Surface immobilization of biomolecules by click sulfonamide reaction†

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Received (in Cambridge, UK) 21st April 2008, Accepted 25th June 2008
First published as an Advance Article on the web 15th July 2008
DOI: 10.1039/b806764c

Alkyne-modified biomolecules can be immobilized site- and chemoselectively on sulfonylazide slides under very mild conditions by means of the click sulfonamide reaction.

The use of surface-immobilized biomolecules plays an increasingly important role in life science research.[1–3] In general, the site- and chemoselective immobilization leading to a defined orientation of biomolecules is preferable or even mandatory because it generates homogeneous surface coverage and guarantees accessibility to the biomolecules' binding site.[2] The chemistry used for this purpose must be compatible with multiple functional groups found in biomolecules. It should proceed chemoselectively under mild conditions and in aqueous solution. Consequently, different types of bioorthogonal reactions were developed to meet this growing demand.[4,5] Among these, cycloaddition reactions including the Diels–Alder reaction and the 1,3-dipolar cycloaddition have attracted considerable attention.[5]

Very recently Chang *et al.*[6a,c] and Fokin *et al.*[6b] reported that sulfonylazides and terminal alkynes react in the presence of catalytic amounts of Cu(I) and H_2O to form stable *N*-acyl sulfonamides. The reaction proceeds with liberation of N_2 under very mild conditions with complete regioselectivity and is accelerated in aqueous solvents and even in pure water. Here we demonstrate that this "click sulfonamide reaction"[7] (CSR) is a versatile and efficient method for the surface immobilization of different types of biomolecules including biotin, a carbohydrate, phosphopeptides and proteins. In order to determine the functional group tolerance of the CSR, alkyne-functionalized biotin **1**, α-D-mannose **2**, and phosphopeptides **3** and **4** (Fig. 1) were treated with Z-protected tauryl sulfonylazide **5** in aqueous solution in the presence of different cosolvents, Cu-catalyst and additives. These initial experiments revealed that the reaction proceeds rapidly (90–95% conversions are reached within 2–4 h, see ESI† Fig. S2) and without undesired side reactions (*e.g.* with the amino acid side chains) in the presence of 2 mol% of $Cu(CH_3CN)_4PF_6$, 2 mol% of the tertiary amine TBTA (see ESI†), 4 mol% sodium ascorbate and 1 equiv. $NaHCO_3$ in aqueous conditions by analogy to the results of Fokin *et al.*[6b] for mainly aromatic and aliphatic reaction partners. These results indicated that the CSR should provide a suitable technique for immobilization of different biomolecules to appropriately functionalized surfaces.

For immobilization, glass slides initially modified with an intermediate layer of generation 4 PAMAM dendrimers and equipped with an additional glutaric acid linker were chosen as carriers to allow maximum surface coverage (Fig. 2, H-slides obtained from Chimera Biotec, Dortmund).[8] *N*-deprotected tauryl sulfonylazide **7** was coupled to the surface (see ESI†). In initial experiments biotin-alkyne **1** and α-D-mannose-alkyne **2** were spotted at different concentrations in H_2O–DMF (2 : 1) and in the presence of the Cu(I)-catalyst as well as the additives detailed above. After storage in a humidity chamber (washing and blocking, see ESI† for details), immobilized biotin and mannose were detected by treating the slides with Cy5-labelled streptavidin and Alexa-647-labelled Concanavalin A, respectively (see ESI†) and fluorescence scanning. The results for the immobilization of biotin (see ESI†, Fig. S3) and mannose (Fig. 2B) revealed that both biomolecules were immobilized with remarkable efficiency. Thus, well resolved spots were detected if 0.1 mM biotin-alkyne **1** (see ESI†, Fig. S3) and 1 mM alkynylated mannose **2**[9] were used (Fig. 2B). In both cases analogues lacking an alkyne group were used as negative controls (NC, see ESI† for structures, Fig. S1)

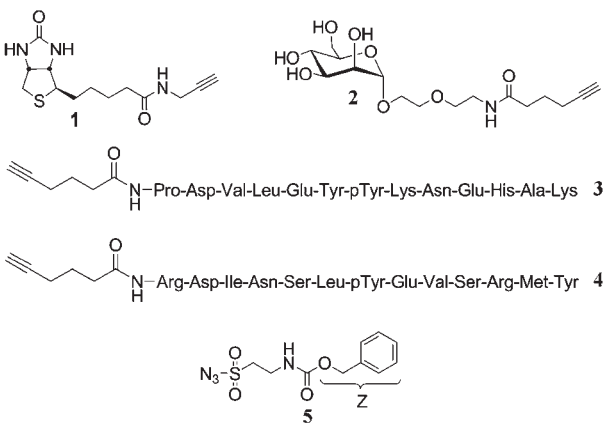

Fig. 1 Structures of alkyne-modified biomolecules.

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† Electronic supplementary information (ESI) available: Experimental details and additional microarray and spectroscopic data. See DOI: 10.1039/b806764c

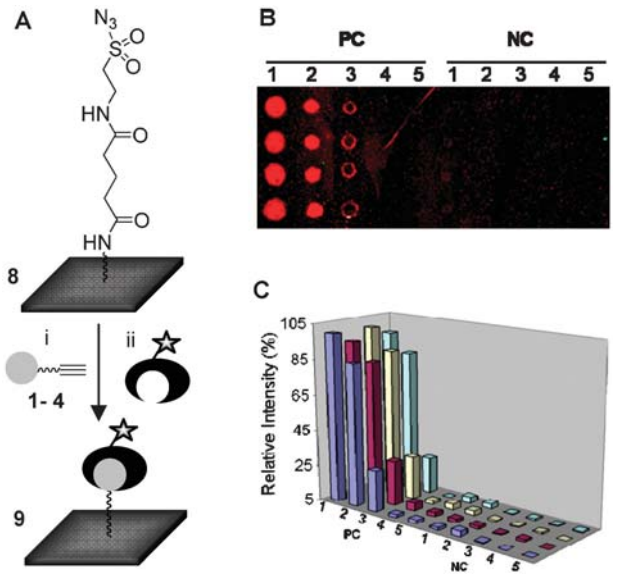

Fig. 2 (A): Immobilization of biomolecules onto sulfonylazide-functionalized glass slides, (i) alkyne-modified biomolecules (**1–4**), (ii) fluorescent dye-labelled protein. (B): Microarray of α-D-mannose-alkyne **2** (PC) immobilized onto a sulfonylazide-functionalized slide by CSR. 10 mM (1), 1 mM (2), 0.1 mM (3), 0.01 mM (4), and 0.001 mM (5) solutions were used for spotting. α-D-Mannose without alkyne was used as a negative control (NC). The slide was incubated with 100 nM Alexa-647-labelled Concanavalin A. After washing, the slide was scanned for fluorescence. (C): Plot of relative fluorescence intensity.

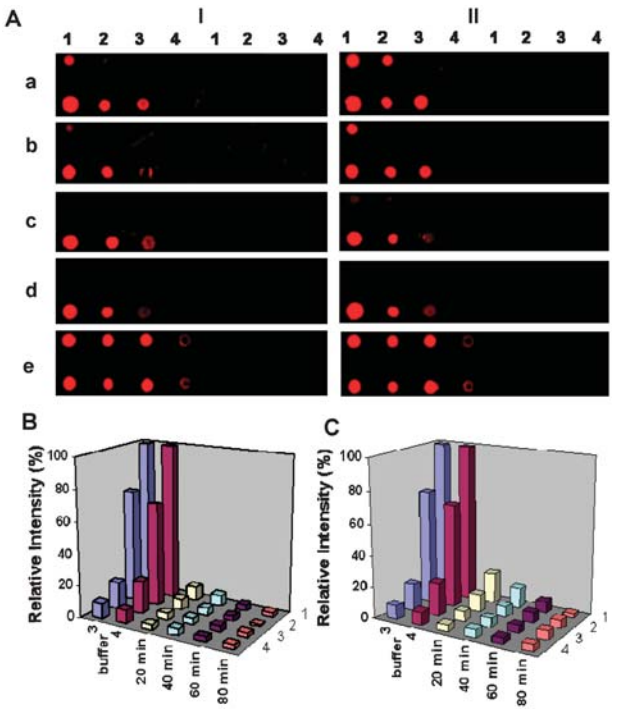

Fig. 3 Phosphopeptide array. Phosphopeptides **3** (i) and **4** (ii) were immobilized onto sulfonylazide slides *via* CSR at four different concentrations, 1 mM (1), 0.1 mM (2), 0.01 mM (3) and 0.001 mM (4), along with negative controls (see ESI†, Fig. S4). Phosphatase assay (A): Peptide array slides were incubated with two concentrations of PTP1B, (I) 5 μg mL^{-1} and (II) 0.5 μg mL^{-1}, over four time intervals, (a) 20, (b) 40, (c) 60 and (d) 80 min, and (e) peptide array region incubated with buffer only was used as a control region. The slides were then incubated with Ab-Biotin-Strep-Cy5 conjugate for 30 min and slides were scanned for fluorescence. (B) and (C): Relative fluorescence intensity plotted against phosphatase (PTP1B) incubation time at 5 and 0.5 μg mL^{-1} of PTP1B, respectively.

and were not immobilized, demonstrating that the immobilization occurred by means of the described click sulfonamide reaction. A time course experiment monitoring immobilization of biotin-alkyne **1** for 4–12 h revealed that good results were already obtained after 4 h (see ESI†, Fig. S3).

In a further experiment alkyne-derivatized phosphopeptides **3** and **4** (for their synthesis see ESI†) were immobilized onto sulfonylazide slides at different concentrations as described in Fig. 3. Immobilization efficiency was determined by treatment with an anti-phosphotyrosine antibody–biotin–streptavidin–Cy5 (Ab-Biotin-Strep-Cy5) conjugate and fluorescence detection (Fig. 3A). The results shown in Fig. 3e (see ESI†, Fig. S4) demonstrate that both phosphopeptides can be readily detected at concentrations of 0.01 mM. Peptides not containing an alkyne moiety (NC, see ESI† for the structures of the negative controls) were not immobilized, demonstrating that the CSR reaction is compatible with the side chain functionalities found in the immobilized peptides and the peptide backbone itself. The phosphopeptide arrays of **3** and **4** were also used for a dephosphorylation assay with phosphatase PTP1B which resulted in a time dependent rapid dephosphorylation of substrate peptide **3**, whereas peptide **4** was hydrolyzed by the enzyme with much lower efficiency (Fig. 3). These results demonstrate that phosphopeptide arrays prepared by means of the CSR reaction qualitatively reproduce the results obtained for PTP1B substrate mapping obtained with other immobilization techniques and in solution.[4f]

In order to demonstrate applicability to the immobilization of entire proteins, a fluorescent Cherry-Ypt7 protein, the yeast analogue of the human Rab7 protein, equipped with an alkyne was immobilized. Ypt proteins are a class of small GTPases critically involved in vesicular transport processes.[10] The required alkyne-modified Cherry-Ypt7 **11** was prepared by means of expressed protein ligation (Fig. 4A, see ESI†).[11] Cherry-Ypt7-alkyne **11** did react with Z-tauryl sulfonylazide **5** under click sulfonamide reaction conditions in solution to give Cherry-Ypt7-sulfonamide (see ESI†, Fig. S5).

For immobilization, the alkyne-modified Cherry-Ypt7 was spotted at different concentrations in PBS buffer as described above (see ESI†). After washing with PBS buffer containing 0.5% Tween 20 and water, reading of the fluorescence signal clearly revealed that the protein had been immobilized successfully (Fig. 4B). The minimum concentration yielding clear fluorescent signals was found to be 2 μM (see ESI†, Fig. S6). Cherry-Ypt7 without any alkyne attached (see Fig. 4B, NC) was not immobilized at all, demonstrating that the CSR is selective and compatible with the functional groups of all accessible amino acids on the Cherry-Ypt7 protein.

Successful immobilization of functional proteins *via* this approach was demonstrated by immobilizing an alkyne-modified Ras-binding domain (RBD, see Fig. 4 and ESI† for the synthesis of the tagged protein) of cRaf1 on a

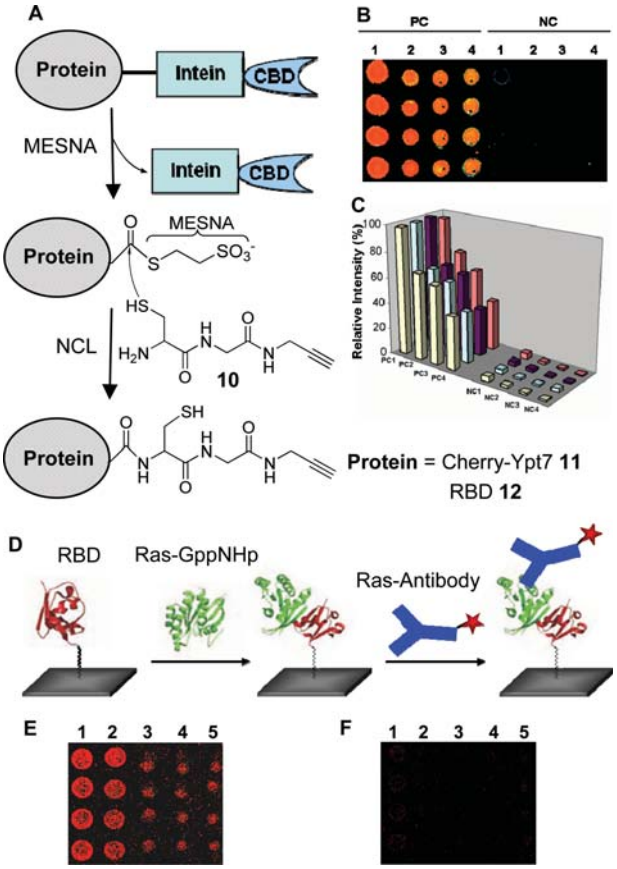

Fig. 4 Protein immobilization. (A): Preparation of alkyne-modified Cherry-Ypt7 **11** and RBD **12** *via* expressed protein ligation (EPL). (B): Cherry-Ypt7-alkyne (PC) was immobilized onto a sulfonylazide slide at four concentrations, 100 μM (1), 50 μM (2), 25 μM (3) and 12.5 μM (4), by CSR (Cherry-Ypt7 without alkyne functionality was used as negative control, NC). (C): plot of relative fluorescence intensity. (D): Schematic representation of RBD-alkyne immobilization onto sulfonylazide slide by CSR and Ras:GppNHp binding to RBD followed by a Ras-antibody. (E): RBD immobilized (at five concentrations, 1: 50 μM, 2: 25 μM, 3: 12.5 μM, 4: 6.2 μM, 5: 3.1 μM) on a slide was treated with activated Ras:GppNHp followed by Cy5-labelled Ras-antibody and scanning for fluorescence. (F): RBD slide incubated with Ras:GDP followed by Cy5-labelled Ras antibody. MESNA = sodium 2-mercaptoethanesulfonate. NCL = Native chemical ligation.

sulfonylazide slide. The interaction of the Raf kinase and Ras plays a prominent role in the Ras-MAP kinase signalling pathway.[12] RBD immobilized on the slide was treated with the Ras GTPase (aa 1–180) protein in its active state (*i.e.* bound to the non-hydrolyzable GTP analogue GppNHp) as well as with inactive Ras (Fig. 4E and F). Ras bound to immobilized RBD-alkyne was detected by subsequent incubation with a Cy5-labelled Ras-specific antibody. Only in the case of incubation with active Ras:GppNHp was a fluorescence signal dependent on the concentration of immobilized **12** observed. Thus immobilization *via* a C-terminal alkyne leads to fully functional RBD protein on the surface, which can discriminate between active and inactive Ras.

This research was supported by the Max Planck Gesellschaft, the Fonds der Chemischen Industrie and Zentrum für Angewandte Chemische Genomik (ZACG). TG and PJ are grateful to the Alexander von Humboldt Stiftung for research fellowships. The authors would like to thank K. Alexandrov for providing the Cherry-Ypt7 construct.

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