Fluorescent 'keep-on' type pharmacophore obtained from dynamic combinatorial library of Schiff bases

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Abstract

We established a novel principle for fluorescence detection of a target protein. A low-molecular-weight fluorescent pharmacophore, as a targeted probe, was selected from a dynamic combinatorial library of Schiff bases. The pharmacophore retains its fluorescence when bound to the hydrophobic site of the target, whereas it loses it because of hydrolysis when unbound.

Keywords

Fluorescent HSA binder, dynamic combinatorial library, Schiff base, size-exclusion chromatography, hydrolysis, protein-ligand docking simulation

Introduction

Fluorescence-based molecular targeted probes are widely used both *in vivo* and *in vitro* for sensing and quantification of target biologics, such as proteins(1, 2) or nucleic acids(3, 4). Among them, low-molecular-weight probes(5-7) usually possess several advantages such as cell permeability(8-10) and feasibility in large-scale production. Generally, a typical low-molecular-weight fluorescent probe contains three components: a pharmacophore, a fluorophore and a linker(8, 11). However, attaching such fluorophores, especially big ones, often perturbs biological property of the pharmacophore. Hopefully, the fluorophore would be one part of the pharmacophore(11). More preferably, the fluorophore itself would be an 'all-in-one' type pharmacophore(12, 13).

Until now, practical 'all-in-one' fluorescent pharmacophores can be generally categorized into two major groups: always-on probes (Fig. 1A), and turn-on ones (Fig. 1B). In case of the former, the probes always give a fluorescent signal no matter if they are bound to the target biologics. A major problem of the always-on probes is that meaningful signal only arises if unbound probes can be thoroughly washed out from the system. This wash-out process is tedious, and even after that, it often results in huge background fluorescence signals. In some cases, detection by changing of fluorescence polarization(14-16) is alternatively used for avoiding the wash-out process(17). In case of the latter group, turn-on probes can instantly detect biologics by increasing of fluorescence intensity, without suffering from the huge background or tedious washing process(11). For the turning-on, conspicuous solvatochromic property together with target selectivity / affinity of the pharmacophore are simultaneously required upon molecular designing. Because of this limitation of molecular diversity in the rational designing, the scientific community is gradually moving to obtain such targeted turn-on probes by combinatorial methods using solvatochromic fluorophore libraries (8, 9, 18, 19).

Independent to these two principles, we hereby propose the 3rd category of all-in-one fluorescent pharmacophore and define it as 'keep-on' type; the principle is that the fluorescent pharmacophore would be kept intact when bound to the target protein, whereas quenched by degradation when unbound (Fig. 1C).

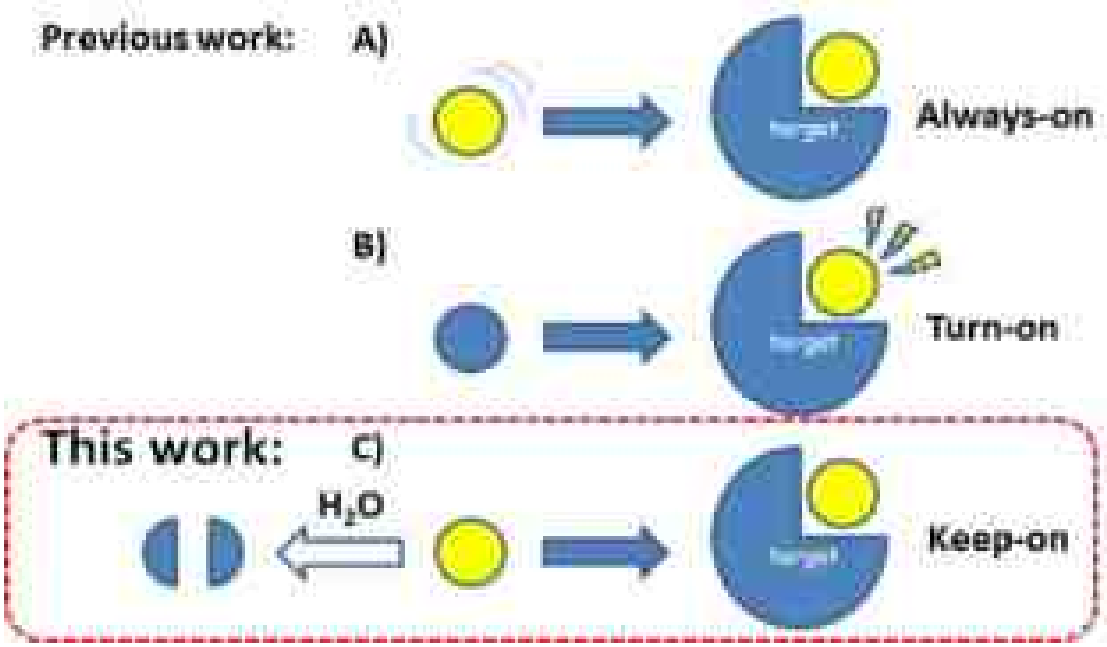

Fig. 1. Principles for detecting target biologics by 'all-in-one' type fluorescent pharmacophores without wash-out process. A) For the always-on probe, detection by fluorescence polarization is often used on the basis of rotational mobility changing of the pharmacophore. B) For the turn-on probe, solvatochromism upon binding is observed by changing of microenvironment polarity of the pharmacophore. C) For the keep-on probe, the fluorescence of the pharmacophore remains intact upon binding, otherwise it is quenched by degradation when unbound.

Materials and methods

A dynamic combinatorial library (DCL) of Schiff bases was synthesized by mixing 14 aryl amines and 13 aryl aldehydes (Fig. 2). It was incubated with a target protein (i.e., human serum albumin; HSA), and treated with sodium cyanoborohydride. Then, the HSA-bound candidates were selected by size-exclusion chromatography (SEC) followed by ethanol treatment, and deduced by liquid chromatography-mass spectrometry (LC-MS) equipped with photo diode array (PDA). The deduced candidates were synthesized in parallel, and a fluorescent keep-on-type pharmacophore for HSA was selected from the candidates by fluorescence analysis. The identification of the selected keep-on pharmacophore was shown in Fig. S3. For the details, see the Electronic Supplementary Material (ESM).

Results and discussion

To establish a methodology to obtain the keep-on-type fluorescent pharmacophore, we synthesized a DCL(20-24) of (hetero)aryl-conjugated Schiff bases(25) because of the following reasons. First, a number of precursors possessing aryl amino- or aldehydes / ketones are commercially available, so as some of the coupled Schiff base tend to possess fluorescence property(26) when excited in the long ultraviolet or visible spectral region. Second, the fluorescence of the Schiff base would vanish because of hydrolysis when it is not bound to a target protein and exposed to aqueous environment; only when the Schiff base is bound in a hydrophobic site of the target, the hydrolysis would be avoided to keep its fluorescence on.

The DCL of Schiff base library was made as shown in Fig. 2, and it was screened against a target protein, on the basis of SEC followed by LC-MS(27, 28). As the model target protein, we chose HSA because it is commercially available and structural analysis such as binding-site determination is established and easily performed(29). After incubation of the DCL with HSA, the library mixture was reduced by sodium cyanoborohydride.(25) Then, it was passed through a SEC column to retain all of the unbound library compounds. The eluent was denatured by mixing with ethanol, to release the reduced HSA binders from the protein. The released mixture was then analyzed by LC-MS equipped with PDA for measurement of absorption spectra (Fig. S1). Obviously, total numbers of the peaks were reduced after the SEC selection, because HSA-unbound compounds were adsorbed on the column and eliminated from the library. As shown in the bottom of Fig. 2, seven Schiff bases (i.e., potential HSA binders) from the starting 182 compounds were deduced as candidates by combination analysis of absorption and mass number. Among them, an already-known HSA binder (**A12L13**)(30) was successfully recovered. This means that the selection system using SEC was appropriately working. Because this binder is known to be a turn-on-type pharmacophore for HSA(30), we excluded it for further experiment.

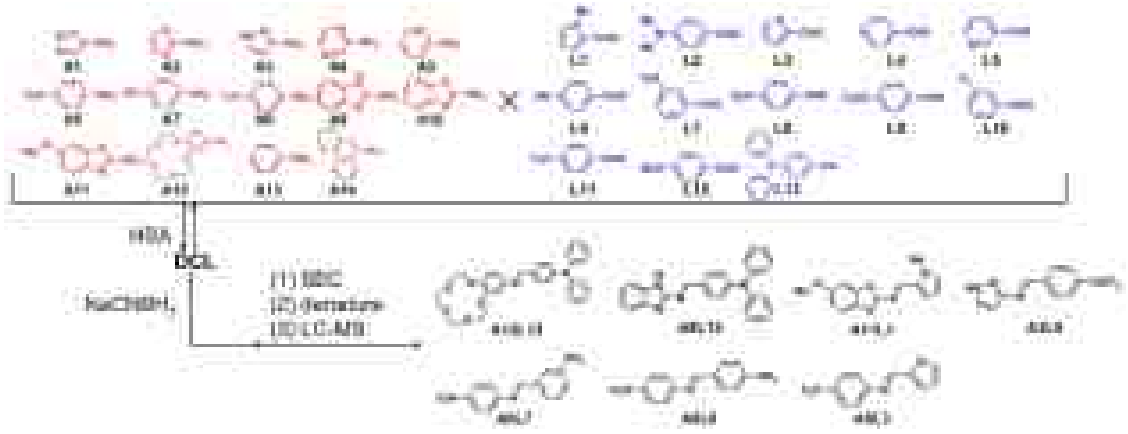

Fig. 2. Selection of HSA binders from a dynamic combinatorial library (DCL) of Schiff

bases by combination of size-exclusion chromatography (SEC) / LC-MS methods. The mixture of 182 different Schiff bases (top) was once incubated with the target protein (i.e., HSA), and then reduced with sodium cyanoborohydride ($NaCNBH_3$). The protein-*bound* candidates were separated by SEC. Seven candidates, shown in the bottom, as potential HSA binders were deduced by LC-MS.

Next, the remaining six candidates were independently synthesized, and each fluorescence property was evaluated. As shown in Fig. S2, only one candidate (i.e., **A9L13**) from the six Schiff bases showed intense fluorescence when excited at 365 nm. When the candidate was mixed with HSA, it showed solvatochromism, and more importantly, it retained its fluorescence intensity even after 100 minutes incubation at room temperature (Fig. 3A). Of course, the fluorescence of this HSA binder gradually disappeared because of hydrolysis when HSA was absent (Fig. 3B). These results clearly suggest that we could successfully obtain a fluorescent keep-on-type pharmacophore from DCL of Schiff bases.

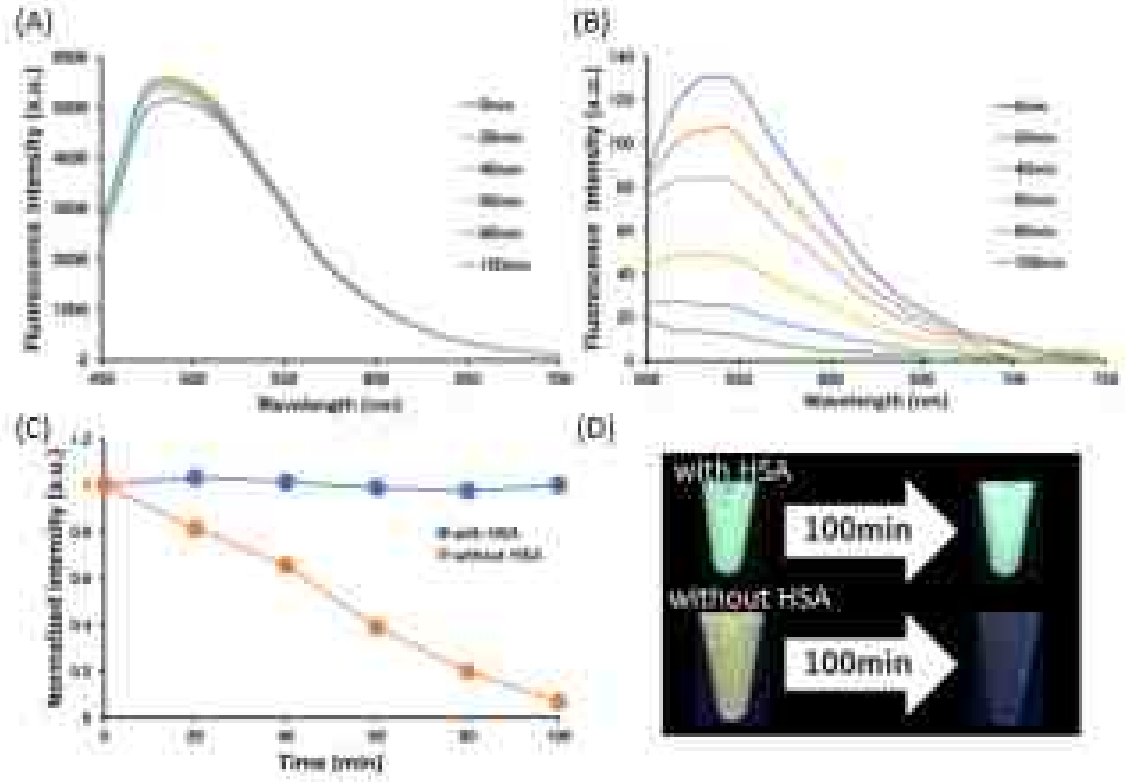

Fig. 3. Fluorescence emission titration of 'keep-on' type pharmacophore (**A9L13**) in the (A) presence or (B) absence of HSA when excited at 365 nm in D-PBS / 4% DMSO using NanoDrop 3300 fluorospectrometer. A 70 nm hypochromic shift of the emission peak-top was observed when the pharmacophore was bound to HSA. (C) The normalized fluorescence intensity-changing at 600 nm was plotted against the incubation time. (D)

Images of the buffered solution of **A9L13** in each state were taken by a smartphone under the irradiation at 365 nm by a UV hand lamp.

The pharmacophore-binding site of HSA was determined by a competition assay in the presence of salicylic acid or tryptophan, which is known as a site I or II HSA binder, respectively(31). As shown in Fig. S4, the interaction between tryptophan and HSA was disrupted in the presence of the keep-on pharmacophore, whereas that between salicylic acid and HSA was not. This clearly suggests that the keep-on pharmacophore bound to site II.

The site II-favorable binding of the pharmacophore was also rationalized by a protein-ligand docking simulation using sievgene(32) of myPresto(33). Thirty separate poses resulted in docking to site II with free energy in the range -9.75 to -7.47 kcal/mol, whereas those to site I in -8.51 to -5.62 kcal/mol. As shown in Fig. S5, the docking model of site II with the lowest energy suggested that the pharmacophore was buried inside the deep hydrophobic pocket and shielded from the aqueous environment, which could have prevented hydrolysis.

Finally, we evaluated binding affinity and target selectivity of the keep-on pharmacophore toward HSA. Judging from fluorescence titration, the affinity of the keep-on pharmacophore was similar to that of the previously discovered HSA binder possessing a triphenylamine moiety(30); the dissociation constant (K_D) and detection limit against HSA were estimated to be 41 μM and 0.12 μM, respectively, and it did not bind to several different target-unrelated proteins (Fig. 4). In addition, the pharmacophore could discriminate between HSA and bovine serum albumin (BSA) by color-changing, although the dissociation constant of the pharmacophore against BSA was similar order to that against HSA (Fig. S6). Compared with the BSA-bound state, a 44 nm hypochromic shift of the emission peak-top was observed when it was bound to HSA (Fig. S8). This hypochromic shift can be explained by that the twisted intramolecular charge transfer (TICT)-based fluorescence(34) of the binder was less frequently occurred; the microenvironment around the pharmacophore in the HSA-bound state seems more hydrophobic than that in the BSA (Fig. S8, also see Fig. S7 for TICT property of **A9L13**).

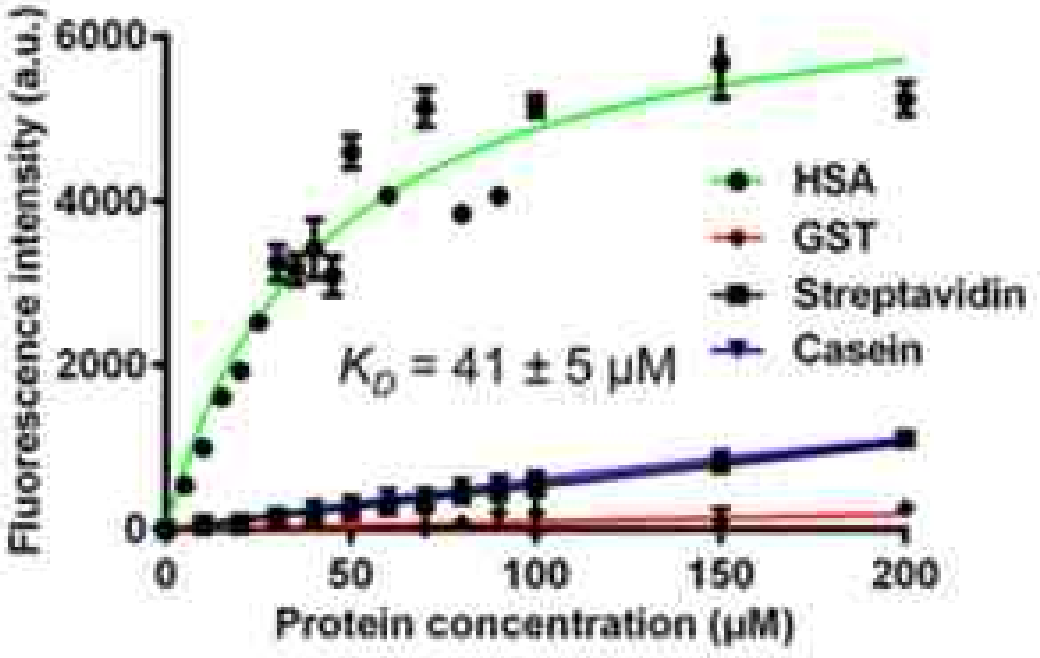

Fig. 4. Evaluation of HSA-binding affinity (dissociation constant; K_D) and selectivity of the keep-on-type pharmacophore (**A9L13**, 25 μM) by relative fluorescence intensity change at 485 nm in D-PBS / 10% DMSO.

Conclusions

In conclusion, a novel fluorescent 'keep-on' pharmacophore was obtained for the first time from a DCL of Schiff bases, by combination of SEC / LC-MS screening methods. Among various screening techniques(35), we envision that the demonstrated methodology would be generally applicable for obtaining a variety of keep-on pharmacophores for different target biologics in one of the easiest ways, because hit compound(s) can be readily available by simple column screening using instantly-prepared mixed compounds with commercial availability.

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Compliance with ethical standards

There are no conflicts to declare.

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