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Dual-targeting peptide probe for sequence- and structure-sensitive sensing of serum albumin

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ABSTRACT

Peptide-protein interactions mediate numerous biologic processes and provide great opportunity for developing peptide probes and analytical approaches for detecting and interfering with recognition events. Molecular interactions usually take place on the heterogeneous surface of proteins, and the spatial distribution and arrangement of probes are therefore crucial for achieving high specificity and sensitivity in the bioassays. In this study, small linear peptides, homogenous peptide dimers and hetero bivalent peptides were designed for site-specific recognition of human serum albumin (HSA). Three hydrophilic regions located at different subdomains of HSA were chosen as targets for the molecular design. The binding affinity, selectivity and kinetics of the candidates were screened with surface plasmon resonance imaging (SPRi) and fluoroimmuno assays. Benefiting from the synergistic effect from the surface-targeted peptide binders and the flexible spacer, a heterogenetic dimer peptide (**heter-7**) with fast binding and slow dissociation behavior was identified as the optimized probe. **Heter-7** specifically recognizes the target protein HSA, and effectively blocks the binding of antibody to HSA. Its inhibitory activity was estimated as 83 nM. It is noteworthy that **heter-7** can distinguish serum albumins from different species despite high similarities in sequence and structure of these proteins. This hetero bivalent peptide shows promise for use in serum proteomics, disease detection and drug transport, and provides an effective approach for promoting the affinity and selectivity of ligands to achieve desirable chemical and biological outcomes.

1. Introduction

Peptide-protein interactions mediate a wide range of biological processes including signaling pathways, DNA replication machinery, protein trafficking and immune response, and also control many protein-protein interactions (Pawson and Nash, 2003; Wu et al., 2010; Xiong et al., 2002). Based on such multifarious function, peptide-protein interactions have generated interest in the fields of chemistry, biology and medicine (Ding et al., 2016; Hossain et al., 2016; Lewin et al., 2000). Understanding the principles of these interactions as they occur naturally, and discovery of new interaction partners is critical for drug screening, disease treatment, as well as for development of effective bioanalytical techniques (An et al., 2015; Gray and Brown, 2014; Healy et al., 2015).

Considerable effort has been made to advance understanding of basic and applied aspects of peptide-protein binding events. Peptide libraries (Larman et al., 2011; Wu et al., 2014; Gray and Brown, 2014; Peng et al., 2006) and array technologies (Doran and Kodadek, 2014) provide plentiful resources to identify peptide binders to target proteins. Progress in analytical methods provides effective strategies for discovery and characterization of these interactions (Debnath et al., 2011; Diana et al., 2015; Frei et al., 2013; Goh et al., 2014). With increasing knowledge, peptide-mediated interactions have been shown to have small binding interfaces, fast response to stimuli, and operation through short linear sequences on the protein surface (Petsalaki and Russell et al., 2008). These are beneficial for manipulating cellular processes, blocking disease pathways and detecting target molecules (Hu et al., 2014; Zhou et al., 2016; Lipp et al., 2015; Hwang et al., 2017). However, the above features also bring moderate affinity and specificity as compared with protein-protein interactions, and may be difficult to handle biochemically. This makes application of these peptides in bioassays of complex biosystems challenging.

To address the problems of affinity and specificity in peptide probes, mimicking natural protein interactions is an attractive way (Pelay-Gimeno et al., 2015; Shiba, 2010; Chen et al., 2014; Grigoryan et al., 2009; Lawson et al., 2013). Multimetric ligands in particular are

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effective due to the benefit of synergistic effects (Janssen et al., 2013; Joshi et al, 2008; Vance et al., 2009). Covalently attaching multiple copies of recognition elements to a molecular scaffold yields peptide dendrimers which possess enhanced binding ability and functionality. Successful application of multimeric peptides has been demonstrated in small antibody mimetics, bioimaging and drug delivery (Cai et al., 2014; Chu et al., 2015; Rao et al., 1998; Wan and Alewood, 2016). Currently various branched structures such as polylysine, polyglycerol and poly(amidoamine) are available, and afford diverse functional surface groups for chemical ligation and fine control of conjugation chemistry (Cheng et al., 2011; Choi et al., 2000). The spatial distribution and surface arrangement of these ligands are critical for achieving the desired binding behavior towards different target proteins (Kojima et al., 2014; Kwok et al., 2013; Wan et al., 2015; Xu et al., 2016). Moreover, compared to well-characterized homomeric dendrimers with multiple identical peptide sequences, branched peptides with heterogeneous targeting ability still need further exploration.

Human serum albumin (HSA) is the most abundant protein in plasma, and plays crucial roles in maintaining blood pressure, buffering plasma pH and transporting various endogenous and exogenous species (Fanali et al., 2012; He and Carter, 1992; Yu et al., 2016; Zhang et al., 2008). HSA is also frequently used as a therapeutic reagent in patients during the restoration of lost fluid and blood (Fan et al., 2014). Recent studies have revealed HSA has a long half-life and an intrinsic capability to extravasate and accumulate in solid tumors. These findings render HSA useful for cancer treatment (Byeon et al., 2014; Li et al., 2016). BSA with high structural similarity to HSA but much cheaper is usually used as an alternative to HSA in many biochemical applications. However, misuse of BSA instead of HSA to patients can lead to life-threatening injury (Fan et al., 2014; Sohl and Splittgerber, 1991; Bujacz, 2012). Therefore, it is of great importance to develop targeting probes and biosensors which recognize HSA but not BSA (Reja et al., 2016; Fan et al., 2014; Prasad et al., 2013).

In this study, small linear peptides, homo peptide dimers and hetero targeting peptides were designed to recognize three hydrophilic regions located on the surface of different HSA subdomains. The affinity and selectivity of these molecules were screened with surface plasmon resonance imaging (SPRi) and fluoroimmuno assays. To give more insight into these peptide-protein interactions, binding kinetics were also monitored in real-time. A hetero dimer peptide was identified as the optimum probe. Its binding specificity, structure sensitivity and inhibitory activity against antibody binding to HSA was also examined. The interactions between the hetero dimer and serum albumins were further analyzed with molecular modeling.

2. Experimental

2.1. Peptide synthesis

Small linear peptides (**mono-1**–**mono-3**), homo peptide dimers (**homo-4, homo-5**) and a hetero targeting peptide (**heter-6**) were synthesized manually using FMOC solid phase peptide synthesis. FMOC-amino acid-Wang resins were used as the starting material. 4-Methylmorpholine was used as activating reagents. HBTU was used as the coupling agent. After elongation, peptides were cleaved from the resins and analyzed with HPLC and MS. The cleavage conditions, yields and purities of the peptides were provided in Supplementary Material.

2.2. Synthesis of the hetero bivalent dimer (*heter-7*)

The copper-catalyzed azide-alkyne cycloaddition reaction was employed for synthesis of hetero bivalent dimer (**heter-7**). FMOC-propargyl-Gly-OH was attached to the N-terminal of **mono-2** during the solid phase peptide synthesis. Azido-PEG(24) carboxylic acid was coupled to the N-terminal amino group of **mono-3** using the on-resin condensation reaction. After FMOC deprotection and cleavage, **propargyl-Gly-2** and **azido-PEG-3** were obtained and characterized (the cleavage conditions were provided in Supplementary Material). Click reaction was carried out with 1 equiv of **azido-PEG-3** (3.0 mg, 2.5 mM) and 1.2 equiv of **propargyl-Gly-2** (1.5 mg, 3 mM) in DMF containing 1% (v/v) trimethylamine. $CuSO_4$ (0.3 mg, 3 mM) and sodium ascorbate (6 mM) were added to catalyze the reaction. The mixture was degassed and purged with nitrogen. The reaction vessel was sealed and the mixture was stirred for 2 h at room temperature. After purification, **heter-7** was obtained as a white solid in 10.7% yield, and characterized with HPLC and high resolution mass spectrometry (Table S1 and Fig. S14).

2.3. SPRi sensing

SPRi analysis was measured on a Plexera PlexArray HT system (Plexera LLC, Bothell, WA). The system was operated at a flow rate of 2 μL/s. PBS buffer was employed as the running buffer throughout given its compatibility with the analysis of serum albumins (Anees et al., 2014; Canoa et al., 2015; Shim and Reaney, 2015; Jiang et al., 2014). The chip surface was first balanced with PBS to obtain a baseline. HSA sample solutions of different concentrations were then injected for interaction with immobilized peptides. After 300 s, dissociation was brought about by injection of PBS. The sensor chip surface was regenerated with 0.5% (v/v) H_3PO_4. Real-time binding signals were recorded and analyzed with PlexArray HT software. Kinetic analysis was performed using BIA evaluation.

2.4. Fluorescence-Linked Immunosorbent Assay (FLISA)

In a 96-well plate, aliquots (100 μL) of 5% HSA (w/v) in sodium carbonate buffer (0.1 M, pH 9.6) were added to each well. After incubation at 4 °C overnight, the plates were washed with PBST (PBS with 0.05% Tween-20) once, and then washed twice with PBS. Different peptides (0.15 mM, in PBS) were then added into the wells and incubated for 2 h at 37 °C. After washing with PBS three times, 100 μL FITC-labeled antibody against HSA (10 μg/mL) was added and incubated for 1 h at 37 °C to allow binding with HSA. After the final washing step, fluorescence was measured using a microplate reader. The excitation wavelength was set at 490 nm, and the emission intensity at 525 nm was recorded. A control experiment was carried out following the same procedure except for the addition of peptide. These experiments were repeated, and all data were obtained in triplicate.

3. Results and discussion

3.1. Design of peptide monomers

HSA contains three homologous domains (I-III), and each of these is further divided into two separate helical subdomains (A and B) (Carter et al., 1989). This modular structural organization of HSA is the basis of its unique ligand binding ability and biological function. As peptide-protein interactions are mediated by short linear sequences (London et al., 2010; Stein and Aloy, 2008), three 6-mer hydrophilic fragments distributed in different domains of HSA were chosen as the targets (Scheme 1). Residues 82–87 (ETYGEM) located in domain IA were defined as target 1 (T1); Residues 268–273 (QDSISS) and 499–504 (PKEFNA) are distributed in domain IIA and III and were selected as target 2 (T2) and target 3 (T3) respectively.

Evidence suggests that antisense peptides can interact specifically with their corresponding sense peptides (Heal et al., 2002; Miller, 2015). Considering the extended conformation T1-T3, use of antisense peptides provides a suitable means for the effective design of affinity peptide binders. Based on the genetic sequences of T1-T3, three hexapeptides **mono-1** (HFTIGF), **mono-2** (TGDRIL) and **mono-3** (SIKLFG), were readily designed by direct reading of corresponding

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