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Site-specific immobilization of biomolecules by a biocompatible reaction between terminal cysteine and 2-cyanobenzothiazole†

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We report herein a new site-specific microarray immobilization method based on a biocompatible reaction between terminal cysteine and 2-cyanobenzothiazole (CBT). This immobilization strategy has been successfully applied to anchor small molecules, peptides and proteins onto microarrays.

Microarray technology has transformed biomedical research in recent years.[1] Over the last two decades, it has become a robust and powerful screening platform for a wide range of biological applications, including studies of molecular interactions, drug discovery and disease diagnosis.[2] The compound immobilization strategy employed is crucial for successful implementation of microarray screening. Most common immobilization strategies could be grouped into those based on non-covalent immobilization, random covalent immobilization or site-specific covalent immobilization. Among them, site-specific covalent immobilization approaches have captured much attention because they ensure uniform display and optimum orientation of biomolecules upon immobilization, thereby facilitating subsequent interactions of these biomolecules and their analytes during screening.

Over the years, a number of chemoselective reactions, such as Diels–Alder reaction,[3a] native chemical ligation,[3b] click chemistry,[3c] Staudinger ligation[3d,e] and thiol–ene chemistry,[3f] have been applied for site-specific immobilization of biomolecules in a microarray. A recent strategy developed by Waldmann's group also involved the use of oxime ligation, in which oxyamine-functionalized proteins were successfully anchored onto ketone-derivatized glass slides chemoselectively under physiological conditions (pH = 7.0).[4]

Despite the aforementioned advancement, there is still growing interest in the development of novel immobilization methods for continuous expansion of microarray applications. An ideal immobilization method should fulfil a number of criteria. First, the conditions for immobilization should be mild, *e.g.* room temperature, physiological pH and without organic co-solvents. Biomolecules such as proteins are rather fragile, and both their tertiary structure and biological functions may be perturbed under harsh immobilization conditions. Secondly, the immobilization method should be highly selective. Given that multiple functional groups are present in peptides, small molecules and proteins, a non-selective method could result in inhomogeneous immobilization, leading to multiple orientations of the biomolecules. Consequently, the binding site of the biomolecules may be blocked and their molecular recognition can be disrupted. Finally, the immobilization method should be efficient. Since the immobilization process is performed on the solid surface, where reaction kinetics is usually lower than that in solution, only a highly efficient method would ensure maximum compound immobilization.

With these criteria in mind, we sought to develop a new approach for general immobilization under mild conditions. In 2009, Rao's group reported an efficient biocompatible reaction between a terminal cysteine and 2-cyanobenzothiazole (CBT).[5] The group demonstrated that the reaction could proceed in PBS buffer without the use of any catalyst. It was reported that the overall rate of this reaction was much faster than that of the well-established copper-free click reaction.[5] The reaction has been successfully employed in selective labeling of terminal cysteine-containing proteins on cell membranes. This useful reaction was later extended to several other applications, such as controlled assembly of nanostructures in live cells, site-specific incorporation of ^{18}F for PET imaging, *etc.*[6] In this study, we report, for the first time, that this reaction could serve as a versatile and robust immobilization approach for site-specific attachment of different biomolecules on microarrays (Scheme 1). We want to point out that, although copper(I) catalyzed click chemistry (CuAAC) has been widely utilized in microarray immobilization,[1,3c] the usage of the copper(I) catalyst can lead to the damage of biomolecules.[7] While in our method, the immobilization conditions are very mild without using any catalyst, which eliminate the possibility of damaging biomolecules.

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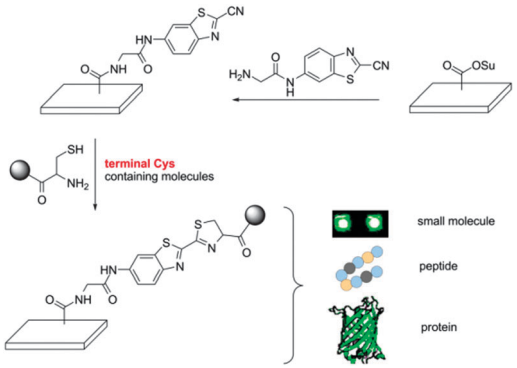
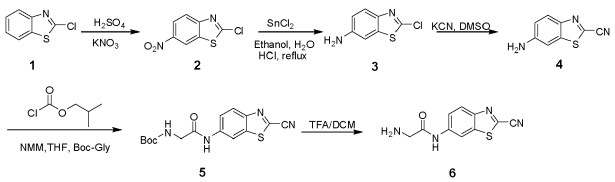


Scheme 1 Schematic illustration of the immobilization of terminal cysteine-containing biomolecules onto 2-cyanobenzothiazole (CBT)-functionalized slides in this study. Su = succinimide.



Scheme 2 Synthetic scheme of a glycine–CBT molecule. (a) KNO_3·H_2SO_4, ice, 1 h, 78.8%; (b) $SnCl_2$, ethanol, HCl, reflux, 57.9%; (c) KCN, DMSO, 24.9%; (d) isobutyl chlorformate, Boc-Gly, NMM, 57.8%; (e) TFA–DCM, 22.6%.

In our setup, synthesis of glycine–CBT (**6**) was first carried out based on the reported method.[8] As depicted in Scheme 2, nitration of 2-chlorobenzothiazole, **1**, was performed under acidic conditions. The resulting nitro derivative, **2**, was then reduced to its amine form, **3**, using $SnCl_2$ (57.9% yield). The installation of a cyanide group was achieved by reacting **3** with KCN at high temperature. Subsequently, a Boc-glycine linker was introduced at the amine group of **4** using isobutyl chloroformate as the activating reagent. Finally, the Boc group was removed with TFA to yield the target molecule, **6**. The structural identification of glycine–CBT **6** was confirmed by ^{1}H NMR, ^{13}C NMR and ESI-MS respectively (ESI†).

In the next step, we moved forward to produce CBT-functionalized slides. *N*-Hydroxysuccinimide (NHS) ester-functionalized slides were first fabricated following the previously established protocol (ESI†). A solution of glycine–CBT in DMF was subsequently applied to NHS-coated slides under basic conditions to produce CBT-functionalized slides.

To establish the method, we first tested the immobilization efficiency of this strategy using small molecule dyes (Fig. 1). A cysteine-containing dye, cysteine–tetraethylrhodamine (Cys–TER) (Fig. 1b), was synthesized and reacted with glycine–CBT in PBS buffer (pH = 7.4). LC-MS analysis indicated that the reaction occurred rapidly and was complete within 1 hour (see ESI,† Fig. S1). No side reaction was observed. Encouraged by this result, we carried out small molecule immobilization in our preliminary experiments. Different concentrations of Cys–TER ranging from 0.2 to 2000 μM were spotted in duplicate onto CBT-functionalized slides, followed by incubation in a humid chamber for 3 hours. Excess dyes were then washed away, and the slides were imaged using a microarray fluorescence scanner. As shown in Fig. 1a, the fluorescence intensity of the spots increased

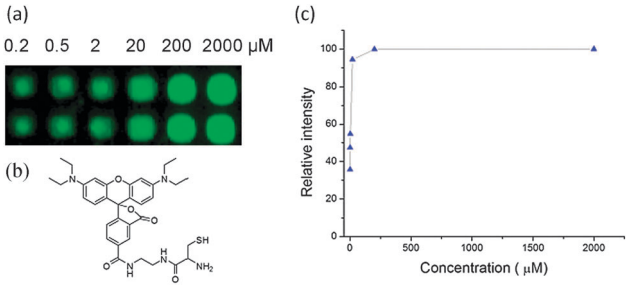


Fig. 1 Various concentrations of Cys–TER were applied to CBT-functionalized slides (0.2, 0.5, 2, 20, 200 and 2000 μM). (a) Fluorescence microarray images. (b) The structure of Cys–TER used in this study. (c) The plot of relative fluorescence intensity of (a).

with increasing concentrations of Cys–TER. Further data analysis revealed that the surface of CBT-coated slides started to become saturated at a concentration of around 20 μM. When the concentration was above 200 μM, the slide surface became completely saturated. To rule out the possibility of non-specific binding, a negative control, amine-TER was included in our experiments. No noticeable fluorescence signal for amine-TER was observed under the same experimental conditions (see ESI,† Fig. S2), indicating that this newly developed microarray immobilization method was highly selective.

We next studied the immobilization of more delicate biomolecules, *i.e.* proteins. We used EGFP as our model protein in the study. EGFP with an N-terminal cysteine was expressed and purified by reported procedures (Fig. 2a).[9] Specifically, EGFP was cloned into the pTwin1 vector to generate pTwin1-intein-EGFP, which contains an intein fused to the N-terminal residue of the EGFP. An extra N-terminal cysteine residue was introduced into EGFP through the PCR method. pH-induced intein cleavage resulted in the release of the EGFP containing a cysteine residue at its N-terminus. Coomassie staining analysis revealed that the protein was sufficiently pure after purification (Fig. 2b and Fig. S5, ESI†). Various concentrations of EGFP were then immobilized on CBT-functionalized slides in a humid chamber. Microarray images clearly

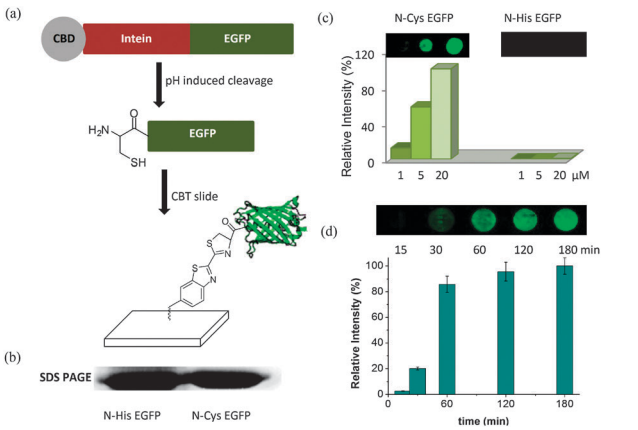


Fig. 2 Immobilization of EGFP onto a CBT-functionalized slide. (a) Preparation of N-terminal cysteine-containing EGFP *via* the intein-mediated cleavage method. (b) SDS-PAGE analysis of N-His-EGFP and N-Cys-EGFP. (c) Different concentrations of N-Cys-EGFP and N-His-EGFP were immobilized onto CBT-functionalized slides. (d) Time-dependent immobilization of N-terminal cysteine-containing EGFP onto microarrays.

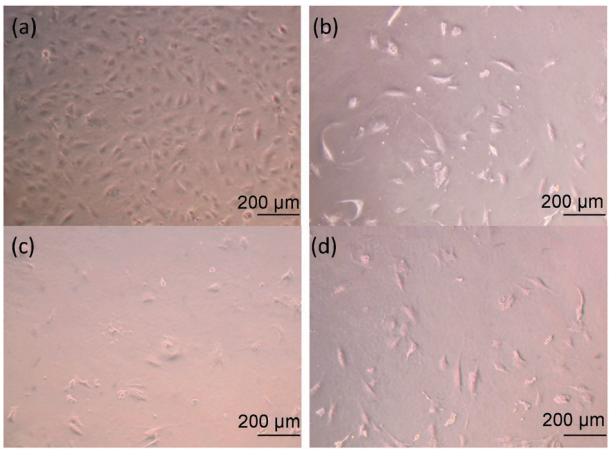


Fig. 3 Cell adhesion study of slides immobilized with different peptide substrates. Microscopic images of cells grown on slides (a) coated with CRGD peptides, (b) coated with RGD peptides, (c) coated with CGGG peptides, (d) CBT-functionalized slides.

indicated that the fluorescence intensity increased with increasing concentrations of the protein (Fig. 2c). In order to prove that the reaction was site-specific toward only the N-terminal cysteine in the protein, a negative control, N-His-EGFP, which does not have an N-terminal cysteine in the protein, was prepared (Fig. 2b), and subsequently immobilized on the same slide (Fig. 2c); to our delight, no noticeable immobilization was observed under the same experimental conditions. These results thus unequivocally demonstrated that this CBT immobilization method is highly selective towards the N-terminal cysteine residue of the target protein. To acquire a better understanding of the reaction kinetics of CBT and N-Cys-EGFP on microarrays, we also carried out time-course experiments. 50 μM of the protein was incubated with CBT-functionalized slides in PBS buffer (pH = 7.4) for different lengths of time, followed by fluorescence scanning (Fig. 2d); the fluorescence signals gradually increased before reaching ~80% saturation after around 1 hour, indicating highly efficient protein immobilization.

Finally, we evaluated whether the immobilization method could be used to fabricate peptide microarrays suitable for cell adhesion studies. Two N-cysteine-containing peptides, CRGD and CGGG, were synthesized with a microwave peptide synthesizer and site-specifically immobilized onto CBT-functionalized glass slides. After extensive washings to remove excess peptides, the resulting peptide microarrays were seeded with NIH-3T3 fibroblast cells. The cells were allowed to adhere for 48 hours in a CO_2 incubator before being imaged using a fluorescence microscopy (Fig. 3); it is apparent that the slide coated with CRGD peptides, which contain the well-established cell-adhesion RGD motif, exhibited significantly more efficient cell adhesion and growth than the one coated with CGGG peptides (a negative control peptide sequence). The number of cells grown on the CRGD-immobilized slide was comparatively higher (Fig. 3a), whilst cells grown on the CGGG-coated slide displayed smaller and rounded morphology (Fig. 3c). These phenomena are in agreement with those in the reported literature.[10] It is noted that cells can grow on CBT-functionalized slides, although not as well as those grown on CRGD-immobilized slides. The negative control slides coated with non-cysteine-containing RGD peptides display a similar growth pattern to CBT-functionalized slides (Fig. 3b and d), proving that there is no immobilization of this peptide. The method described herein thus proves that cysteine-containing peptide substrates can be covalently linked to CBT-coated slides and used for cell adhesion studies. It can provide a facile approach to screen peptide ligands capable of promoting cellular adhesion and growth.

In summary, we have developed a new method for site-specific microarray immobilization of biomolecules, including small molecules, peptides and proteins. The reaction can take place under very mild conditions, and it is highly selective towards terminal cysteine-containing molecules. We believe that this new method will be a useful tool to current and future microarray applications.

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