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Fluorescent molecularly imprinted polymer thin films for specific protein detection prepared with dansyl ethylenediamine-conjugated *O*-acryloyl L-hydroxyproline

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ABSTRACT

Protein-imprinted polymers, capable of specific transduction of protein binding events into fluorescent signal change, were designed and synthesized by using dansyl ethylenediamine-conjugated *O*-acryloyl L-hydroxyproline (Hyp-En-Dans). Human serum albumin (HSA) was used as a model target protein and HSA-imprinted polymers (HSA-IP) were prepared on glass substrates. Specific fluorescence change was observed for HSA binding on the imprinted polymer thin film, whereas a weaker response was observed for other proteins, including bovine serum albumin, chymotrypsin, lysozyme, and avidin. The binding specificity was found to derive from the rigid structure of the hydrogen-bondable pyrrolidine moiety. Compared with SPR measurements, the non-specific binding caused by the polymer matrix and/or randomly located fluorescent monomer residues that did not compose specific binding sites did not contribute to the observed fluorescence change. These results revealed that the proposed protein-imprinting technique using Hyp-En-Dans could provide a highly selective protein-sensing platform, in which only specific binding events would be detected by fluorescent measurements.

1. Introduction

Protein recognition-based analytical techniques, which frequently employ antibodies as protein-recognizing molecules, have been intensively studied as tools for proteomics and diagnosis. However, the difficulties and high costs associated with the preparation of specific antibodies, as well as their limited stability dependant on external conditions, pose a challenge with the development of antibody-based analyses. In addition, unless complicated label-free detection systems are used, preparation of labeled antibodies/antigens is necessary for the detection of binding events, and this is not always easy without alteration of their binding properties. Therefore, conjugated synthetic materials possessing selective binding activity and facilitating detection of the binding events are desirable as substitutes.

Molecular imprinting is a promising method for preparing artificial receptors bearing pre-determined binding cavities capable of recognizing target molecules (Wulff, 1995; Sellergren, 2000; Haupt and Mosbach, 2000; Zimmerman et al., 2004; Komiyama et al., 2003; Takeuchi et al., 2005; Bonini et al., 2007; Hansen 2007; Takeuchi and Hishiya, 2008; Whitcombe et al., 2011; Menaker et al., 2009). Molecularly imprinted polymers (MIPs) are prepared by co-polymerization of functional monomers with crosslinkers in the presence of a template molecule. After the removal of the template molecule, binding cavities complementary to the template can be created. Furthermore, signaling functionality towards the binding events can be easily introduced when functional monomers conjugated to a reporter molecule are used during polymerization. In such cases, the molecular design of the functional monomers is critical to obtain highly specific signaling MIPs. Usually, signaling functions can be introduced into MIPs by using fluorescent functional monomers, where the fluorophore moiety is located close to the binding sites and the binding event can be transduced into the fluorescence change (Leung et al., 2001; Kubo et al., 2005; Tao et al., 2006).

Recently, binding targets of MIPs have been shifted from small molecules to proteins, and much effort has already been made to prepare MIPs for proteins (Bossi et al., 2007; Linares et al., 2009; Zhao et al., 2009). Generally, proteins are soft and flexible, and thus a variety of conformations on the protein-functional monomer complexes might be formed in a pre-polymerization mixture. This leads to creating numerous heterogeneous types of binding cavities in the resultant MIPs, which often yields lowered selectivity of protein binding. In order to obtain highly selective protein binding cavities, it is important to devise functional monomers that minimize the formation of complex conformers with target proteins.

In this study, in order to prepare signal-producing protein-imprinted polymers, we designed and synthesized a novel functional monomer, *O*-acryloyl L-hydroxyproline conjugated with

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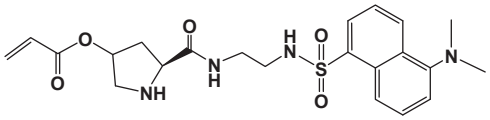

Fig. 1. Chemical structure of the functional monomer: *O*-Acryloyl L-hydroxyproline conjugated with dansyl ethylenediamine (Hyp-En-Dans).

dansyl ethylenediamine (Hyp-En-Dans), bearing a rigid hydrogen-bondable site for proteins (pyrrolidine moiety) and a fluorescent probe (dansyl moiety) for detecting hydrophobic regions (Fig. 1). The rigid structure of the proposed monomer may provide better molecular recognition ability compared with flexible linear alkyl chains. Additionally, the presence of a cyclic secondary amine in the pyrrolidine moiety, which can form a hydrogen bond with a certain substructure, where the rigid cyclic amine can be fit to interact in target proteins, may enhance the specificity of the binding sites in MIPs. Dansyl compounds, when moved to a microenvironment with a decreased polarity around the dansyl moiety, are known to have enhanced emission intensity together with a blue shift in the emission wavelength (Hill et al., 1996; Hayashida et al., 2007). In this context, if the dansyl moiety in the binding cavity of the proposed fluorescent functional monomer were oriented towards hydrophobic regions of the bound protein, the binding event would result in a measurable emission intensity change. Herein, we prepared MIP thin films for human serum albumin (HSA) by using this fluorescent functional monomer. HSA is a well-known biomarker of diabetic nephropathy and is reported to have several hydrophobic regions; it is therefore worth adopting HSA as a model protein in order to explore the signaling functions of the designed functional monomer and the resulting MIPs.

2. Experimental

2.1. Materials

Ethylenediamine, *N*,*N*′-diisopropylethylamine (DIEA), triethylamine (TEA), 5-(dimethylamino)-naphthalene-1-sulfonyl (dansyl) chloride, citric acid, sodium hydrogen carbonate, diethylether, methanol, dichloromethane, sodium chloride, trimethylchlorosilane, avidin from egg white, and human serum albumin (HSA, Fraction-V) were purchased from Nacalai Tesque Co. (Kyoto, Japan). Dichloromethane was distilled prior to use. Acrylamide, 2, 2'-azobis(2-methylpropione amidine) dihydrochloride (V-50), *N*, *N*′-methylenebisacrylamide, tris(hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), Boc-3-hydroxypyrrolidine, and magnesium sulfate (anhydrous) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 1-Hydroxybenzotriazole hydrate, di-*tert*-butyldicarbonate, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC•HCl), 4-hydroxy-pyrrolidine-1,2-dicarboxylic acid 1-*tert*-butyl ester were purchased from Watanabe Chemical Industries (Hiroshima, Japan). *N*-Hydroxysuccinimide (NHS), bovine serum albumin (BSA, Fraction-V), lysozyme, chymotrypsin from bovine pancreas, 11-mercaptoundecanoic acid, and 3-methylamino-1-propanol were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Trifluoroacetic acid (TFA), 4 M HCl in dioxane, (S)-3-hydroxy-1-methylpyrrolidine, 3-(dimethylamino)-1-propanol, and acryloyl chloride were purchased from Tokyo Chemical Industries, Co (Tokyo, Japan). Triethoxysilylpropyl maleamic acid was purchased from AZmax (Chiba, Japan). 2-Morpholinoethanesulfonic acid, monohydrate (MES) was obtained from Dojindo (Kumamoto, Japan).

2.2. Synthesis of Hyp-En-Dans

2.2.1. 5-Dimethylamino-naphthalene-1-sulfonic acid (2-amino-ethyl)-amide (dansyl ethylenediamine) (**1**)

Dansyl chloride (2.0 g 7.42 mmoL) dissolved in dichloromethane (20 mL) was slowly added into a solution of ethylenediamine (10 mL 20 eq) dissolved in dichloromethane (30 mL) using a dropping funnel with stirring at 0 °C over 30 min. After checking the disappearance of a spot of the 5-dimethylamino-naphthalene-1-sulfonyl chloride on a TLC plate (developing solvent: ethyl acetate), the solvent was removed by evaporation and then dissolved in ethyl acetate. The solution was washed with NaCl (aq) and dried over $MgSO_4$. The solvent was evaporated and removed in vacuo, giving **1** as a yellow–green solid. (1.82 g, 91.2%).

^{1}H NMR (300.40 MHz, $CDCl_3$) δ=2.68–2.72 (t, NCH_2, 2H, 2.92) (s, SO_2NCH_2, 2H, 7.18–7.21) (d, J=9 Hz, naphthalene, 1H), 7.50–7.60 (m, naphthalene, 2H), 8.25–8.31 (m, naphthalene, 2H), 8.53–8.56 (d, J=9 Hz, naphthalene, 1H) MALDI–TOF–MS (matrix: CHCA) m/z=292.83 [M], 293.89 [M+H], 316.79 [M+Na].

2.2.2. 4-Acryloyloxy-pyrrolidine-1,2-dicarboxylic acid 1-tert-butyl ester (**2**)

Acryloyl chloride (1.0 mL, 13.0 mmol) dissolved in dichloromethane (20 mL) was slowly added into a solution of 4-hydroxy-pyrrolidine-1,2-dicarboxylic acid 1-tert-butyl ester (2.0 g, 8.65 mmol) and triethylamine (2.4 mL, 17.3 mmol) dissolved in dichloromethane (30 mL), using a dropping funnel with stirring at 0 °C over 30 min. The reaction was terminated by checking TLC (developing solvent: ethyl acetate). The reaction mixture was evaporated and the obtained residue was dissolved in ethyl acetate. The solution was washed with citric acid (aq) and NaCl (aq). Then, the organic solution was back-extracted to $NaHCO_3$ (aq), followed by extraction to ethyl acetate layer with citric acid (aq). After drying over $MgSO_4$, the solvent was removed by azeotropic evaporation with methylene chloride, giving **2** as a colorless and clear liquid. (0.96 g, 39.2%).

^{1}H NMR (300.40 MHz, $CDCl_3$) δ=1.49 (s, *t*-Bu, 9H), 4.40–4.55 (t, J=7.5 Hz, CHCOOH, 1H), 5.88–5.91 (m, C=C,1H), 6.06–6.16 (m, C=C, 1 H), 6.40–6.46 (m, C=C, 1 H).

2.2.3. 4-Acryloyloxy-2-[2-(5-dimethylamino-naphthalene-1-sulfonylamino)-ethylcarbamoyl]-pyrrolidine-1-carboxylic acid tert-butyl ester (**3**)

Compound **1** (773.6 mg, 2.63 mmol), compound **2** (623.4 mg, 2.19 mmol), EDC·HCl (560 mg, 3.86 mmol), 1-hydroxybenzotriazole (370.0 mg, 3.86 mmol) and *N*,*N*′-diisopropylethylamine (1.0 mL 5.48 mmol) were added to 50 mL of dichloromethane, and the mixture was stirred overnight at room temperature under light shielding. The reaction was terminated by checking TLC (developing solvent: ethyl acetate). The reaction mixture was evaporated and the obtained residue was dissolved in ethyl acetate. The solution was washed with citric acid (aq), $NaHCO_3$ (aq) and NaCl (aq) and dried over $MgSO_4$. The solvent was removed by evaporation, and the obtained product was purified by silica gel column chromatography (eluent: ethyl acetate), giving **3** as a yellow–green solid. (0.82 g, 67.2%).

^{1}H NMR (300.40 MHz, $CDCl_3$) δ=1.45 (s, t-Bu, 9H), 2.89 (s, N $(CH_3)_2$, 6H), 3.11 (br, SO_2N CH_2, 2H), 3.33 (br,$CONCH_2$, 2H), 4.18 (s,NCOCH, 1H), 5.86–5.89 (d, J=9 Hz, C=C, 1H),6.07–6.16 (m, C=C, 1H), 6.39–6.45 (d, J=9 Hz,C=C, 1H), 7.17–7.19 (d, J=9 Hz, naphthalene, 1H), 7.49–7.60 (m, naphthalene, 2H), 8.21–8.31 (d, J=9 Hz, naphthalene, 2H), 8.52–8.55 (d, J=9 Hz, naphthalene, 1H) MALDI–TOF–MS (matrix: CHCA) m/z: 462.46 [M+H-Boc],m/z: 561.51 [M+H].