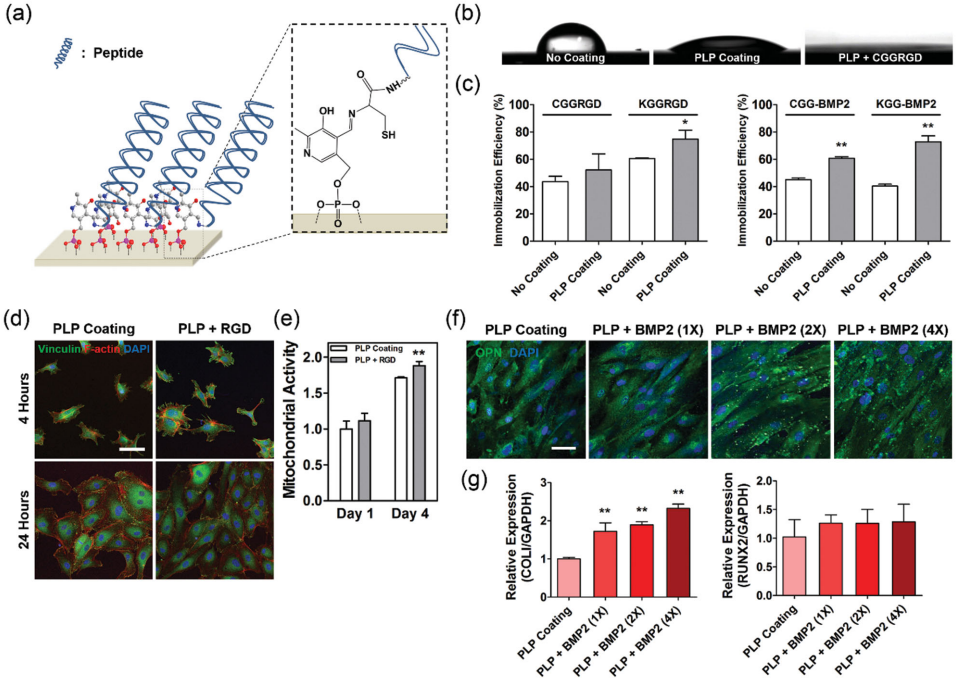

Figure 5. a) The schematic represents the chemical reactivity of PLP to peptides. b) Water contact angles are altered in noncoated (left), PLP-coated (middle), and peptide (CGGRGD) immobilized PLP-coated (right) TiO_2 surfaces (n = 3). c) The immobilization efficiency of peptides (CGGRGD, KGGRGD, CGG-BMP2, and KGG-BMP2) on TiO_2 substrates was determined with or without the PLP coating (n = 3, $*p < 0.05$ and $**p < 0.01$, compared to the no coating group). d) Staining of the focal adhesion vinculin and cytoskeleton protein F-actin was performed in HUVECs on PLP-coated TiO_2 substrates with and without peptide (CGGRGD) coatings 4 and 24 h after cell seeding, scale bar = 50 μm. e) The mitochondrial metabolic activity of HUVECs was measured by MTT assay (n = 3, $**p < 0.01$, compared to the no coating group). f) Immunofluorescent staining for the protein osteopontin (OPN) was performed in hADSCs differentiated for 21 d on PLP-coated TiO_2 surfaces with and without BMP2 peptide immobilization, scale bar = 50 μm. g) Gene expression levels of COLI and RUNX2 were quantified in the BMP2-PLP-coated group using qRT-PCR. Levels were normalized to that of the PLP coating alone group (n = 3, $**p < 0.01$, compared to the no coating group).

PLP coating alone (Figure 5f). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was used to quantify gene expression of osteogenic markers and indicated that collagen type I (COLI) expression was significantly upregulated in hADSCs on BMP2-PLP-coated surfaces in a peptide concentration-dependent manner (Figure 5g). Although there was no significance, expression of runt-related transcription factor 2 (RUNX2) also increased in cells cultured on BMP2-PLP-coated surfaces (Figure 5g). From these results, we concluded that functional bioactive peptides were efficiently immobilized onto the PLP-coated TiO_2 surfaces, further enhancing cellular adhesion and proliferation of human ECs and osteogenic differentiation of human stem cells.

3. Conclusion

In summary, we developed a vitamin B_6-based novel method for surface functionalization of TiO_2 medical implants. The increased hydrophilicity caused by PLP coating promoted adhesion, migration, and proliferation of diverse cell types (including ECs, EPCs, osteoblasts, and ADSCs—which are critically important to the success of stents and dental implants) on the TiO_2 surfaces. Highly hemophilic, PLP-functionalized TiO_2 implants exhibited enhanced bone-to-implant integration in vivo. Moreover, PLP makes the implant surfaces more functional and bioactive by mediating surface modification of bioactive peptides that promote cell adhesion, proliferation, and stem cell differentiation. Our bio-inspired, vitamin-based chemical approach for surface functionalization could be useful for improving the functionality and biocompatibility of various biomedical implants and devices.

Supporting Information

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

Acknowledgements

J.S.L. and K.K. contributed equally to this work. This research was supported by the National Research Foundation (NRF) of the Republic of Korea: Mid-career scientist (Grant No. 2014002855, H.L.) and the Basic Science Research Program (Grant No. NRF-2013R1A1A2A10061422, S.-W.C.). This work was also supported by the World Premier Material Development Program from the Ministry of Industry, Trade, and Natural Resources (Grant No. 10037915, H.L.) and by a grant (Grant No. 2009-0083522, S.-W.C.) from the Translational Research Center for Protein Function Control, Republic of Korea. All animal care and experimental procedures were performed with the approval of the Animal Care Committee of Korea Advanced Institute of Science and Technology (KAIST).

Received: April 13, 2015
Revised: May 18, 2015
Published online: June 11, 2015

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