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Letter

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Sustained Immobilization of Growth Factor Proteins Based on Functionalized Parylenes

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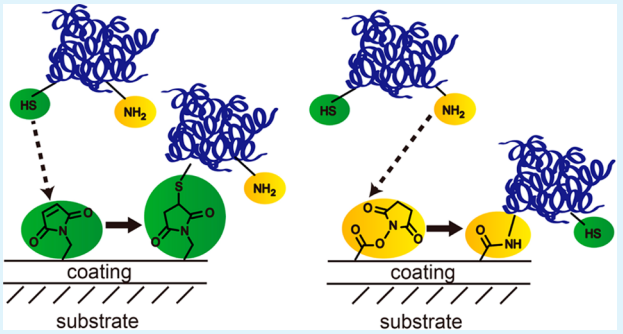
Supporting Information

ABSTRACT: Protein molecules immobilized on biomaterial surfaces are performed based on oriented conjugation or replaced mimicking peptides. The sustainable immobilization of growth factor proteins using functionalized parylene coatings is demonstrated in this study. Site-specific and nonspecific immobilization approaches are realized to conjugate bone morphogenetic protein (BMP-2). The binding affinities and conformational changes of BMP-2 are confirmed by QCM and SPR characterizations. Osteoinduction of stem cells is examined by ALP activity on the BMP-2 modified surfaces. Finally, immobilizations and equally sustained biological functions of vascular endothelial growth factor (VEGF) and a mimicking peptide of KLTWQELYQLKYKG (QK) are also examined and confirmed.



KEYWORDS: *protein immobilization, growth factor, biomaterial, bioconjugation, surface modification*

With recent advances, bioconjugation reactions have become essential tools in both fundamental research discovery and practical therapeutics. Stemming from engineering approaches toward modifying the nature of protein molecules,[1] studies of these reactions have extended beyond natural biomolecules[2] and are now successfully used in pharmaceutical molecules, synthetic biomolecules, biofunctional linkers, and newly designed polymer conjugates.[3] Development in this field has, during the past decade, moved into an era of exploring orthogonal and regioselective conjugations.[4] However, these advances hardly address the challenges of modifying protein molecules; a perfect strategy for protein engineering may yet be discovered.[5,6] Modification procedures require extra attention to retain the protein's native conformation and to control the aligned orientation,[6] and multiple steps are needed to introduce binding tags[7] or to execute recombinant approaches.[5] Methods have been developed to complete a specific task, but their applicability and versatility are limited for a wide range of applications, and the associated high cost has impeded their practical use in market products.[8] Although the above-mentioned challenges remain unsolved, the immobilization of proteins on biomaterials is still attempted due to the promising biological incentives for modified biomaterials.[8,9] In the future, ideal surfaces for immobilizing functional protein molecules are envisioned to be created based on (i) site-specific conjugation and oriented active sites for the immobilized protein molecules; (ii) the employment of mimetic peptide derivatives from proteins rather than large proteins; and (iii) simple approaches and minimized modification procedures on the proteins and material surfaces. The immobilization approaches developed should be broadly applicable for a variety of studies and practical uses. In this letter, we report the use of functionalized parylene coatings for the study of growth factor protein immobilization via both site-specific and nonspecific approaches. *N*-Hydroxysuccinimide (NHS) ester and maleimide functionalized parylenes are used as interlayers to provide anchoring sites for substrate materials. These polymer coatings belong to the parylene family, which are conformal coatings with excellent biocompatibility for biotechnological applications.[10] Their application on various materials, including metals, polymers, and silicon, has also shown promise.[11,12] The proposed approaches to immobilize growth factor proteins are illustrated in Figure 1. The site-specific approach defined herein is demonstrated using maleimide groups to which protein molecules are tethered via maleimide−thiol coupling reaction through native and accessible disulfides, and the use of mild conditions enables a preferential reduction of the exposed disulfide bonds without destroying the tertiary structure of the protein or abolishing its biological activities.[13,14] On the other

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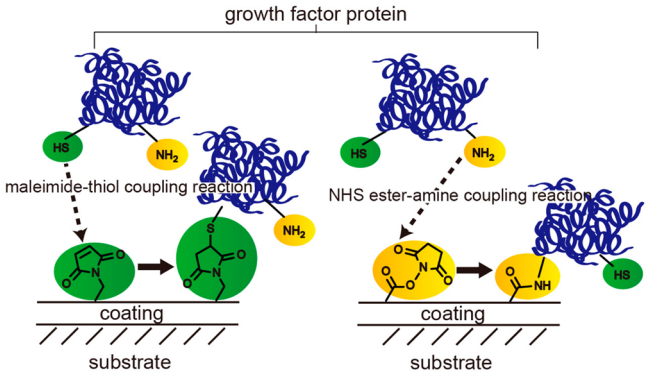

Figure 1. Schematic illustration of immobilizing growth factor proteins using site-specific and nonspecific coupling approaches based on maleimide and NHS ester functionalized coatings.

hand, when proteins are immobilized nonspecifically via NHS ester-amine coupling reaction, the technique that has provided the most straightforward route relies on the formation of an amide bond that is accessible for conjugation without denaturing the protein, although this is performed in a heterogeneous manner with respect to the number and location of the conjugated proteins.[15] Bone morphogenetic protein 2 (BMP-2) was used in this study, and the conformational changes of the immobilized BMP-2 with respect to site-specific and nonspecific approaches were characterized and compared using a combination of surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) analysis. The osteoinductive activity of the BMP-2 immobilized by the two approaches is expected to exert palpable modulatory effects in vitro on human mesenchymal stem cells (hMSCs), and distinct levels of osteocalcin production and bone nodule formation are anticipated. For reasons of cost effectiveness and processing feasibility, immobilized mimicking peptides may potentially and practically be introduced as replacements for growth factor proteins.[5] With this regard, immobilizations were also attempted herein by using the peptide KLTWQELYQLKYKG (QK) and vascular endothelial growth factor (VEGF) as models, and the resulting cellular responses toward endothelial cells were studied.

In the experiments, NHS ester-substituted [2.2]-paracyclophane was designed and synthesized as the starting material for chemical vapor deposition (CVD) polymerization to prepare a parylene coating containing NHS ester side groups, namely, poly[(*p*-xylylene-2-carboxylic acid *N*-hydroxysuccinimide ester-*co*-*p*-xylylene)] (hereafter referred to as NHS ester coating). The characterization data for the coating, including X-ray photoelectron spectroscopy (XPS) and infrared reflection absorption spectroscopy (IRRAS), are included in the Supporting Information. In addition, a maleimide-functionalized parylene coating, poly[(4-N-maleimidomethyl-*p*-xylylene)-*co*-(*p*-xylylene)] (hereafter referred to as maleimide coating), was prepared following previous reports, also by a CVD polymerization process.[16] The resulting NHS ester coating and maleimide coating provide NHS ester and maleimide anchoring sites that are readily available for orthogonal conjugations of NHS ester−amine and maleimide−thiol coupling reactions, respectively.

The immobilization of BMP-2 on NHS ester- and maleimide-coated substrates was first verified by IRRAS to show the characteristic band vibrations of the coatings and the conjugated BMP-2 molecules; two separate control experiments including the examination of nonspecifically adsorbed BMP-2 on a parylene coating that that does not contain any functional group (parylene N) as well as using a fluorescently labeled antibody to detect the immobilized or physically adsorbed BMP-2, were also performed, and the data are included in the Supporting Information. The binding capacities and affinities of the immobilized BMP-2 molecules was further addressed using a quartz crystal microbalance (QCM) system, which can dynamically monitor the mass of molecules adsorbed or conjugated on such crystals.[17] NHS ester and maleimide coatings were deposited on the crystals of a QCM system, and BMP-2 proteins were immobilized on the coated crystal surfaces accordingly. The influence on the sensitivity of modified crystal can be neglected from the fact that the coatings are well-adhered rigid films on the crystals,[18] and the coating thickness is in the range of approximately 150 nm reportedly with good accuracy for characterizations.[19] Two separate experiments were conducted for site-specific (maleimide coating) and nonspecific (NHS ester coating) approaches to BMP-2 immobilization, and both were compared

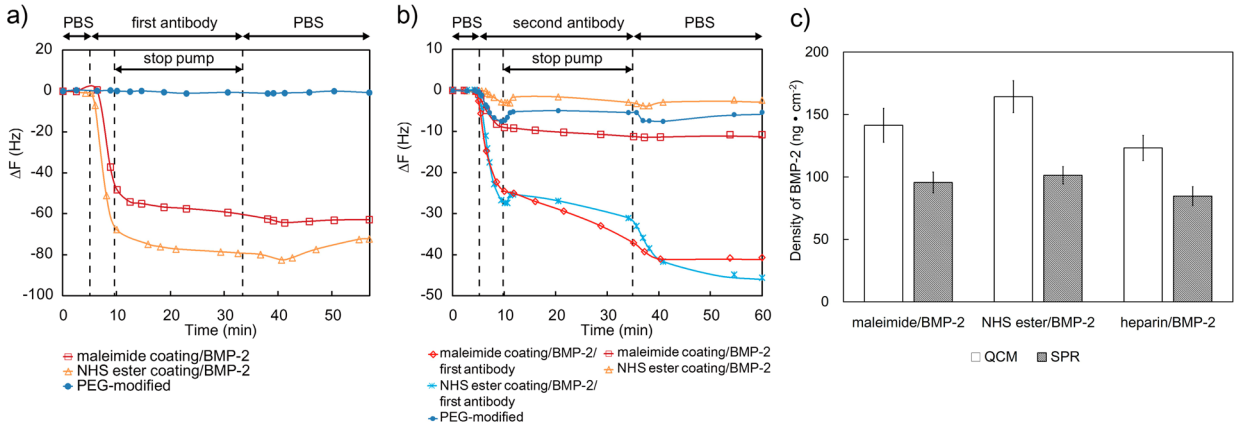

Figure 2. (a) QCM dynamic analysis of BMP-2 binding affinity toward primary antibody. (b) QCM dynamic analysis of binding affinity toward secondary antibody. (c) BMP-2 conformational analysis using a combination of QCM and SPR. Immobilization of BMP-2 protein was performed either by maleimide−thiol coupling reaction on maleimide coating or by NHS ester-amine coupling reaction on NHS ester coating, and the binding of BMP-2 on a heparin surface was used as a control.

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to a polyethylene glycol (PEG)-modified surface (control surface). Characterizations were performed by introducing human BMP-2 antibody (primary antibody) and a goat antimouse IgG antibody (secondary antibody) to the QCM system, and the binding of these antibodies was monitored and calculated following the Sauerbrey equation.[20] As indicated in Figure 2a, BMP-2 immobilized by either maleimide or NHS ester coating showed the anticipated high affinity toward BMP-2 antibody, and reduced frequencies of 62.1 ± 5.8 Hz (mean ± standard deviation, n = 3) and 72.6 ± 3.7 Hz were observed, which correspond to approximately 338.6 ± 31.8 ng cm^{-2} and 393.1 ± 20.4 ng cm^{-2} of bound BMP-2 antibodies, respectively. In contrast, the protein-resistant PEG-modified surface showed particularly low binding of the antibody of approximately 2.7 ± 2.2 ng·cm^{-2} (0.5 ± 0.4 Hz). Further experiments were conducted by introducing a secondary antibody to the system (Figure 2b), and as anticipated, specific binding affinities were observed for the surfaces with previously attached primary antibodies: approximately 224.9 ± 17.3 ng cm^{-2} and 251.2 ± 18.6 ng cm^{-2} were found for the maleimide and NHS ester coatings, respectively. Low binding affinities were observed on the control surfaces, including the PEG-modified surface (31.9 ± 6.6 ng·cm^{-2}) and the BMP-2-immobilized surfaces using maleimide or NHS ester coating (61.7 ± 5.0 ng cm^{-2} and 15.6 ± 4.9 ng cm^{-2}, respectively). The differences between site-specific and nonspecific BMP-2 immobilization were found to be less prominent; nevertheless, the binding affinities/activities (toward antibodies) of the immobilized BMP-2 of both approaches were confirmed.

The above results may be satisfactory, and a combination of QCM and surface plasmon resonance (SPR) analysis was thus used for further study. Compared to the acoustic measurements of QCM, the SPR technique measures and converts optical signals to the mass of bound molecules of interest, and the results are independent of molecular morphology and of other adsorbed molecules.[21] Thus, the mass difference between the two techniques is attributed to trapped water molecules within proteins,[22] and it was found that a denatured protein film contains higher amounts of water and shows higher dissipation values.[23] Characterization of immobilized BMP-2 by QCM and SPR are compared in Figure 2c. A higher level of trapped water molecules was discovered for BMP-2 immobilized nonspecifically via NHS using the ester-amine coupling route, and 63.0 ± 5.8 ng·cm^{-2} of water molecules was extrapolated. A lower level of water coupling (45.8 ± 5.3 ng·cm^{-2}) occurred on the other surface, with BMP-2 site-specifically immobilized via tethering to maleimide groups, which a more sustained protein structure of BMP-2 was hypothesized to exist on such a surface. A control experiment was also conducted to create a BMP-2 surface using a previously modified heparin layer, which has been shown to assist the binding of various growth factors with retained and activated biological activity.[24] The control surface was examined in parallel in this study, and the result indicated a lower water molecule content of 38.6 ± 2.5 ng cm^{-2}, similar to the site-specific immobilization approach, which unambiguously supports the hypothesis that a more sustained BMP-2 structure is possible using this approach.

In addition to characterizing the resulting biological responses on BMP-2 immobilized surfaces, osteoblastic differentiation of human mesenchymal stem cells (hMSCs) was further examined on both immobilized BMP-2 surfaces using the two approaches, and the activity of alkaline phosphatase (ALP), which is an early marker of osteogenesis,[25] was analyzed in the experiment. After 14 days of culture, hMSCs exhibited significantly higher ALP expression and cell morphological changes (Figure 3a, b) on both BMP-2 immobilized surfaces; in

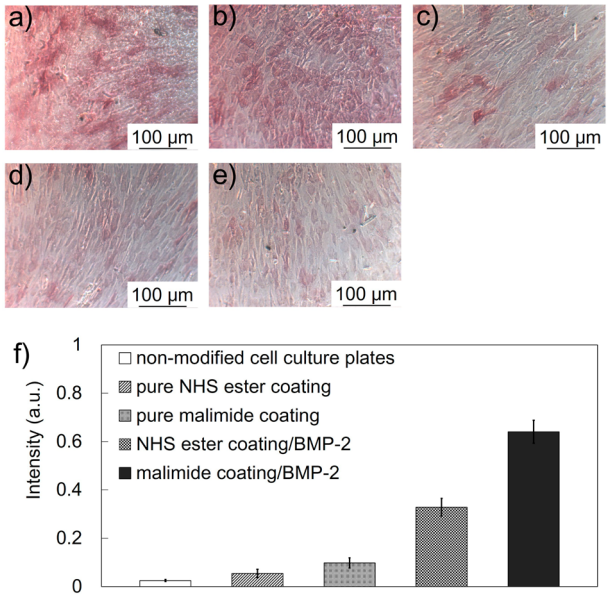

Figure 3. Induced ALP activity of hMSCs cultured on a surface of (a) immobilized BMP-2 by using maleimide coating; (b) immobilized BMP-2 by using NHS ester; (c) bare maleimide coating; (d) bare NHS ester coating; (e) nonmodified cell culture plates. (f) Statistical analysis of ALP results. The samples were analyzed after 14 days of cell culture, and each experimental group was evaluated in triplicate.

comparison, suppressed ALP expression was found on surfaces such as the bare maleimide coating, bare NHS ester coating, and nonmodified cell culture plates (Figure 3c–e) in which BMP-2 was not present. The quantification of the ALP results is presented in Figure 3f, and interestingly, a noticeably higher level of expression was found on the site-specific immobilized BMP-2 maleimide coating (intensity 0.64 ± 0.05) compared to the nonspecific NHS ester coating (intensity 0.32 ± 0.04); 50% more ALP was expressed on the former surface. In contrast, intensities below 0.12 were observed for the control groups in which no BMP-2 was immobilized on the surface. Consistent osteoinduction was also discovered by culturing porcine adipose derived stem cells (pADSCs) on such modified surfaces, and the results are included in the Supporting Information. The findings are showing consistency with data from QCM and SPR discussed above, and they indicate that the site-specific immobilization approach can better retain the structure of BMP-2 and provides effective binding domains that were better recognized by hMSCs and show stronger osteogenic differentiation.

Finally, attempts were made to immobilize functional peptide motifs of growth factors proven to maintain efficacious therapeutic dosage at the treated site with high stability and specificity. In addition, their low cost and ease of synthesis are attractive for practical use and mass production.[26] QK peptide (KLTWQELYQLKYKG), a VEGF-mimicking peptide,[27,28] was used as a model for immobilization on NHS ester coated surfaces, and cultured bovine arterial endothelial cells (BAECs) were subsequently studied on such VEGF- and QK-immobilized surfaces. Other control surfaces were tested in parallel, including a PEG-modified surface and a nonmodified

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cell culture plates. As shown in Figure 4a–e, after 2 days of culturing BAECs, higher rates of cell growth were observed on

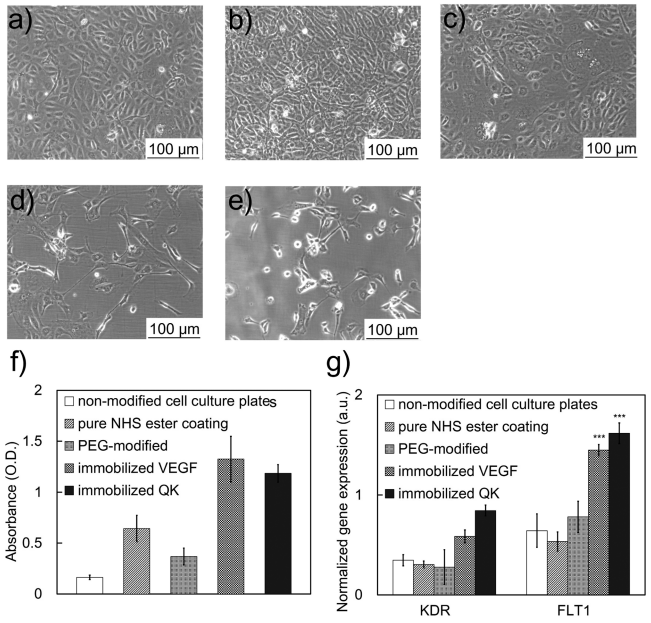

Figure 4. Cell culture study of BAECs on growth factor and mimicking peptide immobilized surfaces. Cell growth patterns after 2 days on (a) VEGF protein-immobilized surface; (b) QK peptide-immobilized surface; (c) bare NHS ester coating; (d) PEG-modified surface; and (e) nonmodified cell culture plates. (f) MTT assay. (g) Gene expression (*** $P < 0.001$).

surfaces where VEGF or QK were immobilized and confluent cell growth patterns were observed. Comparably, cell-adherent patterns were suppressed on the PEG-modified surface and on nonmodified cell culture plates. The MTT assay results shown in Figure 4f confirm the observations from these images quantitatively and show consistent cell viability on these surfaces; approximately 2-fold more viable cells were found on the VEGF- and QK-immobilized surfaces compared to the PEG- and nonmodified surfaces, indicating significantly enhanced cell proliferation and mitochondrion activity on the former surfaces. The angiogenic potential of the endothelial cells was further examined by reverse transcription polymerase chain reaction (RT-PCR) experiments. Two vascular related genes, KDR (VEGF receptor 1) and FLT1 (VEGF receptor 2), were chosen as angiogenesis markers.[29] The results were normalized using GAPDH as a reference gene[30] (results of GAPDH expression are shown in Supporting Information), and they were quantitatively analyzed for the tested surfaces. As summarized in Figure 4g, significantly enhanced expression of KDR and FLT1 was found for the surfaces with VEGF and QK immobilization, compared to surfaces with neither growth factors nor mimicking peptides. Although certain attributes were found to be slightly different between VEGF and QK, and verifying the differences may require detailed investigations in future studies, the combined cellular responses in terms of cell adhesion, cell growth, and angiogenesis patterns are improved with the VEGF and QK surfaces.

In summary, growth factor proteins were immobilized on material surfaces by exploiting both site-specific and nonspecific conjugation strategies via functionalized parylene coating. Biochemical characteristics with respect to binding affinities for corresponding primary and secondary antibodies and conformational changes upon conjugation were all found to be significantly enhanced on surfaces with immobilized BMP-2 protein, as was the resulting osteoinductive activity. The concept of immobilizing a biomimetic peptide such as QK to provide therapeutic effectiveness also demonstrated great potential as a replacement for its counterpart, the large protein VEGF. Taken together, the site-specific approach to immobilizing growth factor proteins and the concept of using peptide motifs of growth factors both showed encouraging results and may be useful in future strategies for installing functional proteins on material surfaces. Nevertheless, maleimide- and NHS ester-functionalized parylenes have been demonstrated to be a solid modification pathway for protein immobilization on the surface of biomaterials. Because of the high biocompatibility, a proven FDA track record, and good accessibility for conjugation with protein molecules without the need for postchemical modifications to the molecules or the material's surface, this protein immobilization platform not only is scientifically significant in new biomaterials design but also provides economic advantages in the downstream processes of industrial products.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details; XPS high-resolution C_{1s} spectra of NHS ester coating; IRRAS characterization of NHS ester coating; confirmations of protein immobilization on the coating surface; gene expression of GADPH, KDR, and FLT1 on each experimental surface; quantitative analysis of GADPH. 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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