Bioconjugate Chemistry

Communication

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Single-Step Immobilization of Cell Adhesive Peptides on a Variety of Biomaterial Substrates via Tyrosine Oxidation with Copper Catalyst and Hydrogen Peroxide

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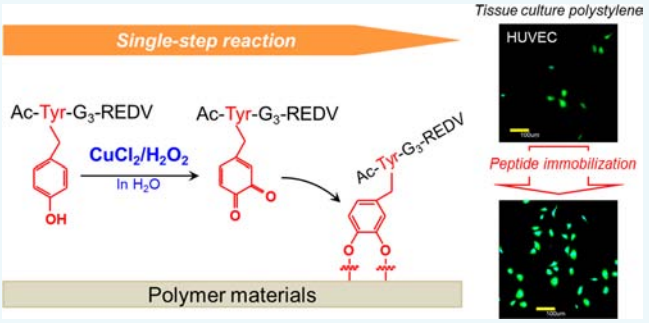
Supporting Information

ABSTRACT: Immobilization of biologically active peptides which were isolated from extracellular matrix proteins is a powerful strategy for the design and functionalization of biomaterial substrates. However, the method of peptide immobilization was restricted, that is, peptide is often immobilized through the reactive groups inherent in substrates with multistep reactions. Here, we report a single-step immobilization of fibronectin-derived cell adhesive peptide (Arg-Glu-Asp-Val; REDV) onto polymer materials by use of tyrosine oxidation with copper catalyst and hydrogen peroxide. REDV peptide was successfully immobilized on tissue culture polystyrene, poly(ethylene terephthalate), poly(vinyl chloride), expanded-poly(tetrafluoroethylene), and poly(L-lactic acid), resulting in enhanced adhesion of human umbilical vein endothelial cells. This method is a single-step reaction under very mild conditions and is available for the biological functionalization of various medical devices.



Biomaterials interact with proteins, blood, cells, and tissues through their interface; therefore, their surface features are crucial for controlling biological responses.[1] Immobilization of bioactive peptides and proteins is a useful strategy for functionalizing biomaterial surfaces.[2,3] Numerous methods for immobilization of bioactive peptides onto biomaterials have been investigated, including physical adsorption and chemical binding.[4,5] However, the usage of each method is limited because it was developed for a specific material. In addition, the methods often involve multistep reactions comprising the introduction of functional groups onto the materials, activation of the functional group, and immobilization of the peptide by condensation. Messersmith et al. have reported the modification of biomaterial surfaces using 3,4-dihydroxy-L-phenylalanine (DOPA), which is known for its contribution to the strong adhesive properties of marine mussel proteins.[6−9] Poly(ethylene glycol) has been successfully immobilized on a titanium surface by using DOPA as an anchor molecule.[6] DOPA *o*-quinones produced by catechol oxidation can bind to metal substrates through chelate formation and can react with primary amines, carboxylic acids, and thiols by Michael addition.[10] Since catecholic compounds including DOPA and dopamine can bind to a variety of biomaterials on the basis of these reactions, this reaction is becoming a popular strategy for modifying biomaterial surfaces.[11−13] However, this method has some restrictions when used for peptide immobilization. For example, catechol hydroxyl groups must be capped with special protecting groups such as *tert*-butyldimethylsilyl ester and cyclic ethyl orthoformate during the synthesis of peptides containing DOPA residues.[14−16]

Park et al. reported peptide immobilization through tyrosinase-catalyzed oxidation of tyrosine to DOPA. They successfully immobilized cell adhesive peptides containing a tyrosine residue (PRGDGGGGGY) on a titanium oxide substrate, thereby enhancing cell adhesion.[17] This method could immobilize peptides containing tyrosine residues without the use of special dihydroxyphenyl derivatives. However, since quinones can react with tyrosinase in addition to reacting with the substrate, this approach will result in the undesirable immobilization of tyrosinase onto the biomaterial surface.

To address the aforementioned problems, we have developed a novel peptide immobilization method for various polymeric substrates used in medical devices. This method is based on the copper-catalyzed oxidation of tyrosine residues (Scheme 1). It has been reported that tyrosine can be converted to DOPA quinones by oxidation with copper chloride (II) ($CuCl_2$) and hydrogen peroxide (H_2O_2).[18] Quinones attach to the radicals made by oxidation and polymerize themselves, allowing the imobilization of bioactive peptides containing tyrosine residues on biomaterial surfaces.[19] Endothelial cell adhesive Arg-Glu-Asp-Val (REDV) peptide, which was isolated from the human fibronectin IIICS region, was chosen as the representative cell

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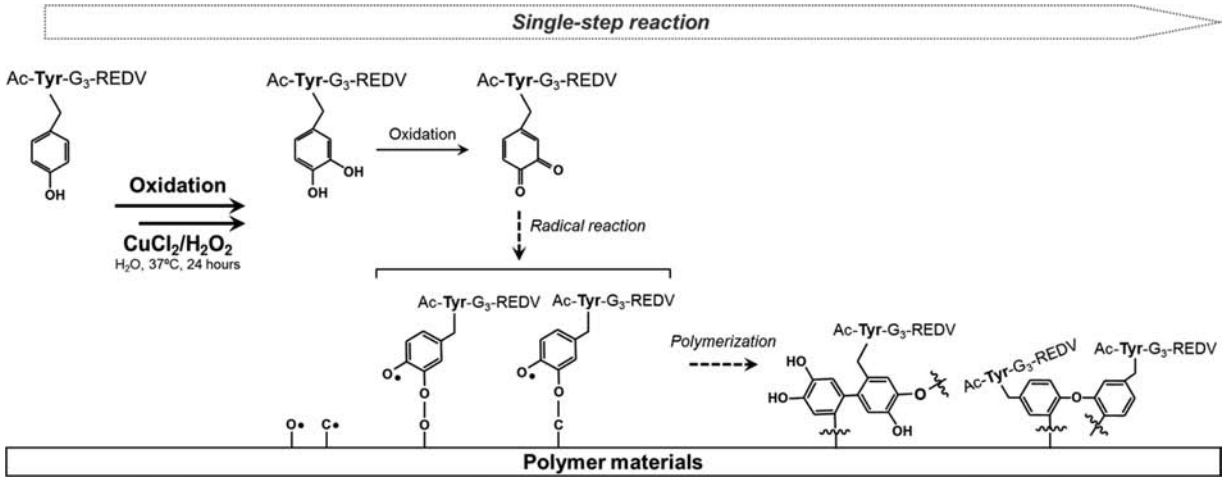
Scheme 1. Single-Step Peptide Immobilization through Oxidation of Tyrosine Residue Catalyzed by Copper Chloride (II) and Hydrogen Peroxide[a]



[a]Dotted arrows indicate predicted reactions between tyrosine residues of peptide and polymer materials.

adhesive peptide.[20,21] Ac-YGGGREDV (Y-REDV) was synthesized by the typical Fmoc solid phase procedure without any special protecting groups and was characterized by MALDI-TOF mass spectrometry (MS) after RF-HPLC purification (Figure S1).

Specimens (φ = 13 mm or 10 × 10 mm^2) were immersed in Y-REDV aqueous solution (0.5 mM), which was followed by the addition of $CuCl_2$ (0.04 equiv) and H_2O_2 (4.4 equiv). After incubation at 37 °C for 24 h, substrates were washed by Milli-Q water. Cover glass (as a control), tissue culture polystyrene (TCPS), poly(ethylene terephthalate) (PET), poly(vinyl chloride) (PVC), expanded-poly(tetrafluoroethylene) (ePTFE), and poly(L-lactic acid) (PLLA) were selected as substrates. Among these substrates, PET, PVC, ePTFE, and PLLA are commonly used in medical devices.

Surface wettability of specimens was determined before reaction (unmodified), after immersion in $CuCl_2$ and H_2O_2 mixture ($CuCl_2/H_2O_2$-treated) and in Y-REDV solution without any reagents (Y-REDV-treated), and after reaction in Y-REDV solution containing $CuCl_2$ and H_2O_2 (Y-REDV/$CuCl_2/H_2O_2$-treated) using the water-in-air contact angle (Figure 1). For all substrates, no significant differences were found in the water contact angles between unmodified, $CuCl_2/H_2O_2$-treated, and Y-REDV-treated surfaces. However, the water contact angles decreased by about 15−20° for the Y-REDV/$CuCl_2/H_2O_2$-treated surfaces, suggesting that Y-REDV peptides were successfully immobilized on all substrates. The reason behind the water contact angles of the substrates not remaining the same after Y-REDV immobilization could be that the substrate surfaces were not fully covered by Y-REDV.

X-ray photoelectron spectroscopy (XPS) measurements were taken on all substrates to confirm the existence of nitrogen derived from Y-REDV (Figure 2). Since none of the substrates contained nitrogen, the detection of nitrogen indicates the existence of Y-REDV on the surface. Nitrogen peaks were clearly higher for the Y-REDV/$CuCl_2/H_2O_2$-treated surfaces of glass, TCPS, PET, and ePTFE. The small nitrogen peaks for the Y-REDV-treated surfaces of TCPS, PET, and PVC might be due to the small amounts of nonspecific adsorption of Y-REDV peptides, which does not affect the surface wettability. Nitrogen peaks for the Y-REDV/$CuCl_2/H_2O_2$-treated surfaces of PVC

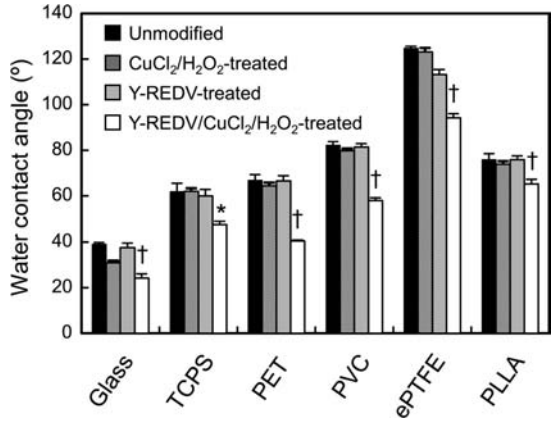


Figure 1. Water contact angle of unmodified, $CuCl_2/H_2O_2$-treated, Y-REDV-treated, and Y-REDV/$CuCl_2/H_2O_2$-treated specimens. (n = 3; $^*p < 0.05$ and $^\dagger p < 0.01$ compared to unmodified surface, one-way ANOVA followed by Tukey's posthoc test).

and PLLA were smaller than those for other substrates, suggesting that while this novel method is applicable to all tested substrates, it is less effective on PVC and PLLA. On PVC, radical production might be inhibited resulting in the low efficacy of Y-REDV binding because it contains a stabilizer such as zinc carboxylate.[22] In the case of PLLA, a part of its surface was probably degraded to low-molecular-weight oligomer by $CuCl_2/H_2O_2$ oxidation.[23] Even if Y-REDV bound to the PLLA surface, the stability of immobilized Y-REDV might not be so high and it could be removed by washing before XPS measurement. The reactive efficiency of tyrosine residues might be different depending on the type of substrate and the reaction conditions (temperature, time, pH, etc.). The amount of Y-REDV-immobilized on the glass surface was quantified by detecting amino acids after acidic hydrolysis (Figure S2). The amount of immobilized Y-REDV increased with increase in reaction time and temperature. When the reaction on glass was conducted under conditions used for the polymer substrates (i.e., at 37 °C for 24 h), the density of immobilized Y-REDV was about 8.3 pmol/mm^2. In addition, copper (Cu 2p) was not detected on the Y-REDV-immobilized surfaces for any of the substrates (data not shown), indicating that $CuCl_2$ was

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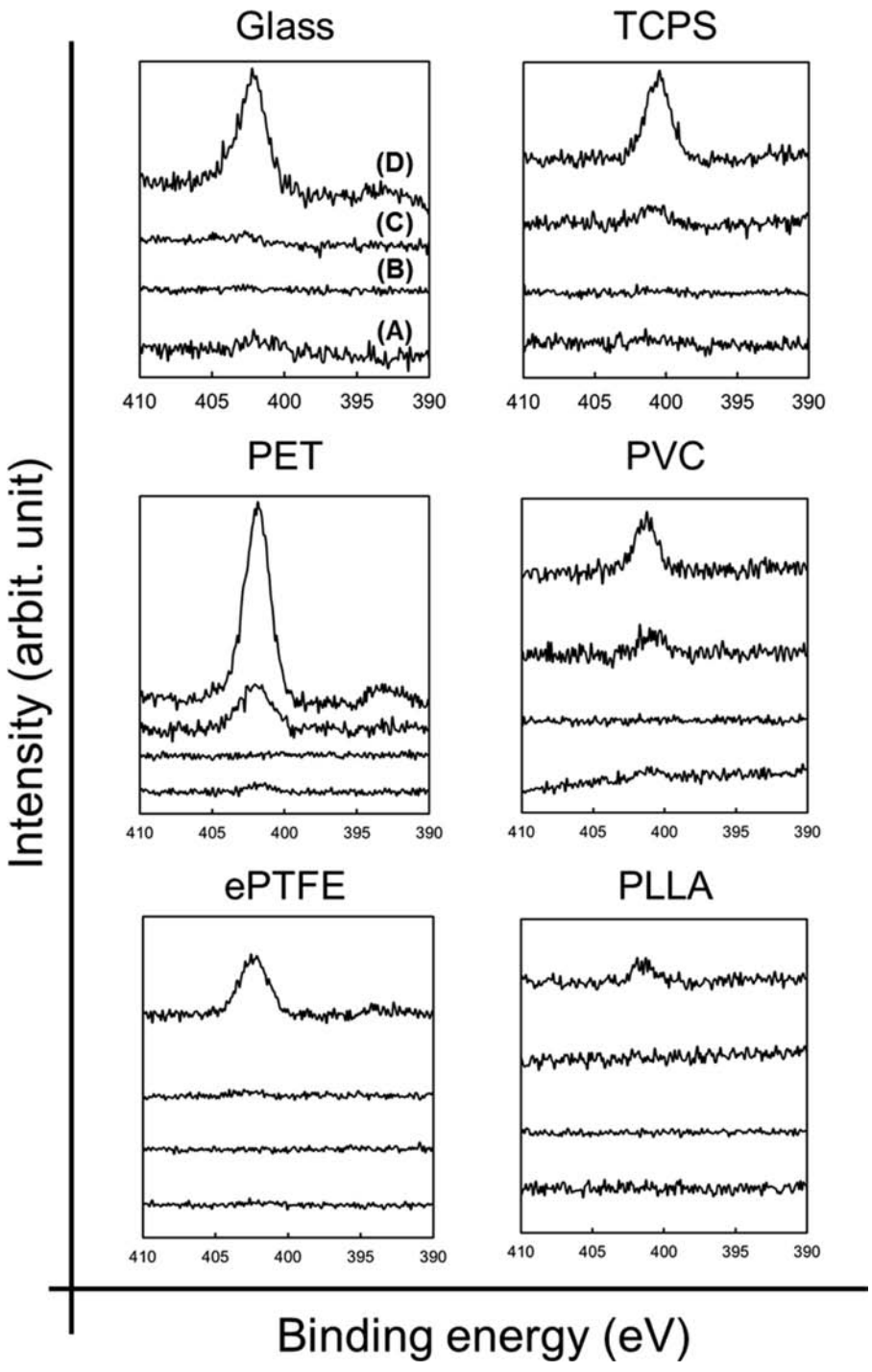

Figure 2. XPS spectra in the N 1s region of material surfaces: (A) unmodified, (B) $CuCl_2/H_2O_2$-treated, (C) Y-REDV-treated, and (D) Y-REDV/$CuCl_2/H_2O_2$-treated specimens.

removed after washing. Since copper is a cytotoxic metal, it should be removed completely.[24] The results of the surface analyses demonstrate that Y-REDV was successfully immobilized on all substrates through the single-step oxidation of tyrosine residues to DOPA using $CuCl_2$ and H_2O_2.

Figure 3 shows the adhesive behavior of human umbilical vein endothelial cells (HUVECs) in vitro in the absence and presence of 2% fetal bovine serum (FBS) and growth factors (GFs). In FBS/GFs-free medium, HUVECs adhered on the unmodified surfaces according to the inherent surface property of each substrate (Figure 3A). HUVECs were spread when adhered to glass, TCPS, PET, and PVC, but they were poorly spread when adhered on ePTFE and PLLA. This tendency was also shown on $CuCl_2/H_2O_2$-treated surfaces. On the Y-REDV-treated surfaces, it seems that HUVEC adhesion and spreading were slightly enhanced due to the nonspecifically adsorbed Y-REDV peptide. On the Y-REDV/$CuCl_2/H_2O_2$-treated surfaces, the adhesion and spreading of HUVECs were improved in all cases. By the addition of FBS and GFs in medium, the spreading of HUVEC was enhanced on the substrates except for ePTFE (Figure 3B). Adsorption of serum proteins was strongly suppressed on ePTFE, resulting in the low adhesion

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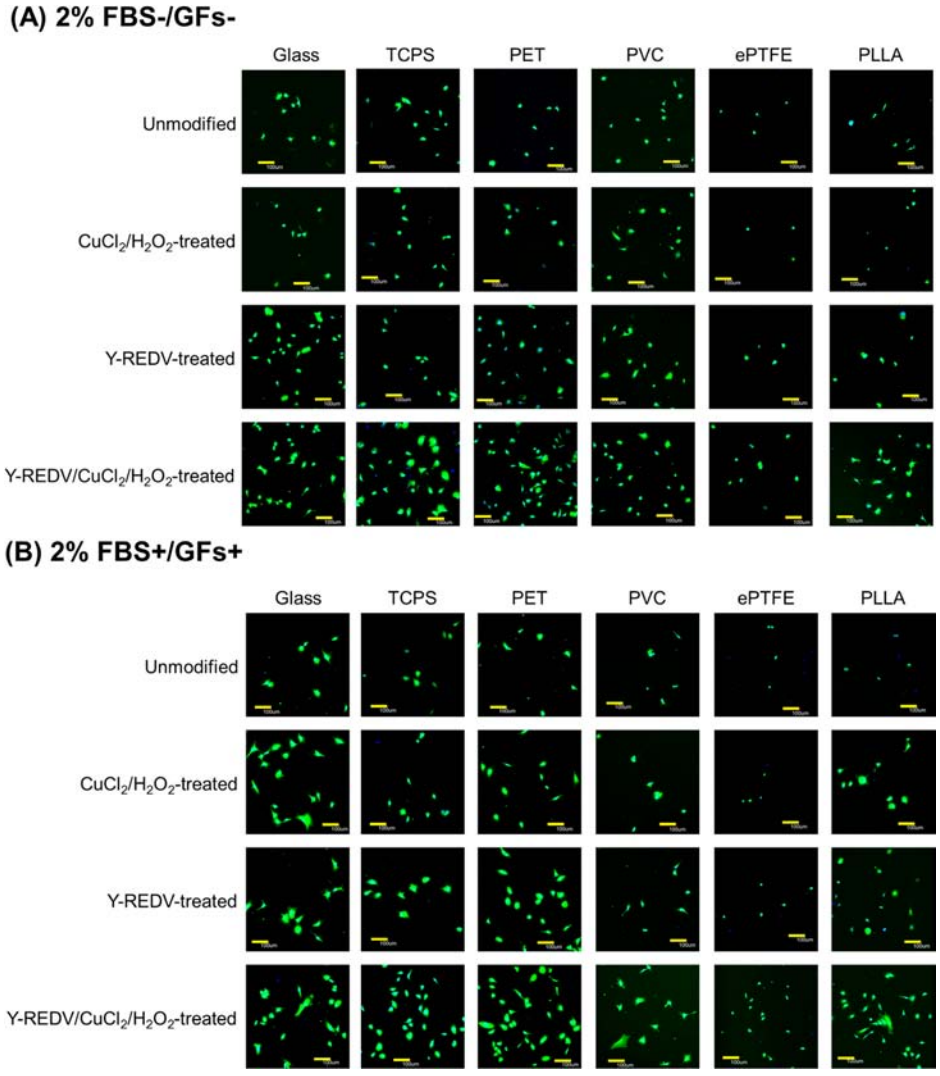

Figure 3. Morphology of adherent HUVECs on unmodified, $CuCl_2/H_2O_2$-treated, Y-REDV-treated, and Y-REDV/$CuCl_2/H_2O_2$-treated material surfaces in the medium (A) with or (B) without 2% FBS and growth factors.

and spreading of HUVECs.[25] The number of adherent HUVECs was directly counted from the entire image of cell seeding area (Figure 4). In FBS/GFs-free medium, the number of adherent HUVEC was similar on unmodified, $CuCl_2/H_2O_2$-treated, and Y-REDV-treated surfaces on each substrate. HUVEC adhesion was enhanced on the Y-REDV/$CuCl_2/H_2O_2$-treated surface of all substrates, especially glass, TCPS, and PET. The low adhesion of HUVECs on Y-REDV/$CuCl_2/H_2O_2$-treated PVC, ePTFE, and PLLA was caused by a low amount of immobilized Y-REDV. If the surface of each substrate was fully covered by Y-REDV, the adhesion behavior of HUVECs should be similar. Hence, this result indirectly indicates the difference in the density of immobilized Y-REDV on each substrate. The number of adherent HUVECs increased on all surfaces by the addition of FBS and GFs. This behavior might be due to the high activity of HUVEC in the FBS/GFs-containing medium, or the efficiency of nonspecific adsorption of serum proteins. From the in vitro experiment, cytotoxicity was not found on Y-REDV/$CuCl_2/H_2O_2$-treated surfaces of all substrates, indicating that copper was removed. Taken together, these results demonstrate that the Y-REDV immobilization through tyrosine oxidation promotes HUVEC adhesion and spreading on all substrates in both FBS/GFs-free and -containing mediums.

■ CONCLUSIONS

We successfully developed a simple and direct method for immobilizing bioactive peptides onto a variety of material surfaces without losing biological function. This was accomplished by the single-step oxidation of tyrosine residues in the presence of a copper catalyst and hydrogen peroxide in aqueous solution under mild conditions (37 °C, 24 h). Y-REDV/$CuCl_2/H_2O_2$-treated glass, TCPS, PET, PVC, ePTFE, and PLLA showed the enhancement of HUVEC adhesion and spreading. The advantage of this method is that the tyrosine residue is converted to DOPA by $CuCl_2$ and H_2O_2. Park et al. reported the immobilization of RGDS peptides onto a titanium oxide substrate through tyrosine oxidation catalyzed by tyrosinase. This reaction allowed the quick immobilization of peptides containing tyrosine residues by a single-step reaction within minutes. However, tyrosinase might also be immobilized on the material surfaces due to its own reactive groups. Although $CuCl_2/H_2O_2$-catalyzed tyrosine oxidation is slower than the tyrosinase-catalyzed reaction, it reduces the undesirable side reactions and yields peptide-immobilized surfaces with fewer

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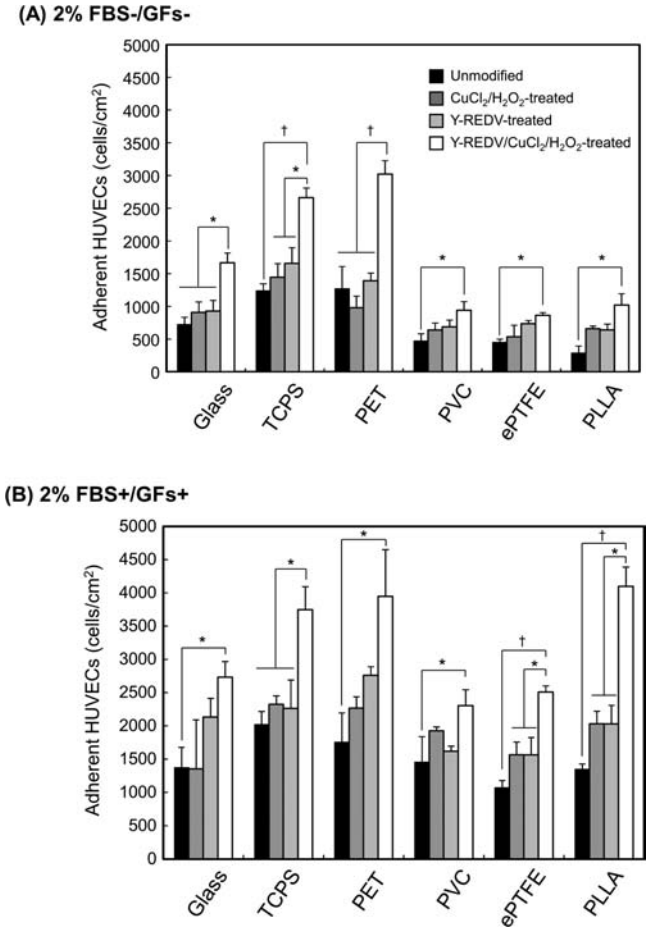

Figure 4. Number of adherent HUVECs on unmodified, $CuCl_2$/H_2O_2-treated, Y-REDV-treated, and Y-REDV/$CuCl_2$/H_2O_2-treated material surfaces in the medium (A) with or (B) without 2% FBS and growth factors. ($n = 3$; $^*p < 0.05$ and $^\dagger p < 0.01$ according to one-way ANOVA followed by Tukey's posthoc test).

impurities. The chemical structure of the covalent binding between tyrosine and substrates, the amount, distribution, and coverage of immobilized Y-REDV on polymer substrates should be further studied. The reaction efficiency could be improved by the optimization of reaction conditions in consideration of the stability of the Y-REDV and materials. Recently, many kinds of polymer materials have been used in medical devices. For instance, the blood-contacting component of an artificial cardiopulmonary device is composed of polymers such as polyethylene, polypropylene, polyurethane, polyvinyl chloride, polycarbonate, and polyester.[26] Our method is useful for immobilization of biological peptides on polymer surfaces, regardless of the presence of functional groups, using a single-step reaction. In addition, because quinones bind to not only polymers, but also inorganic materials such as metals and ceramics, this method could potentially be applied to devices such as stents, artificial joints, prosthetic implants, pins for artificial teeth, bone substitutes, and artificial heart valves.

■ ASSOCIATED CONTENT

Supporting Information

Peptide synthesis. Peptide immobilization and surface characterization. Quantification of immobilized Y-REDV on glass substrate. HUVEC adhesion. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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