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Rational design of biotinylated probes: fluorescent turn-on detection of (strept)avidin and bioimaging in cancer cells†

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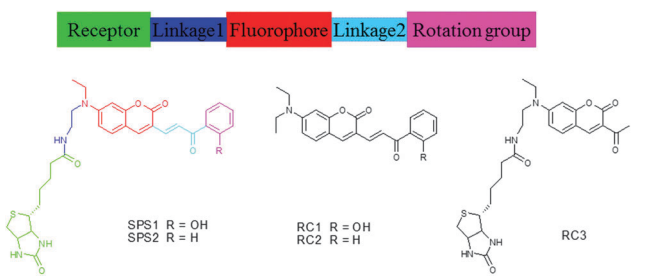
Two fluorescent probes SPS1 and SPS2 were designed by connecting biotin to an environment-sensitive coumarin fluorophore. Streptavidin and avidin induced dramatical fluorescence changes in both probes. SPS2 has potential in fluorescent imaging of biotin receptor-enriched tumor cells.

The strong affinity between biotin and streptavidin (SA) and avidin (AV) ($K_a \sim 10^{13} - 10^{15}$ M^{-1})[1] has found wide applications in bioanalytical areas such as biomolecule recognition,[2] tumor cell diagnosis[3] and immunoassay.[4] Therefore, the development of sensitive and selective probes for (strept)avidin has attracted much attention.[5] Using biotinylated fluorescence probes to monitor relative biomolecules is a simple and common approach.[6] However, most biotin–fluorophore conjugates were found to lose their fluorescence upon binding to (strept)avidin probably because of self-association in complexes with proteins.[7,8] The reported fluorescence off–on probes for (strept)avidin were mainly based on the FRET mechanism,[9] luminescent biotin–transition metal complex conjugates with reduced RET,[10] competition binding[11] or hydrophobic ligand-binding domains of (strept)avidin.[12] The relatively low sensitivity, small Stokes shift and short emission wavelengths, however, may limit their applications.[9e,12b] It is our interest to construct "off–on" fluorescent probes for (strept)-avidin with high sensitivity. In addition, it is reported that the biotin receptors like (strept)avidin occur in tumor cells much more frequently than in normal cells.[13] Finally, we aim to apply our rationally designed probes in living cell imaging.

Recently, many environment-sensitive fluorophores have been exploited to detect certain proteins.[14] Among those, 7-aminocoumarin showed solvent polarity-dependent spectral properties, and its quantum yield decreased with increasing solvent polarity.[15] In view of the aforementioned results, we present here two rationally designed biotinylated coumarin probes SPS1-2 for (strept)avidin (Scheme 1). The probes consist of the following three components. (1) Biotin, a selective (strept)avidin receptor that was also chosen as the cancer targeting unit. (2) The polarity-sensitive fluorophore coumarin to monitor the change in the microenvironmental polarity. An enhanced fluorescence could be anticipated when the probes enter into the hydrophobic region of the protein or permeate into tumor cells. (3) A phenyl group that quenches fluorescence by internal rotation in fluid media that gives rise to an increased fluorescence intensity after the restriction of the rotation. This design is expected to counteract the RET between the fluorophore and the proteins and results in fluorescence enhancement. In addition, a 4-atom spacer was used as the linker between the fluorophore and the receptor to avoid the self-quenching in complexes with (strept)avidin.

Probes SPS1-2 were fully characterized by ^{1}H-NMR, ^{13}C-NMR and ESI-MS data and were employed in the detection of (strept)avidin. Compounds RC1-2 were used as the references to verify the importance of the biotin unit in the detection of (strept)avidin, and RC3 was employed to investigate the role of the rotation group.



Scheme 1 The chemical structures of probes SPS1-2 and their precursors RC1-2 and the reference compound RC3.

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First, the absorption and emission spectra of SPS1-2 in several common solvents were measured to study their potential in protein determination. The biotinylation of RC1-2 does not substantially affect the spectral properties. Similar to their precursors RC1-2, both probes SPS1-2 exhibit clearly solvatochromic absorption and emission spectra (for SPS2, the absorption shifts from 454 nm in toluene to 475 nm in PBS, while the emission shifts from 495 nm to 605 nm in the same series of solvents, Fig. S3 and S4, ESI†). Generally, the absorption and emission maxima as well as the Stokes shifts of both probes increase with the increasing solvent polarity. Their quantum yields are much lower in water than in other solvents (Table S1, ESI†), perhaps due to a quenching mechanism related to hydrogen bonding.

The lower polarity inside the binding pockets and the high affinity of biotin towards SA/AV inspired and promoted us to apply these probes in the detection of SA and AV. We envisioned that the addition of SA/AV would induce dramatic enhancements in the fluorescence intensities of the probes with obvious blue shifts in the emission spectra. The effects of SA and AV on the absorption and emission spectra of the probes turned out to agree with our expectations. SPS1 and SPS2 are weakly fluorescent in phosphate buffer solution (PBS), but they display clear spectral responses towards SA/AV (Fig. 1). SPS1 has maximum fluorescence at 625 nm, the addition of SA shifted the emission band to 587 nm without clear intensity change, while about 50 nm blue shift and 3-fold fluorescence enhancement were induced by AV. The fluorescent responses of SPS2 toward SA/AV are similar to those of SPS1 but more pronounced: both SA and AV triggered evident fluorescence enhancement (>6 folds) and a clear blue shift in the emission spectrum. In order to understand the role of biotin in the detection of SA and AV, the spectral responses of the reference compounds RC1-2 towards SA and AV were measured as well. It can be seen in Fig. S5 (ESI†) that neither SA nor AV exerts much influence on the emission spectra of RC1-2, which implies that there are no strong interactions between RC1-2 and SA/AV. Furthermore, a third reference compound RC3 (Fig. S6, ESI†) without a phenyl moiety was employed to investigate the role of the rotation group in the detection of SA/AV. The results indicate that the rotation group is essential in the determination of SA. The above results suggest that the fluorescence enhancements and blue shifts in the emission spectra of SPS1-2 induced by SA/AV can be ascribed to the specific binding of biotin in the probes to SA/AV. The fluorophores in the probes are supposed to reside in the hydrophobic pocket inside SA/AV upon binding of the biotinylated dyes to the proteins. As a result, the photophysical properties of the dyes are altered greatly.

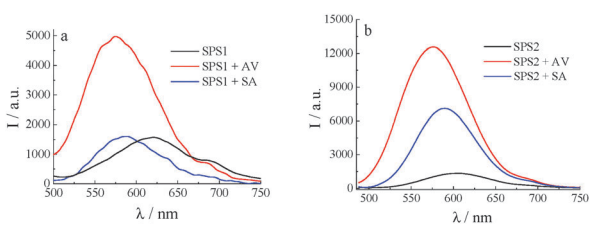

Fig. 1 The emission spectra of SPS1 (a) and SPS2 (b) with or without SA/AV. 10 mM PBS, pH 7.0; [dye] = 3.0 μM, [SA] = [AV] = 1.5 μM; λ_{ex} = 465 nm.

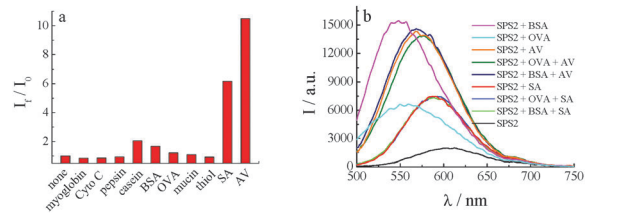

Fig. 2 The relative fluorescence intensity of SPS2 (a) in the presence of 0.1 mg mL^{-1} of various proteins and (b) the competition of SPS2 toward SA/AV over BSA and OVA. [SA] = [AV] = 0.1 mg mL^{-1}, [BSA] = [OVA] = 1.0 mg mL^{-1}. 10 mM PBS, pH 7.0; [SPS2] = 3.0 μM; λ_{ex} = 465 nm, λ_{em} = 576 nm.

In the detection of SA/AV, SPS2 is preferred over SPS1 because of its higher sensitivity. Since the detection of SA/AV with SPS2 is based on the polarity sensitivity of the coumarin, other biomolecules with non-polar cavities may interfere in the detection of SA or AV through non-specific binding. Therefore, the selectivity of SPS2 toward SA/AV over some other proteins including BSA, OVA, mucin, pepsin, casein, cytochrome *C*, myoglobin and biothiols (including cysteine, glutathione and homocysteine) was investigated (Fig. 2). The concentrations of the proteins were 0.1 mg mL^{-1}, and the concentrations of thiols were 0.3 mM. It can be seen in Fig. 2a that SA or AV induces more than 5 times fluorescence enhancement of SPS2, but other proteins, except for casein and BSA, do not induce obvious spectral changes. BSA and casein lead to ~2-fold enhancement in the emission intensity.

Considering the much higher concentrations of OVA and BSA in some biosamples, we also monitored the fluorescent response of SPS2 to OVA and BSA with higher concentrations. The results reveal that in the presence of 1.0 mg mL^{-1} BSA, the emission maximum of SPS2 shifts from 607 nm to 548 nm and about 12-fold enhancement of the fluorescence intensity is observed (Fig. 2b). Similar results were obtained for the reference compound RC2 (Fig. S7, ESI†), indicating that biotin did not exert much influence on the interaction between BSA and the probes. Upon addition of 0.1 mg mL^{-1} AV (or SA) (one tenth of the BSA/OVA concentration) to SPS2-BSA (or OVA) solutions, the emission maximum shifted from 548 nm to 576 nm with slight fluorescence decrement, which was almost the same as that in the absence of BSA/OVA (Fig. 2b). The above results indicate that the presence of 10 times excess of BSA or OVA does not affect the detection of SA/AV. The blue shift and fluorescence enhancement caused by BSA are attributed to the non-specific interaction between the probes and BSA,[15c] which is much weaker than the specific affinity between SPS2 and SA/AV.

In order to gain more insight into the sensing mechanism, the time-resolved fluorescence data of SPS2 in different systems were measured (Fig. S8, ESI†), and the results are summarized in Table S2 (ESI†). Fig. S8 (ESI†) demonstrates that the fluorescence decay time of SPS2 is relatively short in PBS (0.9 ns) and much longer in organic solvents (2.1–7.7 ns). In the presence of 1.0 mg mL^{-1} BSA in PBS, the fluorescence decay of SPS2 has a

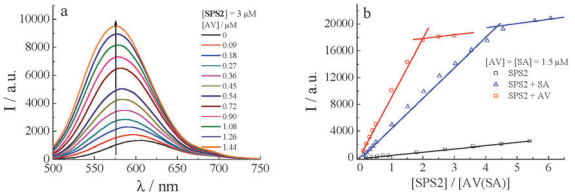

Fig. 3 The emission spectra of SPS2 with different concentrations of AV (a). The fluorescence intensities of SPS2 (b) in the absence (squares) and presence of SA (triangles) or AV (circles). SA/AV was titrated with SPS2. 10 mM PBS, pH 7.0; λ_{ex} = 465 nm, λ_{em} = 576 nm, both slits were 5 nm.

similar type of feature to that in toluene (7.7 ns) and the lifetime becomes longer (3.06 ns in the presence of BSA). The lifetime of SPS2 was a bit longer (1.07/1.79 ns) in the presence of SA/AV (0.1 mg mL^{-1}). Moreover, 10 times excess of BSA hardly changed the lifetime of SPS2 in the presence of SA (1.71 ns, Fig. S8, ESI†). These results reveal that SPS2 can be used for selective identification of SA/AV even under competition from BSA or OVA.

A linear relationship between the concentration of the analyte and the fluorescence signal is very important in the quantitative detection. So, the effects of AV and SA concentrations on the emission spectrum of SPS2 were further studied. It is observed in Fig. 3a (see also Fig. S9, ESI†) that with increasing SA or AV concentration the fluorescence intensity of SPS2 increases progressively accompanied with a clear blue shift. A linear relationship between the fluorescence intensity at 576 nm and AV concentration was found in the range of 0 to 0.84 μM. In the case of SA, the linear range was 0 to 0.54 μM. The detection limits of SA and AV were ~3 nM (S/N = 3). The above results demonstrate the potential of SPS2 for quantitative detection of SA/AV.

The tetrametric SA and AV have four biotin-binding sites, which permits each protein molecule to bind at most four molecules of the probe. In order to obtain the binding ratio of the probe to SA/AV, SA/AV samples with known concentrations were titrated with SPS2. Upon titration of SPS2 to an AV solution, a steep linear rise of the fluorescence signal was observed between 0 and 2 equivalents of SPS2 per AV. Further addition of SPS2 caused only a small rise in fluorescence. Similar results were obtained when SPS2 was titrated to the SA solution, but the break point was at SPS2/SA ≈ 4 (Fig. 3b). The weak linear rise at >2 SPS2/AV or at >4 SPS2/SA was parallel to the control series (squares in Fig. 3b) and clearly from the fluorescence of unbound probe molecules which were in excess, the above results may indicate that the 1 : 2 complex of AV-SPS2 and the 1 : 4 complex of SA-SPS2 were formed.

There are several differences in the structures of SA and AV, which may be the cause of the different binding behaviours of SPS2 to SA and AV. Compared to SA, there are some additional hydrophobic and aromatic groups in the binding sites of AV, and the length of the loop in AV is longer.[1b] We assume that when the biotin moiety in SPS2 is located at a binding site, the benzene ring in SPS2 may act as a cover of the adjacent binding pocket in AV to hamper a second probe molecule entering into this site (Fig. S10a, ESI†). Therefore, the maximum binding ratio of SPS2 to AV is about 2. When the ratio of SPS2/AV < 2, most SPS2 molecules are located at the binding sites of AV (Fig. S10a, ESI†), which results in the steep enhancement of the fluorescence. When SPS2/AV > 2, the excess probe molecules are supposed to be dissolved in the bulk solution, and the increasing trend of fluorescence is similar to that of the SPS2 solution without proteins.

Compared to AV, the loops in SA are shorter. When the biotin moiety of the probe binds to the pocket in SA, the other units including the fluorophore and the benzene ring are relatively flexible, which benefits the entrance of a second probe molecule into the neighbouring pocket. Therefore, the maximum binding ratio of SPS2 to SA is about 4 (Fig. S10b, ESI†). Accordingly, the linear range is 0–4 PSP2/SA. When SPS2/SA > 4 the addition of SPS2 leads to an increment of unbound SPS2 concentration, and the increase of fluorescence is parallel to that of the control series (squares in Fig. 3b).

Finally, we evaluated the possibility of SPS2 for fluorescent imaging of biotin receptor-positive Hela cells (Fig. 4 and Fig. S11, ESI†). After Hela cells were incubated with 10 μM of SPS2 for 1 h, strong intracellular green and red fluorescence could be observed (Fig. 4a and b). Pretreated Hela cells incubated with 10 μM biotin for 1 h to block the receptors in tumor cells followed by incubation with SPS2 for another 1 h led to dramatic decreases in both green and red emissions (Fig. 4e and f). The above results indicate that SPS2 could specifically bind to the biotin-receptors in the cells after permeation into the cell, which verifies the potential application of SPS2 for fluorescent imaging of living cells with biotin receptor-overexpression. The cell viability of HeLa cells pretreated with various concentrations of SPS2 was assessed using a standard MTT assay.[13d] After exposure of cells to SPS2 for 24 h, the viability decreases by 33% at the concentration used for confocal imaging (10 μM, Fig. S12, ESI†), suggesting that SPS2 shows some toxicity to HeLa cells.

In conclusion, two "off–on" fluorescent probes SPS1-2 for SA/AV were designed and synthesized taking advantage of the environment-sensitive solvatochromic coumarin fluorophore and the mobility-sensitive phenyl rotor. SPS2 is highly sensitive

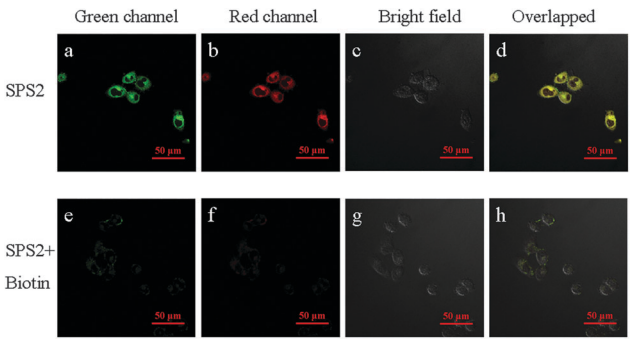

Fig. 4 Confocal laser fluorescence (a and b, e and f), bright-field (c, g) and the merged (d, h) images of living HeLa cells incubated with SPS2 (10 μM) for 60 min (a–d) and pretreated with 10 μM biotin for 1 h (e–h) followed by incubation with SPS2 for another 1 h. Excited at 488 nm; (a, e) green channel; (b, f) red channel.

and selective toward SA and AV with a detection limit of 3 nM. The experimental results further demonstrate that SPS2 can be used for the fluorescent imaging of living cancer cells.

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