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Site-specific, reversible and fluorescent immobilization of proteins on CrAsH-modified surfaces for microarray analytics†

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A novel technique for protein immobilization onto CrAsH-modified surfaces is presented. This approach enables an efficient, reversible and fluorogenic immobilization of proteins. Moreover, expressed proteins can also be directly immobilized from cellular lysates without prior purification. The immobilized proteins are suitable for protein–protein interaction studies and the fluorescence enhancement upon immobilization can be employed for the direct detection of the immobilized protein without the need for secondary detection methods.

In the last decade protein immobilization techniques have become an essential tool for the understanding of protein function and interactions by enabling a rapid profile of proteins with a minimal amount of sample. The major challenge in the generation of protein biochips is the development of suitable conditions for the mild immobilization of proteins onto surfaces. Site-specific attachment is usually preferred to random immobilization because it ensures a homogeneous protein layer with a defined orientation that does not affect the active site of the proteins. A variety of chemical transformations have provided powerful opportunities for the generation of site-specifically oriented chips.[1] Other immobilization strategies exploit the affinity existing between known interacting pairs such as the polyhistidine-Nickel nitriloacetate acid complex or the biotin–avidin interaction.[2] However, the low affinity of the His–NTA complex (K_D 10 μM) may result in undesired protein detachment[3] and the biotin–avidin interaction requires previous protein biotinylation. Hence, the development of novel immobilization methods is still in high demand.

In general, a protein immobilization technique should ideally combine the best features of all the currently existing methods: a high-affinity but reversible binding, a site-specific immobilization under simple and mild conditions and a genetic incorporation of the required tag, thus avoiding additional chemical or enzymatic transformations and enabling a straightforward immobilization of proteins-of interest directly from cell lysates. In addition to these features, an ideal protein immobilization technique would also allow one to directly monitor successful surface binding without the need for secondary detection steps (*i.e.* labelled antibodies or proteins) or without requiring label-free methods such as Surface Plasmon Resonance (SPR) or Mass Spectrometry (MS), not available in every laboratory. Here, we have applied the interaction between biarsenical derivatives of fluorescein and tetracysteine (TC)-motifs as a novel strategy for protein microarray generation which meets all these demands.

Tsien and co-workers reported a method for site-specific protein labeling based on the genetic insertion of a so-called TC motif (CCPGCC) that can selectively form a stable complex with biarsenical-functionalized fluorescent dyes such as FlAsH or CrAsH resulting in a extraordinary enhancement of fluorescence upon binding.[4] This methodology has found numerous applications in fluorescence microscopy localization of proteins,[5] protein imaging, purification,[5,6] labelling and interactions in living cells[7] (recently reviewed in ref. 8). This high affinity interaction (K_D 10^{11} M^{-1}) proceeds fast under mild conditions, it is highly selective for the presence of the TC motif and it can conveniently be reversed by incubation with dithiols. Moreover, since TC-FlAsH binding leads to a strong enhancement of fluorescence, we reasoned that this method might be advantageous for use in protein immobilization because it would make secondary detection steps unnecessary.

To investigate whether a FlAsH-based strategy might be suitable for protein microarray fabrication, a biarsenical dye derived from carboxyfluorescein (CrAsH) was chosen for slide-modification. CrAsH presents a less pH-dependent fluorescence, as compared to FlAsH and contains an additional carboxy group which enables

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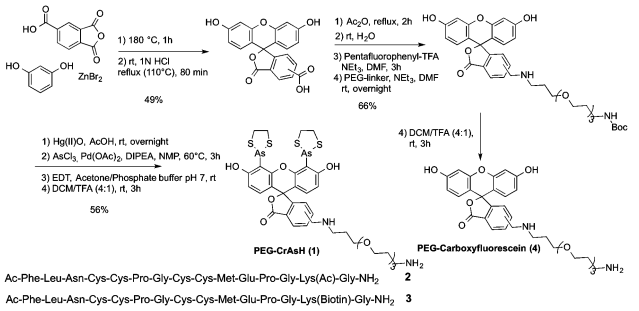

Scheme 1 PEG-CrAsH (**1**), PEG-Fluorescein (**4**) and TC-containing peptides **2**–**3**.

the incorporation of the required linker for surface modification. Hence, a CrAsH derivative conveniently quenched as ethandithiol (EDT)-complex and bearing a polyethyleneglycol (**1**) equipped with an amino group (Scheme 1) was initially prepared in an overall yield of 17% starting from carboxyfluorescein. Interaction with the tetracysteine-containing peptides **2** and **3** was studied in solution confirming that the presence of the linker had no effect on the fluorescence enhancement upon binding that was in alignment with the previously reported data (ESI† Fig. S1).[9]

The immobilization process is depicted in Fig. 1A. To generate the bisarsenical modified slides, glass slides activated with *N*-hydroxysuccinimide (NHS) esters were coated with **1** and subsequently blocked with ethanolamine. As proof-of-principle peptides **2** and **3** were initially spotted onto the modified slides at two different concentrations and incubated for one-hour at room temperature. Direct detection of both immobilized peptides was successfully performed by determining the levels of CrAsH fluorescence intensity upon formation of the complex, thus proving the applicability of the method. The scanning of the slides was performed with white light to reduce the reported photobleaching of FlAsH and other derivatives.[6,10] Repetitive scannings resulted in signals of similar intensity, thus indicating the stability of the complex during the measurements. Additional specific detection of immobilized peptides was achieved for the biotinylated peptide **3** which was subsequently detected with Cy5-labeled streptavidin. No significant signal could be detected on carboxyfluorescein(**4**)-modified slides treated with peptide **3** whereas this same peptide could be detected up to a concentration of 10 μM on CrAsH-modified slides, thereby indicating that the immobilization of the peptide indeed occurred due to specific TC-CrAsH interaction (Fig. 1B and ESI† Fig. S2).

We next investigated the immobilization efficiency at variable incubation times after spotting. Although a major fluorescence signal was already detected after 30 min, a significant increase of the average fluorescence intensities was already remarkable after one-hour incubation (ESI† Fig. S3). Longer incubation times did not result in a substantial increase of the fluorescence.

Since recycling of biarsenical-$(EDT)_2$ modified surfaces would be attractive from an economical point of view, we also investigate the reversibility of peptide attachment. To this end, peptide **3** was spotted and the immobilization efficiency was quantified by fluorescence detection. Subsequently, the slide was incubated with a 2 M solution of EDT in DMSO for different incubation times. Whereas after one-hour the fluorescence intensity could still be detected, the immobilization could be fully reverted after 4 h, as indicated by the absence of a signal, and the regenerated slide could be successfully reused for a second step of spotting achieving similar fluorescence intensities to that in the first spotting round (Fig. 2A and B).

To test whether our method is suitable for mild immobilization of proteins, we next investigated the immobilization of TC-tagged proteins onto biarsenical-coated slides. For a proof-of principle study we chose Ras proteins, which play an essential role in regulating cell signaling and proliferation. To this end, a TC-containing peptide was synthesized and installed C-terminally into an H-Ras protein *via* MIC ligation.[11] The resulting semi-synthetic protein was then spotted onto CrAsH-modified slides together with a H-Ras protein lacking the TC-tag as a negative control. After incubation for one hour the immobilized protein was directly quantified by fluorescence detection of the formed complex. As expected, the H-Ras protein was efficiently immobilized

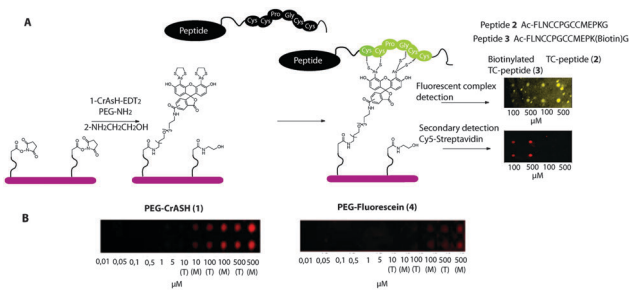

Fig. 1 (A) Immobilization of peptides **2** and **3** onto CrAsH-modified surfaces results in fluorescence enhancement that can be monitored. (B) Peptide **3** in MOPS or Tris buffer was spotted at different concentrations (0.01–500 μM) onto slides functionalized with PEG-CrAsH (**1**, 0.5 mM) or PEG-Fluorescein (**4**, 0.5 mM) and incubated for 1 h. The detection of the immobilized biotinylated peptide (**3**) was performed by incubation with Cy5-streptavidin (200 nM, 30 min).

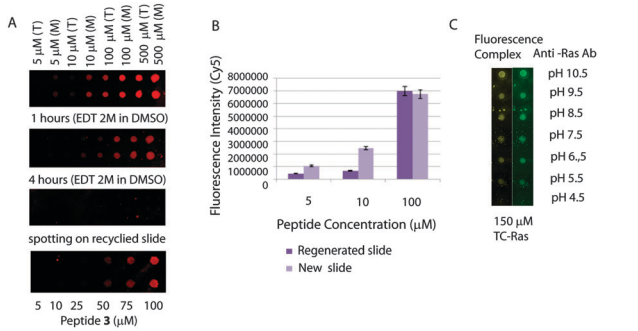

Fig. 2 (A) CrAsH-coated surfaces can be regenerated and reused after 4 h incubation of slides with a 2 M solution of EDT in DMSO. (B) Fluorescence intensities at 100 μM were of the same range in the reused slide compared to a new one; approximately 30–50% of signal loss could be observed at lower concentrations. (C) Immobilization of a TC-containing H-Ras protein. Primary detection through complex formation (yellow) and secondary detection by an anti-Ras antibody (green). The Protein immobilization can be performed at different pH rates (pH 5.5 to 10.5) with similar efficiencies.

in a concentration-dependent manner while only trace amounts of the control protein were detected (ESI† Fig. S5A), thus clearly demonstrating not only the applicability of the approach for the immobilization of proteins but also the suitability of the complex formation as a direct detection method. To prove the correct folding of the immobilized protein we next incubated the slides with a Cy3-labeled antibody directed against Ras isoforms, which recognizes the distinct α-helix of the Ras proteins close to the active site. Therefore binding of this antibody is indicative of correctly folded H-Ras on the slide.[12] Detection by fluorescence scanning showed efficient immobilization of the TC-tagged recombinant Ras protein up to a concentration of 25 μM and a minimal signal in the negative control (signal-to-noise ratio 10 : 1) (ESI† Fig. S5B).

Methods that can be employed in a long range of pH are of most interest. Therefore, we also investigated the pH tolerance of the method by immobilizing the semisynthetic H-Ras protein at different pH rates ranging from 10.5 to 3.5. Although no immobilization was observed at lower pH values, the results indicated that the immobilization strategy works efficiently at a wide range of pH values (10.5 to 5.5) (Fig. 2C).

To further investigate this novel immobilization method, a TC-tag was fused to PDEδ a prenyl-binding protein known to selectively recognize farnesylated proteins, and the recombinant protein was overexpressed in *E. coli*. After protein purification, PDEδ could also be successfully immobilized at different concentrations with a detection limit of 10 μM as observed by scanning for the fluorescent complex formed upon interaction (Fig. 3A).

Direct spotting of the TC-tagged protein from the cell lysates without prior purification would simplify the whole process of protein microchip fabrication and enable a rapid and general approach for the generation of multiprotein microarrays. Hence, we next investigate if a TC-tagged PDEδ could be directly immobilized from the cell lysates. With this aim, cell lysates from PDEδ expressing cells were centrifuged and after determining the total protein concentration were spotted onto CrAsH-modified slides. As shown in Fig. 3C, cell lysates containing the TC-PDEδ could be efficiently immobilized and directly detected by the formation of an interacting complex. No detectable signal was observed when cell lysates lacking the PDEδ-TC protein were employed as a negative control. We also confirmed that the generated protein microarrays can be employed for measuring protein–protein interactions. To this end, PDEδ-containing slides were incubated with a PDEδ antibody and treated with a fluorescently-labeled secondary antibody. Subsequent signal quantification indicated the successful immobilization and the intact folding of all three proteins bound to the surface.

PDEδ is known to specifically bind C-terminal farnesylated proteins.[13] Therefore, as an additional example of protein–protein interaction studies, a semisynthetic bodipy-labeled farnesylated N-Ras protein was prepared and incubated with a slide containing immobilized PDEδ. Detection of the interacting Ras was achieved by fluorescence scanning thus proving that the immobilized PDEδ was functional and that the protein–protein interaction was also taking place on the chip (Fig. 3C).

In summary, we describe here a novel one-step method for protein immobilization based on the stable complex formation

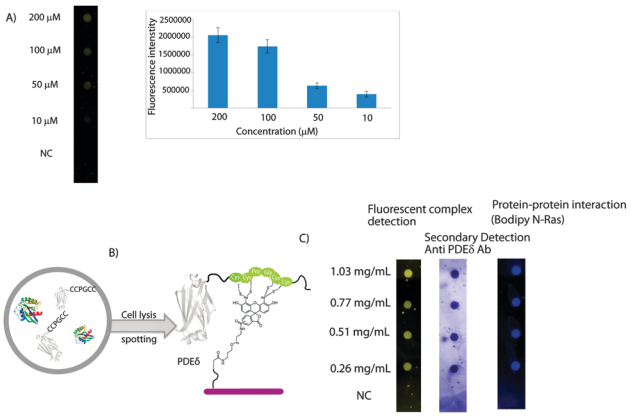

Fig. 3 (A) A purified TC-containing PDEδ can be immobilized and detected up to a 10 μM concentration. (B and C) A TC-containing PDEδ was efficiently immobilized from cell lysates without prior purification (total protein concentration ranges from 1.03 mg ml^{-1} to 0.26 mg ml^{-1}). The immobilized protein could be directly recognized (yellow) as well as indirectly by an anti-PDEδ antibody (purple) and employed to detect protein–protein interactions with a bodipy-labeled farnesylated N-Ras protein (blue).

between peptides or proteins bearing TC-tags and CrAsH-modified surfaces. This immobilization technique is mild, rapid, it requires only one-hour incubation, and it is compatible with the sensitive nature of proteins. Moreover, our approach overcomes important limitations. Briefly, slide reuse and more importantly, direct detection of immobilized proteins have always been challenging issues in the fabrication of protein microarrays. The His–NTA complex enables slide regeneration but the low affinity of this interaction may result in undesired protein detachment. Alternatively, direct detection usually requires label-free methods that are mostly found only in specialized laboratories. Hence, the approach described here presents additional important features compared to the previously described methods, *i.e.* a high affinity but reversible binding that can be employed for slide reuse and a fluorescence enhancement upon immobilization that enables the direct detection of the immobilized proteins. Finally, this strategy can be employed for protein immobilization directly from cell lysates with high efficiency, thus enhancing the practicability of protein microarray fabrication. Our approach expands the repertoire of immobilization methods by providing additional features that can strongly contribute to the application of protein microarrays in life sciences.

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