Journal of Materials Chemistry

Cite this: *J. Mater. Chem.*, 2011, **21**, 15906

www.rsc.org/materials

COMMUNICATION

Facile surface immobilization of cell adhesive peptide onto TiO_2 substrate *via* tyrosinase-catalyzed oxidative reaction†

Kyung Min Park and Ki Dong Park*

Received 10th August 2011, Accepted 30th August 2011
DOI: 10.1039/c1jm13869c

A facile method to immobilize bioactive molecules including phenol molecules onto TiO_2 surfaces *via* tyrosinase-catalyzed oxidative reaction was developed for surface bio-functionalization of biomaterials.

Surface modification of bulk biomaterials is a highly important aspect to biological, chemical, and material sciences for biomedical applications.[1] Various methods, such as physical adsorption and chemical conjugation, have been investigated for the immobilization of bioactive molecules, such as cell adhesive peptides, DNA, growth factors and anticoagulant reagents. However, many researchers are still developing novel methods for site- and chemo-selective immobilization of bioactive molecules to prepare homogeneous and sufficient peptide conjugated biomaterials.[2] Recently, chemical modification of bulk material surfaces using 3,4-dihydroxy-L-phenylalanine (DOPA), which is found in mussel-adhesive proteins, has been widely investigated since this method only requires a simple coating procedure.[1,3–12] Lee *et al.*,[1,3,7] Cha *et al.*,[11,12] and Messersmith *et al.*[1,6,9,10,13] have successfully created multifunctional surfaces, such as PEGylation, cell adhesive peptide conjugation, and DNA immobilization on various surfaces using DOPA molecules. Chemical immobilization using this method simply involves dipping the surfaces in aqueous buffer solution. In the reaction, the DOPA molecules conjugate to surfaces *via* a coordinate covalent bond. Although this method has been shown to result in effective surface conjugation of bioactive molecules, this approach has some limitations, such as long reaction time and DOPA–DOPA conjugation due to oxidation of the molecules during synthesis and storage of polymers. For example, to prepare a PEGylated surface, substrates were immersed in catechol-grafted poly(ethylene glycol) polymer solution for over 12 h. In addition, inert and acidic conditions were required during the synthesis to inhibit oxidation of DOPA molecules.[7]

In the present study, a novel and facile method to immobilize the PRGDGGGGGY (RGD-Y) peptide onto titanium oxide (TiO_2) substrates was developed based on the tyrosinase-catalyzed oxidative reaction. The RGD-Y peptide was selected as a model bioactive molecule. The tripeptide sequence, arginine-glycine-aspartic acid (RGD), which is the signalling domain derived from fibronectin and laminin, has been widely used to improve cell attachment and proliferation through physical or chemical conjugation onto polymers, metals, and ceramics.[14,15] Tyrosinase is an enzyme that catalyzes the oxidation of phenol molecules into *o*-quinones immediately passing the intermediate state (*o*-dihydroxyphenol) in the presence of oxygen.[16,17] When the phenol groups of tyrosine (Tyr) residues in the RGD-Y peptide were converted to *o*-quinone molecules, the RGD-Y peptide was immobilized onto the metal substrate *via* a metal coordinate covalent bond (Fig. 1). The conjugative reaction proceeded under very mild condition and within a few minutes. In order to determine the molecular conversion ratio of phenol moieties to *o*-quinone molecules, the oxidation rate of the phenol groups *via* the tyrosinase-catalyzed reaction was monitored by UV spectroscopy. This initial experiment revealed that the oxidative reaction proceeded rapidly within five minutes and the reaction rates could be controlled by changing the tyrosinase concentration (70–80% conversion was obtained within five minutes using 0.4 KU mL^{-1} of tyrosinase, see ESI†, Fig. S1).

To immobilize the RGD-Y peptide onto TiO_2, the substrates were immersed in a peptide solution, followed by the addition of tyrosinase

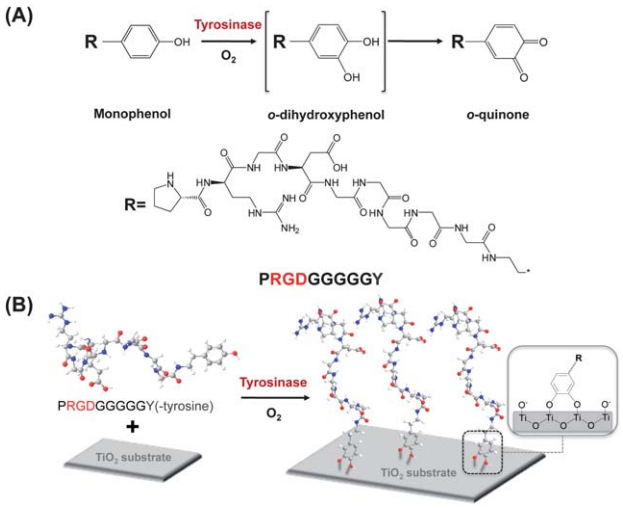

Fig. 1 Schematic representation of molecular conversion (A) and tyrosinase-catalyzed immobilization of RGD-Y peptide onto titanium oxide (TiO_2) substrate (B).

Department of Molecular Science and Technology, Ajou University, 5 Woncheon, Yeongtong, Suwon, 443-749, Republic of Korea. E-mail: kdp@ajou.ac.kr; Fax: +82-31-219-1592; Tel: +82-31-219-2944

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c1jm13869c

solution and the immobilization reaction was carried out for 10 min (see ESI†, Fig. S2 for detail). Before the immobilization, the TiO_2 substrates were washed by sonication in ethanol and acetone for 15 min. The immobilized RGD-Y peptide was quantified using the fluorescamine assay. The surface density of the immobilized peptide is shown in Fig. 2. The peptide density on the surfaces ranged from 0.18 to 0.35 nmol cm^{-2} and the density could be controlled by the tyrosinase concentration. This result can be explained by the fact that the molecular conversion ratio of phenol molecules in tyrosine to *o*-quinone increased at higher concentrations of tyrosinase (see ESI†, Fig. S1).

Surface wettability and chemical atomic composition of the functionalized TiO_2 surfaces were characterized by measuring the static water contact angle and X-ray photoelectron spectroscopy (XPS). Contact angles decreased dramatically from 62° to below 39° after RGD-Y immobilization (see ESI†, Fig. S3), suggesting that the relatively hydrophilic peptides were successfully conjugated on the surfaces *via* the tyrosinase-catalyzed reaction.

XPS analysis indicated that there were differences in surface chemical atom depositions after RGD-Y immobilization onto the TiO_2 substrates (see Fig. 2B and C). Qualitative analysis of the XPS wide scans survey clearly showed successful immobilization of the RGD-Y peptide *via* the tyrosinase-catalyzed oxidation of tyrosine, where an increase in the N1s signal was observed after the peptide immobilization (see Fig. 2B). In the high-resolution N1s spectra of the functionalized TiO_2 surface, an increase in the N–C (400.1 eV) peak was observed due to RGD-Y conjugation to the TiO_2 substrate, suggesting successful RGD-Y immobilization. The quantitative analysis indicated that the chemical atomic compositions of the TiO_2 substrates significantly increased for N1s (3.69% to 7.17%) and C1s (37.87% to 39.55%), which originated from the RGD-Y peptide (see ESI†, Table S1). The surface characterizations demonstrated that the RGD-Y peptide was successfully immobilized onto the TiO_2 substrates *via in situ* molecular conversion through the tyrosinase-catalyzed oxidative reaction.

The cell adhesive properties of the RGD-Y immobilized TiO_2 substrates were investigated *in vitro* using the MC3T3-E1 cell line, which was cultured under standard culture conditions (37 °C and 5% CO_2, see ESI† for detailed culture conditions and methods). *In vitro* cell culture was carried out using medium with or without serum. Serum contains several proteins that can coat the surface and promote cell adhesion; thus, serum free conditions were used to assess the direct effects of RGD on cellular adhesion. The effect of RGD-Y conjugated TiO_2 surfaces using tyrosinase on cellular activities was investigated by F-actin cytoskeleton staining. Fig. 3A shows fluorescence images of the cultured cells on the TiO_2 surfaces. Consistently adhered and spread cells were observed on the RGD-Y immobilized TiO_2, while adherent cells were rarely observed on the bare TiO_2 substrates. When media-containing serum was used (Fig. 3A, first row), the cells were well attached on the unmodified substrates due to protein absorption on the TiO_2 surfaces. As mentioned above, the absorbed proteins allow the cells to recognize their environment, which enhances cellular activity. However, cell spreading on the surfaces was limited due to the absence of adhesion ligands. Very different results were observed in serum free media (Fig. 3A, second row), where the effect of the conjugated RGD-Y on cell adhesion and spreading was more prominent. For instance, cells

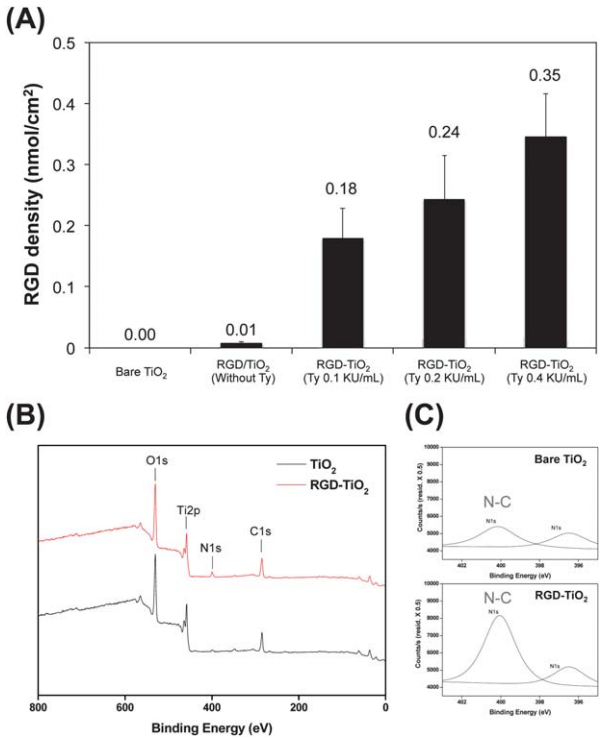

Fig. 2 Density of surface bound RGD-Y peptides at different tyrosinase concentrations onto the TiO_2 substrates (A), XPS spectra; wide scan (B) and high-resolution N1s spectra (C) of TiO_2 surfaces before and after the immobilization of peptide.

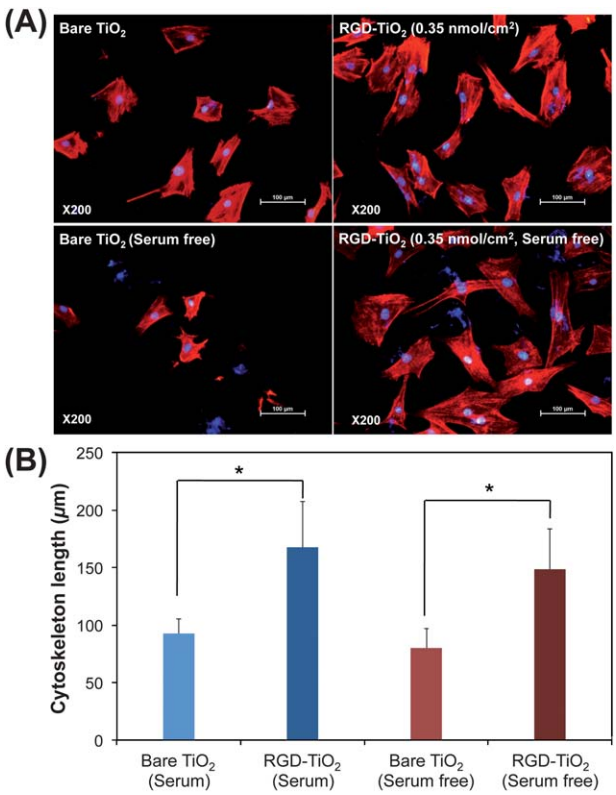

Fig. 3 Fluorescence images (A) and cytoskeleton length (B) of cultured MC3T3-E1 cells on the unmodified TiO_2 and functionalized TiO_2 surfaces with or without serum for 24 h (*$P < 0.001$, $n = 50$).

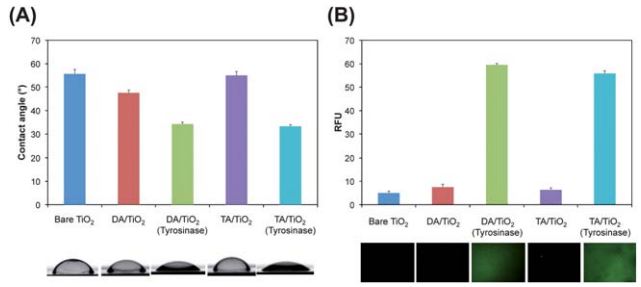

Fig. 4 Immobilization efficiency of the dopamine (DA) and tyramine (TA) with or without tyrosinase: (A) static water contact angles of unmodified and modified TiO_2 substrates and (B) surface distribution of immobilized molecules on the TiO_2 substrates using FITC as a probe.

that had adhered to the unmodified TiO_2 surfaces adopted almost round and polygonal shape, indicating a very low level of adhesion to the surfaces. However, the cells that had adhered onto the RGD-Y immobilized TiO_2 surfaces adopted a spindle-shape and were well spread. The degree of cell spreading was determined by measuring the cytoskeleton length using image analysis (Fig. 3B). As shown in Fig. 3B, the cytoskeleton length of the cells cultured on the RGD-Y conjugated TiO_2 surfaces was longer than that of the cells cultured on the unmodified substrates. This result indicates that the RGD moieties on the surfaces can affect cellular behaviors such as attachment and spreading. Therefore, the results of *in vitro* cell culture demonstrated that the RGD-Y peptide was successfully conjugated *via* the tyrosinase-catalyzed reaction, and the immobilized cell adhesion peptide promoted cell attachment and spreading, which was attributed to the effective affinity of the RGD sequence for the integrin receptors present on the cellular membrane.[2]

One of the key advantages of our system is fast immobilization of bioactive molecules including the phenol moiety when compared with the DOPA coating system. To compare immobilization efficiency, immobilization of dopamine (DA) and tyramine (TA) molecules on the TiO_2 surfaces was compared using tyrosinase (see ESI† for detailed method). The reaction time for immobilization was 10 min. The surface properties of the DA or TA immobilized surface were characterized by static water contact angle and fluorescence observation using FITC. When tyrosinase was used, the water contact angles (Fig. 4A) decreased dramatically, demonstrating that TA molecules were successfully converted and immobilized *via* the oxidative reaction of tyrosinase. The molecular distribution on the surfaces was observed by fluorescence microscopy using a FITC probe that can react with primary amines on poly-dopamine moieties present on the surface due to molecular conversion of TA with tyrosinase (see ESI† for detailed method). Fig. 4B shows the surface distribution of DA or TA molecules tagged with FITC. The relative fluorescence unit (RFU) of the surface immobilized with tyrosinase was dramatically higher than that of the surface immobilized without tyrosinase. When DA molecules were immobilized with tyrosinase, the coating efficiency increased. Although the chemical mechanism of the DOPA system is not fully understood, DOPA molecules may be immobilized onto the TiO_2 surfaces through a coordinate covalent bond during the oxidation of DOPA molecules to DOPA quinone forms. These results can be explained by the fact that addition of tyrosinase allowed the oxidation rate to increase, which resulted in fast immobilization of the molecules to the substrate. These results demonstrated that surface immobilization of TA and DA molecules using tyrosinase was more effective than the conjugation of TA and DOPA molecules without the tyrosinase due to the increased oxidation rate of the molecules.

In summary, we have developed a novel strategy for fast and simple immobilization of the RGD-Y peptide *via* a tyrosinase-catalyzed reaction. The phenol containing RGD peptide was rapidly immobilized within a few minutes and the surface density of conjugated RGD-Y ranged from 0.18 to 0.35 nmol cm^{-2}. In the static water contact angle and XPS analysis, a change in wettability and chemical atom composition were observed after surface functionalization. The *in vitro* cell culture studies demonstrated that the surface immobilized RGD-Y influenced cell adhesion and spreading. Therefore, this study suggests that immobilization of bioactive molecules including phenol moieties using tyrosinase may be an efficient method to prepare functionalized surfaces for various biomedical applications.

This research was supported by the grants from the Fundamental R&D Program for Core Technology of Materials, Ministry of Knowledge Economy, Republic of Korea 10033301 (K00060-282) and the grants from the National Research Foundation of Korea (NRF) grant funded by the Korea Government (2010-0027776).

Notes and references

1 H. Lee, S. M. Dellatore, W. M. Miller and P. B. Messersmith, *Science*, 2007, **318**, 426.
2 L. Perlin, S. Macneil and S. Rimmer, *Soft Matter*, 2008, **4**, 2331.
3 H. Lee, B. P. Lee and P. B. Messersmith, *Nature*, 2007, **448**, 338.
4 S. M. Kang, J. Rho, I. S. Choi, P. B. Messersmith and H. Lee, *J. Am. Chem. Soc.*, 2009, **131**, 13224.
5 H. J. Cha, D. S. Hwang, S. Lim, J. White, C. Matos-Perez and J. Wilker, *Biofouling*, 2009, **25**, 99.
6 H. O. Ham, Z. Liu, K. H. A. Lau, H. Lee and P. B. Messersmith, *Angew. Chem., Int. Ed.*, 2010, **50**, 732.
7 H. Lee, K. D. Lee, K. B. Pyo, S. Y. Park and H. Lee, *Langmuir*, 2010, **26**, 3790.
8 S. H. Ku, J. Ryu, S. K. Hong, H. Lee and C. B. Park, *Biomaterials*, 2011, **31**, 2535.
9 J. L. Dalsin, B. H. Hu, B. P. Lee and P. B. Messersmith, *J. Am. Chem. Soc.*, 2003, **125**, 4253.
10 J. L. Dalsin, L. Lin, S. Tosatti, J. Vörös, M. Textor and P. B. Messersmith, *Langmuir*, 2005, **21**, 640.
11 D. S. Hwang, Y. Gim, D. G. Kang, Y. K. Kim and H. J. Cha, *J. Biotechnol.*, 2007, **127**, 727.
12 D. S. Hwang, S. B. Sim and H. J. Cha, *Biomaterials*, 2007, **28**, 4039.
13 H. Lee, J. Rho and P. B. Messersmith, *Adv. Mater.*, 2009, **21**, 431.
14 K. M. Park, I. Jun, Y. K. Joung, H. Shin and K. D. Park, *Soft Matter*, 2011, **7**, 986.
15 U. Hersel, C. Dahmen and H. Kessler, *Biomaterials*, 2003, **24**, 4385.
16 A. B. Braunschweig, R. Elnathan and I. Willner, *Nano Lett.*, 2007, **7**, 2030.
17 A. Korner and J. Pawelek, *Science*, 1982, **217**, 1163.

