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Investigating fluorescent dyes in fluorescence-assisted screenings†

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Screening of bead-based peptide libraries against fluorescent dye-labeled target proteins was found to be significantly influenced by the dye characteristics. Commercially available red fluorescent dyes with net negative charges adversely showed strong interactions with library beads. The introduction of zwitterionic dyes significantly reduced the unwanted interactions, which sheds light upon using the right fluorescent probe for acquisition of reliable results in various fluorescence-assisted applications.

Combinatorial one-bead-one-compound (OBOC) peptide libraries introduced by Lam *et al.*[1] allow for identification of binding peptides for targets of interest such as proteins. Several methods have been used to obtain positive beads from the screening, such as the use of colorimetric detections by biotinylated proteins[2] or monoclonal antibodies,[3] use of fluorescent dye-labeled proteins[4] and magnetic isolation by magnetic nanoparticle-conjugated antibodies.[5] Amongst them, conjugation of a fluorescent dye to a target protein is simple and straightforward associated with its easy chemical and biochemical platform for application in high-throughput screenings. We previously demonstrated an ultrahigh-throughput screening campaign by employing an automatic bead sorter (COPAS PLUS) that allows for rapid collection of positive beads sorted by the fluorescence intensity.[4,6,7]

However, screening of a bead-based peptide library against a dye-conjugated target protein has been found to be significantly influenced by the fluorescent dye associated with nonspecific interactions such as hydrophobic and charge–charge interactions. As addressed by Camperi *et al.*[4] the true positive beads are difficult to be distinguished from the false positive beads interfered by unwanted interactions between the peptides and the fluorescent dye by the use of the COPAS system because the automatic sorting is only triggered by a certain level of fluorescence intensity. Although Camperi *et al.* could distinguish between the true and the false positive beads by applying two different fluorescence modes (halo appearance *vs.* bright homogeneous fluorescence), their method depends upon the size of the target protein and the nature of the solid support.[4] Herein, we investigate the adverse effects of the commercially available charged red fluorescent dyes on the screening of short peptide libraries and thereby introduce zwitterionic physicochemical properties to minimize the background interactions from the dye.

Initially, commercially available red fluorescent dyes such as AlexaFluor 647 (**AL**) and DyLight 650 (**DY**) were used for labeling the target proteins to avoid strong autofluorescence[4c,8] from TentaGel beads. It is noteworthy that both dyes append multiple sulfate groups for increased solubility and stability in aqueous media. In order to examine background interactions, a portion of 5 mer library beads, comprising 18 D-amino acids excluding methionine and cysteine (*ca.* 100 mg) was prepared and screened against the **AL**-labeled lysine and **AL**-labeled human carbonic anhydrase II (hCAII), respectively (Fig. 1).[9] The results obtained for the **AL**-labeled lysine revealed that positively charged amino acids, *i.e.* arginine and lysine, were dominant in every position of the positive peptides (Fig. 1d). This trend might be caused mainly by strong binding of negatively charged fluorophores to positively charged residues.[10] Upon screening against **AL**-labeled hCAII, a histogram appeared similar to that obtained upon screening against the **AL**-labeled lysine (Fig. 1d and e), implying that the **AL** dye itself is significantly involved in binding with library beads. This trend was also observed with screenings against **DY**-labeled lysine and **DY**-labeled hCAII (see ESI,† Fig. S3). Labeled with **AL**, several other proteins such as bovine carbonic anhydrase II (*b*CAII), C-reactive protein (CRP), α-fetoprotein (AFP) and prostate specific protein (PSA) were also examined and resulted in a high

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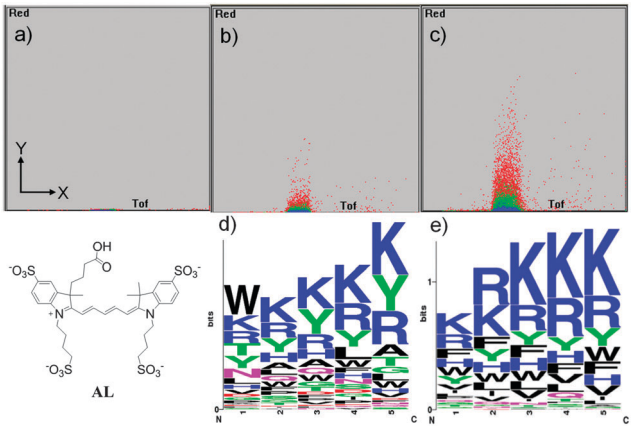

Fig. 1 COPAS sorting images (*X*-axis: time of flight (TOF); *Y*-axis: fluorescence intensity, a.u.) from (a) a 5 mer comprehensive peptide library itself, and from its screenings against (b) **AL**-labeled lysine, and (c) **AL**-labeled hCAII ([dye-labeled target] = 100 nM; detection conditions: excitation at 640 nm with a solid-state laser, Gain = 1, PMT = 450); font histograms of positive peptides for (d) **AL**-labeled lysine, and (e) **AL**-labeled hCAII.

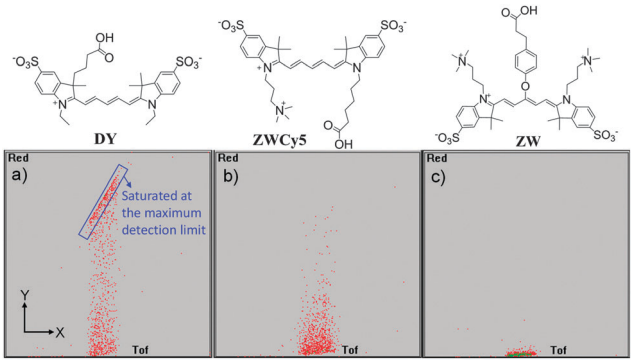

Fig. 2 COPAS sorting images from bead-based screenings of a 5 mer comprehensive peptide library against (a) **DY**-labeled lysine, (b) **ZWCy5**-labeled lysine, and (c) **ZW**-labeled lysine (detection conditions: excitation at 640 nm with a solid-state laser, Gain = 3, PMT = 700). The concentration of each dye-labeled target for screening was adjusted to exhibit an identical emission maximum.

frequency of positively charged entities at each position of the positive peptides (see ESI,† Fig. S4). It is apparent that the undesired strong background bindings of the charged dyes jeopardize the identification of true positive peptides regardless of the protein types.

With an aim to see whether other features also account for the high background bindings, Texas Red dye (**TR**) with net-zero charge was investigated. Although positive peptides identified by screening against **TR**-labeled lysine and **TR**-labeled hCAII showed reduced abundance of positively charged amino acids, these positive peptides contained a considerably increased population of aromatic amino acids such as tryptophan, phenylalanine and tyrosine, presumably due to the high aromaticity of the **TR** dye (see ESI,† Fig. S5). Several positive peptides identified by screening against the **TR**-labeled hCAII still showed strong cross-bindings to the **TR**-labeled lysine (see ESI,† Fig. S6). These observations clearly indicate that the structure of the fluorescent dye has a great influence on fluorescence-assisted screenings.

In an effort to reduce the undesired background bindings from the conventional red dyes, a library without arginine, and another library without both arginine and lysine in diverse elements were investigated in the first place. However, in both cases the dominance of positively charged entities still remained with the most abundant lysine and histidine, respectively (see ESI,† Fig. S7). To achieve reliable screenings, a new type of dye with low background bindings is highly demanded. One possible clue is to modify the Cy5 dye with zwitterionic moieties, well-known for their beneficial role in reducing the nonspecific interactions.[11,12] Although the modified dye (**ZWCy5**) with a zwitterionic moiety introduced on Cy5 showed reduced background bindings compared with the **DY** dye, a high level of background bindings still resided (Fig. 2a and b). When a pair of zwitterionic moieties was symmetrically deployed on the pentamethine cyanine core, the novel dye (**ZW**)[11c] resulted in only a negligible level of background bindings under identical sorting conditions (Fig. 2c). These results are well in concordance with those obtained from an *in vivo* application.[11] The **ZW** dye exhibits excellent optical properties with a high extinction coefficient ($\approx$200 000 M^{-1} cm^{-1}) and a high quantum yield ($\approx$20%), comparable to **AL** and **DY** dyes. The geometrically balanced net zero charge contributes to lowering the adverse background bindings presumably due to the charge shield effect as shown in their previous applications.[11,13]

When a comprehensive 5 mer peptide library was screened against **ZW**-labeled hCAII, the overall fluorescence level in the sorting by COPAS was significantly decreased compared with the screening against **AL**-labeled hCAII under identical conditions (see ESI,† Fig. S8). Analysis of 120 positive beads sorted by the fluorescence intensity produced completely different results from the screenings against the **ZW**-labeled lysine as well as the **AL**-labeled hCAII (Fig. 3d, e and 1e). To obtain the peptides of reasonable binding affinity, a focused library was generated by recruiting only dominant amino acids at each position (see ESI,† Fig. S9). Through another round of screening, 11 peptides were selected, synthesized and evaluated for binding ability. Surface plasmon resonance (SPR) experiments revealed that the dissociation constants (K_D) of fplsk, lpypd and hrtsa were 5.8, 5.3 and 4.3 μM, respectively, which were determined by two-state model fitting found in the Biacore T100 evaluation software (see ESI,† Fig. S10). The micromolar binding affinity was within the acceptable values for short peptides.[14] In particular, fplsk showed excellent specificity for hCAII in the presence of PSA, AFP and CRP (see ESI,† Fig. S11). These results imply that the **ZW** dye can be a great tool for reliable high-throughput screening of OBOC peptide libraries.

To expand the applicability of the **ZW** dye to other targets, we selected Ki-67 as a new target protein, a proliferation marker in early-stage breast cancer. Screening against **AL**-labeled Ki-67 generated a histogram with a great dominance of arginine and lysine at all positions, similar to that obtained with the other targets (see ESI,† Fig. S12). Binding peptides with reasonable affinity could not be identified by validation of positive peptides.

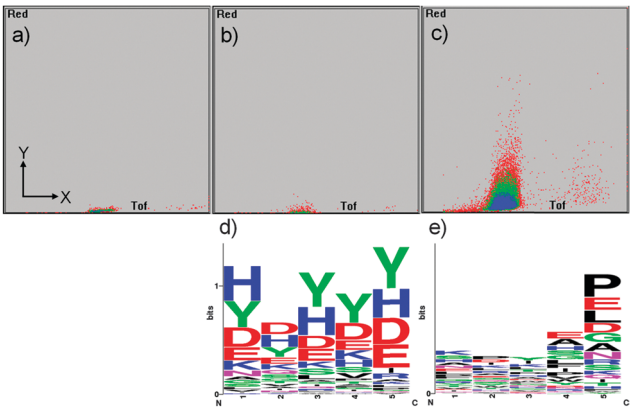

Fig. 3 COPAS sorting images from (a) a 5 mer comprehensive peptide library itself, and its screenings against (b) **ZW**-labeled lysine, and (c) **ZW**-labeled hCAII ([dye-labeled target] = 100 nM; detection conditions: excitation at 640 nm with a solid-state laser, Gain = 3, PMT = 750); font histograms of positive peptides for (d) **ZW**-labeled lysine, and (e) **ZW**-labeled hCAII.

In the meantime, with **ZW**-labeled Ki-67, several binding peptides could be easily identified in conjunction with the negligible influence of the **ZW** dye. The positive 5 mer peptides showed several μM binding affinities observed by SPR. (see ESI,† Fig. S13).

To further elucidate the dye effects, a binding peptide (Ac-fplsk) for hCAII was synthesized on TentaGel beads. Each portion of the beads was incubated with hCAII or lysine, respectively, labeled with 3 different dyes, similarly as in the screening experiments. After thorough washings, the **ZW**-labeled lysine was not detected on the beads under the given image acquisition conditions, confirming that the **ZW** dye involves only a negligible level of background bindings (Fig. 4b). However, the beads incubated with the **AL**-labeled lysine displayed high fluorescence under identical image acquisition conditions stemming from its strong bindings with the peptides (Fig. 4c). Whilst the **AL**- and the **DY**-labeled hCAII resulted in extremely high fluorescent beads, the **ZW**-labeled hCAII brightened only the outer sphere of the beads upon incubation under identical conditions (Fig. 4d and e).[6] In fact, the similar halo fluorescence patterns were also observed with the former two derivatives as the image acquisition parameters were adjusted to reduce the fluorescence level. Therefore, it turned out to be extremely difficult to distinguish the true positive beads from the false ones when the conventional red fluorescent dyes were employed. We envision that it is not generally plausible to develop binding peptides for target proteins by using **AL** for protein labelling, although some previous approaches managed to identify the binding peptides for *b*CAII using **AL** for labeling.[9,15] However, those peptides were also found to interact strongly with the **AL** dye (see ESI,† Fig. S14). In fact, for screenings against the other **AL**-labeled proteins such as AFP, Ki67 and PSA, we could not identify any binding peptides with reasonable affinity. These results clearly illustrate that the **ZW** dye can be an excellent tool for reliable high-throughput screenings assisted by fluorescence.

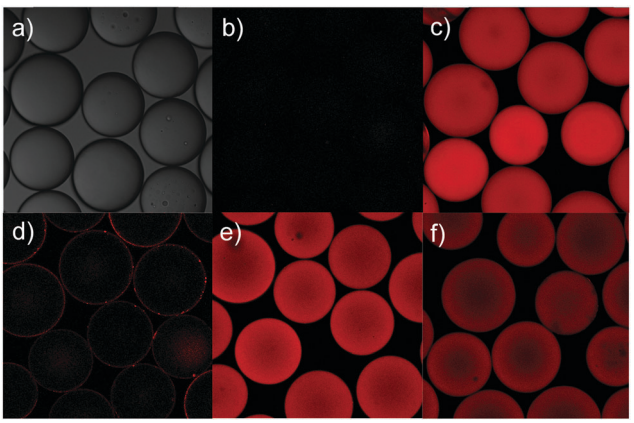

Fig. 4 (a) Microscopic images of TentaGel beads appending Ac-fplsk; confocal images of TentaGel beads modified with Ac-fplsk after incubation with (b) **ZW**-labeled lysine, (c) **AL**-labeled lysine, (d) **ZW**-labeled hCAII, (e) **AL**-labeled hCAII, and (f) **DY**-labeled hCAII. [dye-labeled target] = 100 nM.

In summary, commonly used negatively charged red fluorescent dyes associated with the appending multiple sulfate groups adversely affect the screening of OBOC peptide libraries through strong background interactions with peptides on beads. A symmetric placement of zwitterionic moieties on both ends of the Cy5 dye molecule significantly reduced the background interactions, which could lead to successful identification of short peptides of reasonable binding affinity. Our results suggest that using the appropriate fluorescent dye should be of utmost importance to acquire reliable results in various fluorescence-assisted applications such as screening, assay and imaging.

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