Cell Growth

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A Bioorthogonal Approach for the Preparation of a Titanium-Binding Insulin-like Growth-Factor-1 Derivative by Using Tyrosinase

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Abstract: *The generation of metal surfaces with biological properties, such as cell-growth-enhancing and differentiation-inducing abilities, could be potentially exciting for the development of functional materials for use in humans, including artificial dental implants and joint replacements. However, currently the immobilization of proteins on the surfaces of the metals are limited. In this study, we have used a mussel-inspired bioorthogonal approach to design a 3,4-hydroxyphenalyalanine-containing recombinant insulin-like growth-factor-1 using a combination of recombinant DNA technology and tyrosinase treatment for the surface modification of titanium. The modified growth factor prepared in this study exhibited strong binding affinity to titanium, and significantly enhanced the growth of NIH3T3 cells on the surface of titanium.*

A wide variety of different biomaterials are currently employed in medical applications throughout the world. Research towards the development of new biomaterials has traditionally focused on the preparation of functional materials capable of the simple adhesion of cells or the connection of tissues to metals and ceramics. However, there is a growing interest in the development of biomaterials involving the immobilization of growth factors, which would allow these artificial materials to regulate specific cellular functions, including the gene expression processes associated with cell growth and differentiation.[1] Although numerous studies have been reported pertaining to the use of metallic materials in medical devices such as artificial joints, dental implants and stents, there have been very few reports concerning the surface modification of metal or ceramic materials with biological agents.[2] A biomimetic approach inspired by mussel adhesive activity has recently been developed as a strategy for the surface modification of metals and ceramics with biological materials. Considering that the underwater adhesive protein secreted by mussels contains 3,4-dihydroxyphenethylamine (DOPA) in its active site,[3] it is possible to incorporate proteins containing DOPA or one of its more simplified derivatives dopamine as a strategy for the surface modification of metals and ceramics.[4] Although numerous DOPA-containing peptides and macromolecules have been reported,[5] the site-specific incorporation of DOPA into proteins, such as growth factors, of which tertiary structure are important for the signal transduction activity, have not been reported. It is important to incorporate DOPA without disturbing the tertiary structure and active site of proteins. If such a protein is prepared, it should play a much greater regulatory role in cells, including gene expression for cellular growth and differentiation by binding on various materials. However, because DOPA is a non-canonical amino acid, it cannot be directly incorporated into a protein using conventional protein-engineering (recombinant DNA) techniques.

To overcome this problem, as one of the bioorthogonal approaches,[6] enzymatic incorporation of a non-canonical amino acid after conventional protein engineering was performed. To keep the tertiary structure of the growth factor, we first incorporated tyrosine residues into a growth-factor protein at the terminus and subsequently converted these residues into DOPA using a tyrosinase, which has been used previously for the preparation of DOPA.[7] It is envisaged that DOPA-containing growth factors will exhibit adhesive properties, which would allow them to bind to a wide range of materials. Furthermore, the immobilization of such DOPA-containing growth factors onto a metal or ceramic surface could potentially impart cell-growth-enhancing properties on these materials, representing a significant step forward in the development of functional biomaterials. In this study, we selected insulin-like growth-factor-1 (IGF-1) as a model system to investigate the effects of immobilizing a growth factor on a metal surface. IGF-1 was chosen for this study because it is well established that its native tyrosine residues do not contribute to its binding interaction with the IGF-1 receptor as shown in Figure 1. We incorporated a small pentapeptide tag composed of Tyr-Lys-Tyr-Lys-Tyr (YKYKY) residues at the C-terminal of IGF-1 with the aim of converting this tag into DOPA-Lys-DOPA-Lys-DOPA (XKXKX, X = DOPA) using tyrosinase, as shown in Figure 1. This peptide sequence was originally designed Messersmith's

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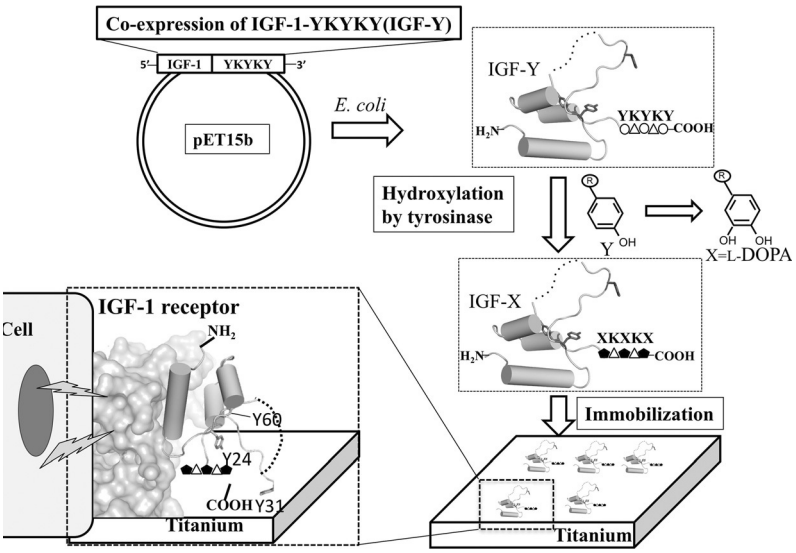


Figure 1. The preparation of the mussel-inspired IGF-1 derivatives. The YKYKY sequence was added to the C-terminal of IGF-1 using conventional protein engineering (IGF-Y). The subsequent conversion of the tyrosine residues into 3,4-dihydroxyphenylalanine (X) residues was achieved by tyrosinase (IGF-X).

group,[8] based on the properties of the underwater adhesive protein secreted from mussels, which was found to contain both DOPA and lysine residues in its binding site.

We initially investigated the properties of several different peptides to determine the best candidate for the modification of IGF-1. All of these experiments were conducted with a FLAG (DYKDDDDK)-tagged IGF-1 protein, as shown in Figure 2. Although the colors of the YKYKY and HHHHHH (H6) proteins remained the same for all of the pH values tested (4.5–8.5), the color of the XKXKX protein was found to be pH-dependent. For example, the XKXKX protein turned from transparent to yellow at high pH (pH > 7). After 24 h the color change completed. The spectral change is similar to that of mixture of catechol and gamine (glycine)

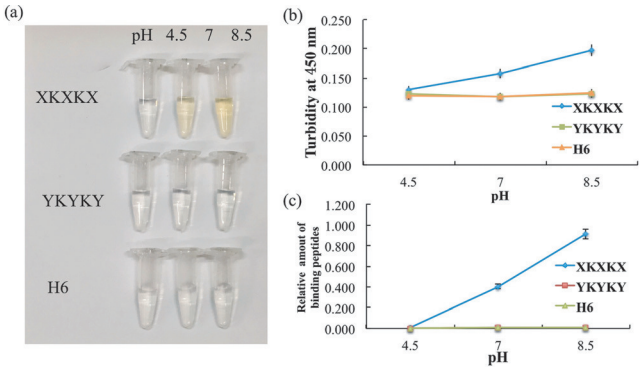


Figure 2. a) Physical appearances of the different peptide solutions (1 mg mL^{-1}) at different pH values. b) Turbidities of the peptide solutions (1 mg mL^{-1}) at different pH values. c) Binding activities of the peptides (1 mg mL^{-1}) onto titanium at different pH values. The binding activities were measured using an *anti*-FLAG antibody, as described in the Supporting Information. XKXKX is short for XKXKX-DYKDDDDK (X = DOPA); YKYKY is short for YKYKY-DYKDDDDK; H6 is short for GSSHHHHHHSSGLVPRGSH-DYKDDDDK.

(Figure S11 in the Supporting Information). The turbidity of a solution of XKXKX in PBS was also found to be dependent on the pH value (Figure 2b), with the mixture becoming increasingly turbid at high pH (pH > 7). It is noteworthy that these properties are similar to those reported for solutions of dopamine and DOPA.[9] This increase in the turbidity of dopamine solutions at high pH has been attributed to the occurrence of Michael addition or Schiff base reactions.[10] Furthermore, the reaction of the catechol moiety of DOPA with the γ-amino groups of the lysine residues in the peptide would make a considerable contribution to reactions of this type and, indeed, the turbidity. The oxidation reaction of catechol is considered to resulted in chemical crosslinking.

The binding ability of XKXKX was found to be heavily dependent on the pH, with this peptide showing much stronger binding affinity than YKYKY or H6 at pH 8.5 (Figure 2c). Based on this result, it was envisaged that the incorporation of XKXKX into IGF-1 would lead to the formation of a protein layer on the surface of titanium in a similar manner to DOPA or dopamine at high pH.

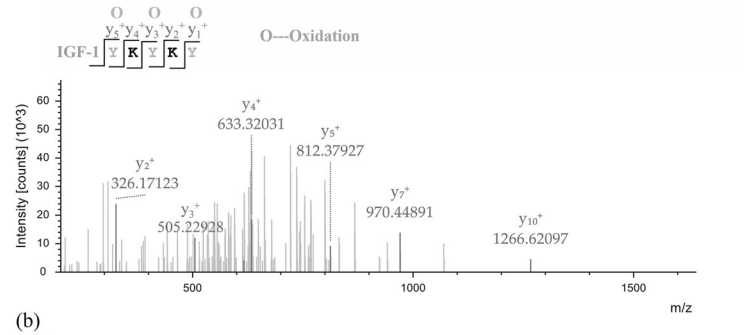
To allow for the incorporation of the peptide into the protein, we initially investigated the incorporation of YKYKY into IGF-1 using a conventional recombinant technology approach (Figure S1). The synthesis of the IGF-1 derivative was confirmed by PAGE (Figure S2) and western blot analysis using an *anti*-IGF-1 antibody (Figure S3). After tyrosinase treatment, the hydroxylation of all of the tyrosine residues in the IGF-1 derivative was confirmed by mass spectrometry (Figure 3a). All of tyrosine residues, including the native tyrosine residues and those introduced by the modification process, were hydrogenated by tyrosinase.

Figure 3b shows that the circular dichroism (CD) spectra of the commercial IGF-1 and the IGF-1 derivative both contained similar positive and negative peaks at 192 and 208 nm, respectively. Only the IGF-1 derivative bearing a YKYKY tag at its C-terminal was in a different conformation prior to the refolding process. This result therefore demonstrated that the refolding process recovered the native conformation of the protein, and that the incorporation of this peptide and its subsequent treatment with tyrosinase did not have an adverse impact on the conformation of IGF-1. The biological activity of IGF-1 was investigated using a cell growth assay (Figure 3c). The results of this assay provided further confirmation that the incorporation of the modified peptide and its subsequent treatment with tyrosinase did not have an adverse impact on the activity of IGF-1. This result is therefore consistent with the fact that the tyrosine residues in IGF-1 do not contribute to its binding interaction with the IGF-1R, explaining why the hydroxylation of these residues had no discernible impact on the biological activity of IGF-1.

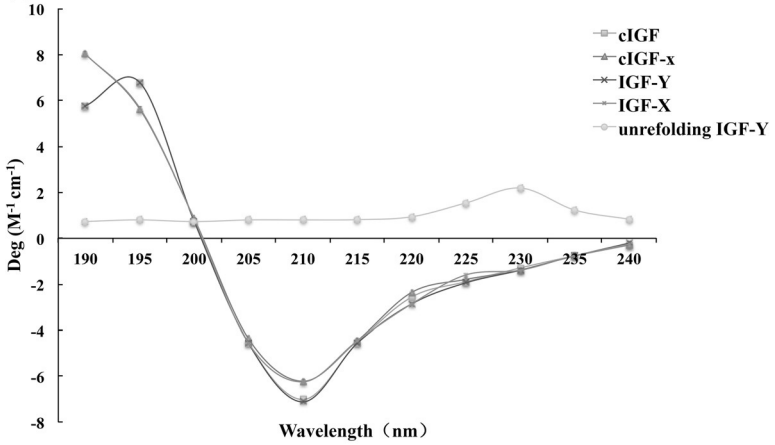
The binding affinities of the different IGF-1 derivatives towards titanium were investigated using quartz crystal

(a)

Seq	y+	Res.Mass
Y-Oxidation	y_5^+ = 812.38249	179.05824
K	y_4^+ = 633.32425	128.09497
Y-Oxidation	y_3^+ = 505.22928	179.05823
K	y_2^+ = 326.17105	128.09497
Y-Oxidation	y_1^+ = 198.07608	198.07608



(b)



(c)

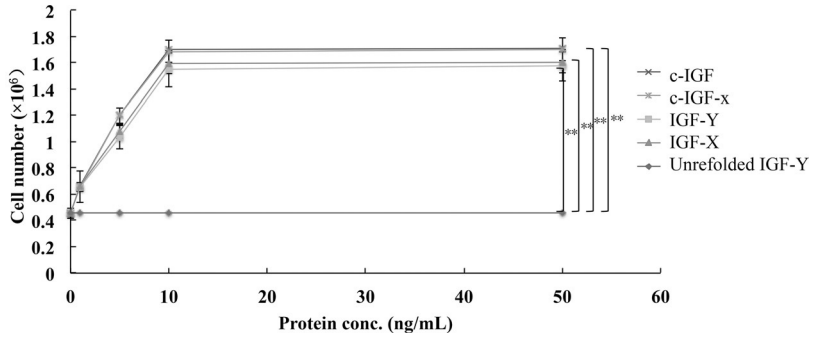


Figure 3. a) LC/MS/MS analysis of IGF-X. b) CD spectra of the IGF-1 derivatives. c) Growth enhancement of NIH3T3 cell by the IGF-1 derivatives in DMEM medium. c-IGF, commercial IGF-1; c-IGF-*x*, c-IGF treated with tyrosinase; unrefolded IGF-Y, IGF-Y before refolding. These data represent the mean values of three independent experiments ± standard deviation. *, $p < 0.05$; **, $p < 0.01$; NS, not significant.

microbalance with dissipation monitoring (QCM-D), as shown in Figure 4. The binding affinity of IGF-X was significantly higher than that of IGF-Y at pH 8.5. Furthermore, bound IGF-X did not dissociate from the surface of the Ti-coated discs, even after they had been washed with PBS. These results indicated that the XKXKX moiety in IGF-X was making a considerable contribution to its ability to bind to the titanium surface. The enhanced binding affinity of IGF-X to titanium was attributed to the reaction between the creosol moieties of the DOPA residues with the ε-amine groups of the lysine residues. The surface concentration of IGF-X on the titanium surface was about five times greater than what would be expected for a theoretical monolayer of IGF-1 (ca. 400 ng cm^{-2}). In contrast, the surface concentration of IGF-Y was consistent with that of a single monolayer of IGF-1. This result suggested that IGF-X was forming multiple layers on the titanium surface by the intermolecular crosslinking of IGF-X.

Figure 5 shows the cell-growth assay results for NIH 3T3 cells in the presence of soluble IGF-Y, commercial IGF-1 (c-IGF), and bound IGF-X. The addition of soluble IGF-1 to the cells led to a slight decrease in cell growth activity compared with the untreated cells. However, the treatment of these proteins with tyrosinase led to a significant increase in their cell growth activity. For example, a very small amount of bound IGF-X led to similar increases in the cell-growth activity as for soluble c-IGF. Notably, the treatment of the cells with IGF-X led to a much greater increase in cell-growth activity than for soluble c-IGF. Similar results to these have also been observed for other immobilized growth factors and attributed to high local concentrations of growth factor, as well as the multivalency and inhibition of down regulation by the bound growth factor. A comparison of the results obtained for the bound and soluble growth factors revealed that the bound growth factors led to a much higher local concentration of growth factor in contact with the cells, which could induce multivalency effect, such as enhancing the dimerization of the growth-factor receptors. In addition, this effect would be further enhanced by inhibiting the internalization of the growth factor, with the bound growth factor resulting in higher levels of growth enhancement compared with the soluble material. Actually phosphorylation of the IGF receptor (IGFR) occurred rapidly and decreased in the presence of soluble IGF, whereas phosphorylation occurred gradually and was maintained in the presence of bound IGF, even after 12 h incubation (Figure 5b). The long activation period of bound IGF-1 is considered to be due to the inhibition of cellular internalization of bound IGF-1, thereby inducing growth enhancement over a sustained period as shown in the

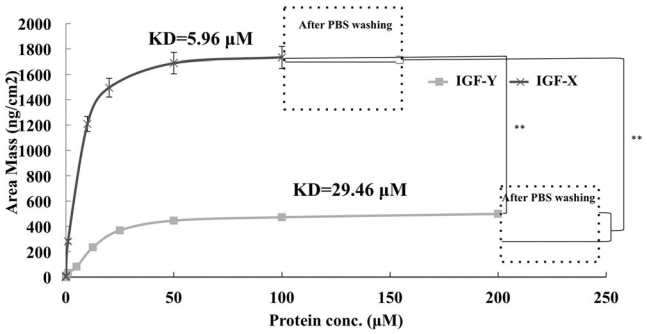


Figure 4. Bound amounts of the IGF-1 derivatives on titanium at pH 8.5 by QCM-D. These data represent the mean values of three independent experiments ± standard deviation. *, $p < 0.05$; **, $p < 0.01$; NS, not significant.

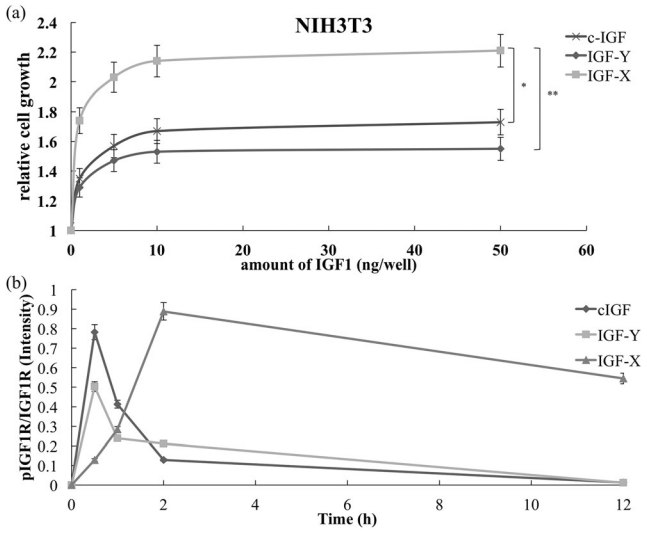


Figure 5. a) Growth of NIH3T3 in the presence of soluble c-IGF, soluble IGF-Y, and bound IGF-X on titanium. Cell numbers were counted after 2 days of culturing using a cell counting kit (absorbance at 450 nm). These data represent the mean values of three independent experiments ± standard deviation. *, $p < 0.05$; **, $p < 0.01$; NS, not significant. b) Time course of activation (phosphorylation) of the IGF receptor in the presence of soluble and bound IGF-1.

Supporting Information (Figure S13). The mechanism of bound growth factors, long-lasting signal transduction has been confirmed by some researchers.[11] Based on these results, we concluded that the increase in cell activity observed for the bound IGF-X could be attributed to a combination of these factors.

In this study, we have successfully achieved the incorporation of DOPA into IGF-1 using tyrosinase after the addition of numerous tyrosine residues using recombinant DNA technology. The binding affinity of IGF-1 to the surface of a Ti-coated disk was enhanced significantly by the hydroxylation of the tyrosine residues with tyrosinase. Furthermore, the bound IGF-1 derivative significantly enhanced cell growth. Considering the universal nature of this mussel-inspired modification process, this new method for the incorporation of DOPA could be used to prepare novel cell-growth-enhancing materials with potential applications in cell-culture systems and regenerative medicine.

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