Methods for Site-Selective Chemical Pretein Immobilization

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Els bioxips de proteïnes (micro arrays) requereixen, per a una implementació eficient, d'un desenvolupament de les tècniques d'immobilització de proteïnes.

Es comenten diverses aproximacions químiques per tal d'assolir la immobilització de proteïnes d'una manera específica amb enllaços covalents. El desenvolupament en aquesta àrea condueix a millores tecnològiques per al desenvolupament de bioxips de proteïnes.

Protein biochips have received increasing attention recently in various areas of research. Simultaneously, the range of methods for their generation has been extended and refined. As a result, many powerful protein immobilization techniques are available to the scientific community today.[1] However, despite this development, most protein biochips continue to be prepared by traditional techniques, which do not always lead to optimal results in terms of protein biochip performance.[2]

Protein immobilization methods can be classified according to their specificity and interaction type. We recently reviewed the state-of-the-art in protein immobilization methods following these criteria.[1] Established, non-specific strategies, such as the immobilization of proteins via free amines of lysine side chains on aldehyde slides, are straightforward to employ since they do not require prior protein modification. Photochemical protein immobilization methods using highly reactive but non-specific intermediates also belong to this group. A drawback of these strategies is their tendency to result in random protein orientation on the biochip surface. This entails the risk of impairing a protein's ability to bind to an analyte, which can negatively affect biochip performance.[2] Specific strategies however, aim at immobilization of proteins in one orientation only and are thus not as susceptible to these problems, but mostly require prior modification of proteins with suitable functional groups or conjugates. These systems take advantage of directing capabilities of e.g. DNA, antibodies, native chemical ligation, enzymatic reactions or certain chemical reactions.

Apart from this criterion, the type of interaction at the heart of the protein immobilization method, i.e. whether it is non-covalent or covalent, is of importance. Proteins functionalized with partners of natural or non-natural affinity pairs, such as biotin or the polyhistidine tag, can be immobilized non-covalently on a surface displaying the corresponding interaction partner. However, these methods are susceptible to reversal of the immobilizing interaction, which can cause gradual protein detachment from the slide surface. Methods based on covalent bond formation do not suffer from this problem.

Thus, when taking into account all of these aspects, site-specific, covalent protein immobilization methods entail the most advantages for biochip performance despite the higher complexity involved in biochip preparation.

We have pursued various chemical approaches to site-specific, covalent protein immobilization. One example is the immobilization of proteins using the synthetically powerful Diels-Alder cycloaddition reaction due to its high tolerance of other functional groups and its ability to proceed in aqueous solvent systems.[3] Dienophile-functionalized streptavidin could be successfully immobilized on maleimide functionalized glass slides (Figure 1). The same approach could also be used to site-specifically modify proteins as was shown by fluorescent labeling of Rab7 through Diels-Alder chemistry.[3] The small Rab GTPases belong to the Ras superfamily of proteins and have been shown to be essential regulators of cellular vesicular transport.[4, 5]

In another approach, the Staudinger ligation was used to site-specifically immobilize the N-Ras protein (Figure 2).[6] Ras is a small GTPase that is involved in cellular signalling and plays a role in many human cancers.[7] N-Ras was C-terminally modified with an azide via expressed protein ligation (EPL) and then successfully immobilized on phosphane-functionalized slides. This system could also be used to site-specifically label Ypt1, a yeast Rab small GPTase, with a Dansyl fluorophore.[6]

We also investigated other types of cycloaddition reactions such as the clicke sulfonamide reaction (CSR), in which sulfonamides and terminal alkynes react to stable N-acyl sulfonamides under very mild conditions and under liberation
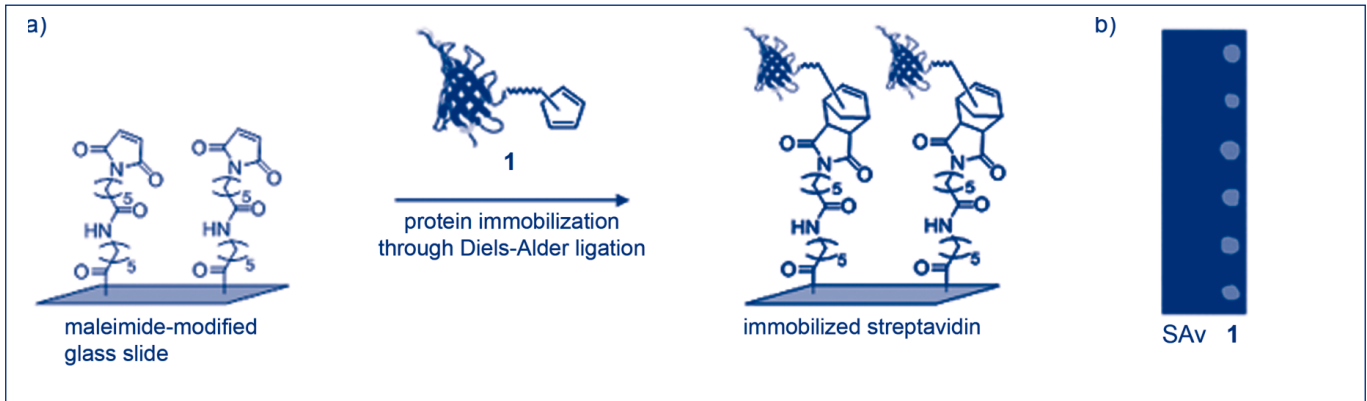
Figura 1. *a*) Diene–functionalized streptavidin is spotted onto maleimide–functionalized slides and is immobilized through Diels–Alder cycloaddition. *b*) Fluorescence scanner image of streptavidin (SAv) microarray after incubation with biotin–Cy5. Spots of diene–modified streptavidin are clearly visible while streptavidin, which has been spotted as a negative control, shows no immobilization.

of nitrogen.[8] The CSR was shown to be tolerant towards many functional groups as could be demonstrated by the ligation of biotin, mannose and a phospopeptide to a sulfonazide building block in solution. A fusion protein of Cherry fluorescent protein and Ypt7 as well as the Rasbinding-domain (RBD) of cRaf1, an effector of Ras, were

C-terminally alkyne-functionalized via EPL and then successfully immobilized in microarrays on sulfonazide slides (Figure 3). While Cherry-Ypt7 was detected directly via Cherry fluorescence, immobilized RBD was subjected to a binding assay using its binding partner Ras, which in turn could be detected by a fluorescently labeled anti-Ras anti-
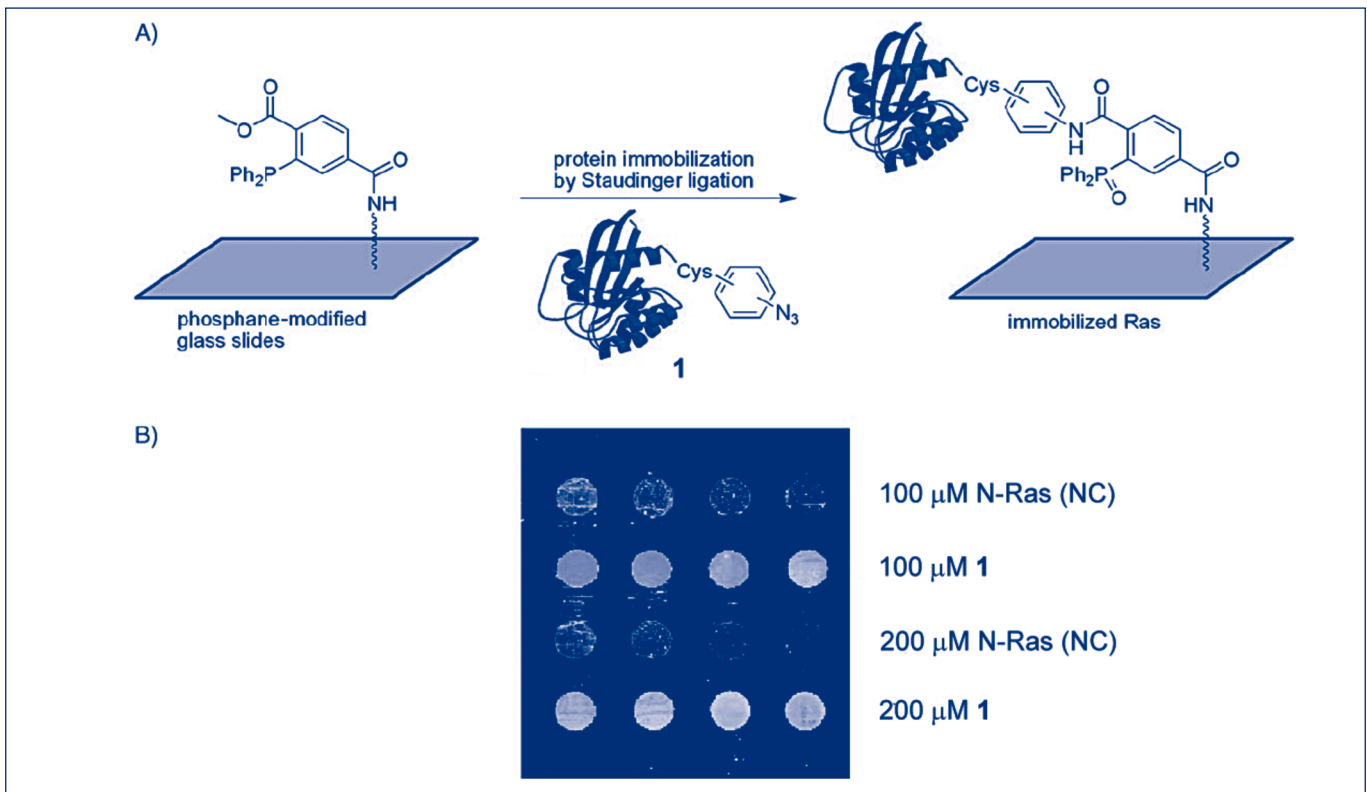

Figura 2. *a*) Azide–functionalized N–Ras is immobilized on phosphane–modified glass slides with the Staudinger ligation. *b*) After incubation of an azide–N–Ras/N–Ras microarray with a fluorescently labeled antibody against Ras, fluorescence is clearly detectable on spots of azide–N–Ras but not on spots of unmodified N–Ras.
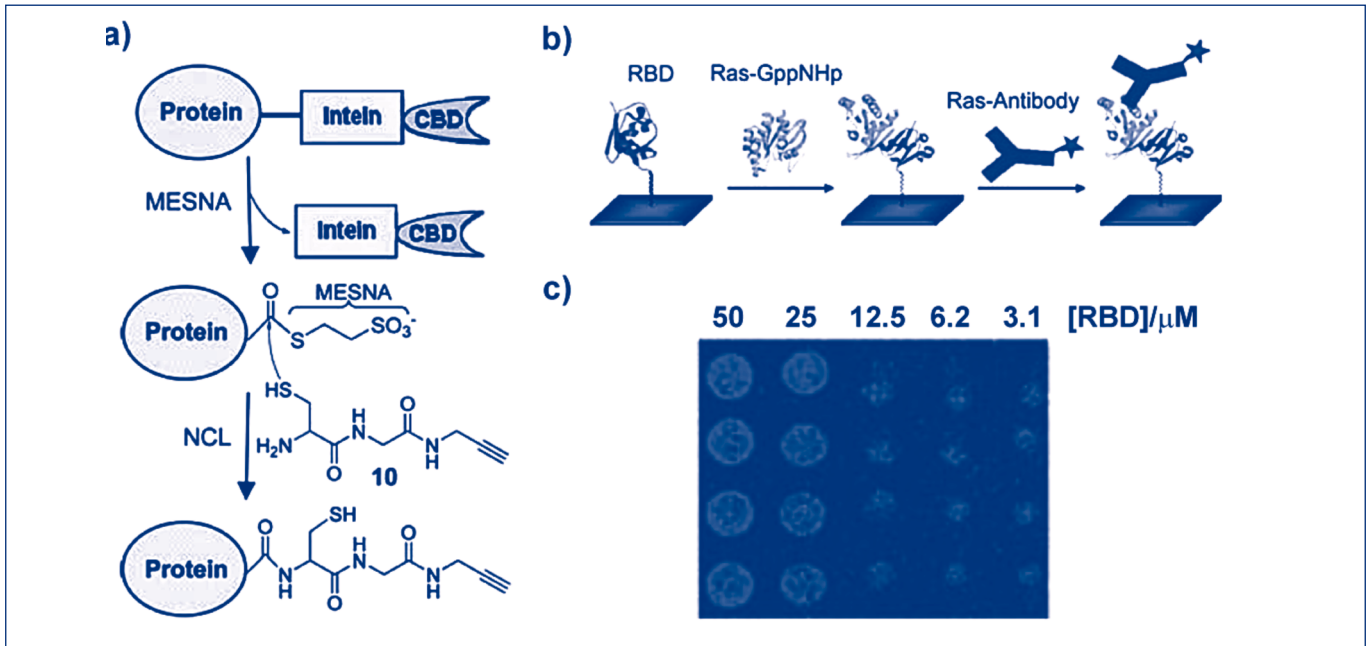
Figura 3. *a)* Terminally alkyne–modified proteins are generated through expressed protein ligation (EPL) by incubating a protein fused to an intein–chitin–binding–domain (CBD) protein with 2–mercaptoethanesulfonate (MESNA) to obtain a reactive protein thioester. The thioester is then reacted with a cysteine–containing alkyne building block in a native chemical ligation (NCL). *b)* A microarray of the Ras–binding domain (RBD) of cRaf1, an effector of GTP–loaded Ras is obtained with the click–sulfonamide reaction (CSR) by spotting alkyne–modified RBD on sulfonamide–functionalized slides. Following CSR immobilization, the microarray is incubated with GppNHp–loaded Ras. GppNHP is a non–hydrolyzable analogue of GTP. Bound Ras is then detected with a fluorescently labeled antibody. *c)* Fluorescence read–out of the RBD microarray processed as in *b)* with the RBD in different concentrations.
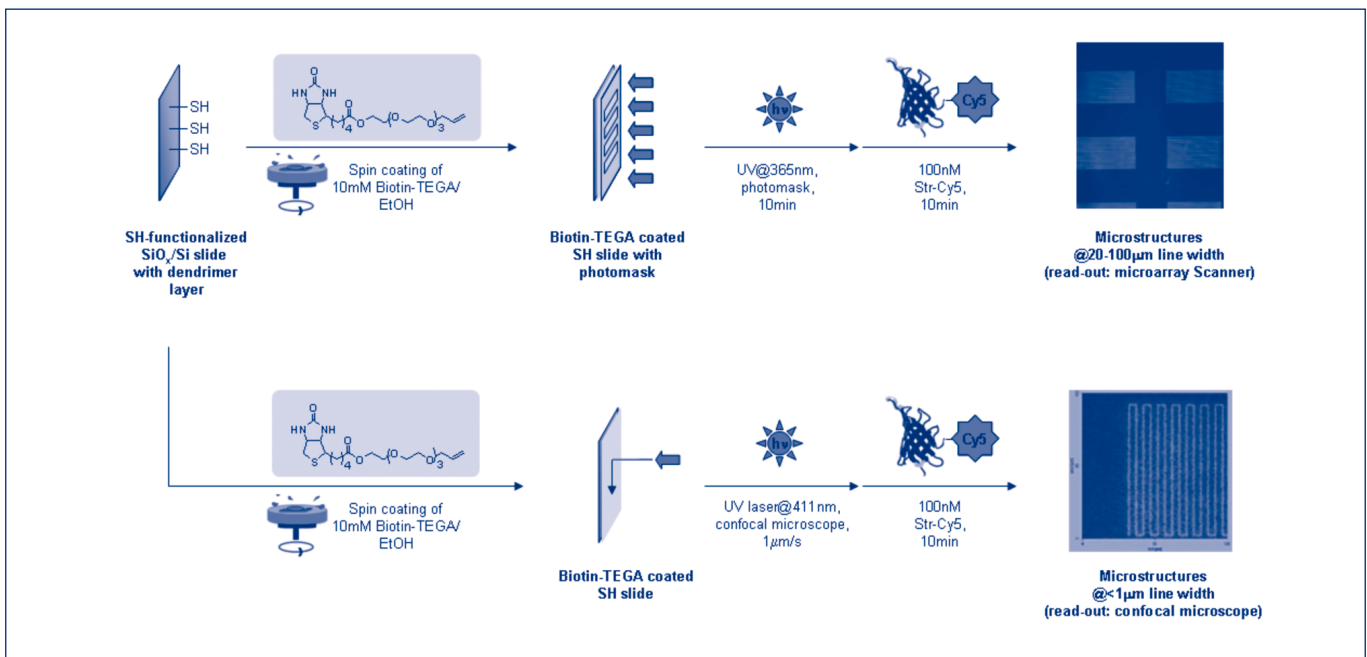

Figura 4. Micrometer– and submicrometer biotin–streptavidin structures obtained via thiol–ene photoimmobilization. Microstructures of biotin are generated by spin–coating olefin–functionalized biotin onto a thiol–functionalized slide and subsequently exposing the slide to UV light at 365 nm through a photomask. After incubation with labeled streptavidin, microstructures are detected with a microarray scanner. To decrease feature size, lines are «written» with a UV laser at 411 nm to obtain a biochip with submicrometer biotin–streptavidin structures.

body. This demonstrated the capability of the CSR-based approach to enable multi-step biological assays involving protein-protein interactions on the biochip surface. In addition, CSR was also used to create phosphopeptide and carbohydrate microarrays.[8]

Further research employs the immobilization of proteins using the light-initiated thiol-ene reaction. Light-based immobilization methods are of particular interest since light exposure is freely controllable using, for example, a photomask or other suitable optical techniques. This grants light-based protein immobilization methods an exceptional amount of flexibility. Protein features of variable size and shape are achievable, which would otherwise require complex and expensive infrastructure. Furthermore, feature sizes from the multi-centimeter down to the nanometer range are possible. In case of the latter, the optical diffraction limit can be overcome with near-field-based techniques for which suitable instrumentation is commercially available.

In the radical thiol-ene reaction, thiols and olefins react to thiolethers when exposed to UV light at 365 nm. In a proof-of-concept study, we have used this reaction for the photomicrostructuring of small molecules.[9] Biotin micro- and nanostructures were used as templates for the immobilization of streptavidin and N-Ras (Figure 4). The obtained protein biochips were shown to retain their functionality in a follow-up protein-protein-interaction assay.[9]

The ongoing development of ever more powerful protein immobilization methods promises a breakthrough in protein biochip technology. We envisage that non-specific protein immobilization strategies will be replaced by specific techniques, which is expected to lead to better protein biochip performance.

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Biografia Prof. Herbert Waldmann

El professor Herbert Waldmann estudià química i va obtenir el grau de doctor l'any 1985. Durant els dos anys següents, va dur a terme una estada postdoctoral a la Universitat de Harvard. L'any 1991 va obtenir l'habilitació a la Universitat de Mainz i poc després va accedir com a professor de química orgànica a la Universitat de Bonn. L'any 1993 esdevingué professor de química orgànica a la Universitat de Karlsruhe.

Des de l'any 1999 és director del Departament de Biologia Química a l'Institut Max Planck de Fisiologia Molecular i, alhora, és professor de bioquímica a la Universitat Tècnica de Dortmund. D'entre els nombrosos premis i distincions pel seu treball, destaquen el Premi Otto Bayer i la Medalla Max Bergmann.

Ha estat autor d'unes cinc-centes publicacions científiques, d'entre les quals destaquen els seixanta-vuit articles a la revista *Angew. Chem. Int. Ed.*, de màxim índex d'impacte en química.

Les seves línies de recerca cobreixen diversos àmbits de la biologia química que abasten des de mètodes per a la immobilització de proteïnes i molècules petites en superfícies fins a la síntesi de col·leccions de compostos inspirades en productes naturals.

Investigador convidat a la Conferència Fèlix Serratosa 2009.