Chapter 11

Chemoselective Strategies to Peptide and Protein Bioprobes Immobilization on Microarray Surfaces

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Abstract

Ordered and reproducible bioprobe immobilization onto sensor surfaces is a critical step in the development of reliable analytical devices. A growing awareness of the impact of the immobilization scheme on the consistency of the generated data is driving the demand for chemoselective approaches to immobilize biofunctional ligands, such as peptides, in a predetermined and uniform fashion. Herein, the most intriguing strategies to selective and oriented peptide immobilization are described and discussed. The aim of the current work is to provide the reader a general picture on recent advances made in this field, highlighting the potential associated with each chemoselective strategy. Case studies are described to provide illustrative examples, and cross-references to more topic-focused and exhaustive reviews are proposed throughout the text.

Key words Peptide microarray, Oriented immobilization, Chemoselective reactions, Click chemistry

1 Introduction

Array-based technologies bring a unique potential for rapid and high-throughput analysis [1, 2]. Nevertheless, in order to guarantee the consistency and the reliability of the analytical data, the full exploitation of such powerful techniques relies on the effective and reproducible immobilization of bioprobe ligands onto the sensor surface. A key aspect in the manufacturing of microarrays is to preserve the biochemical properties of the immobilized biomolecule as well as to ensure its stable binding on the sensor surface throughout the experimental procedures.

Traditional schemes for biomolecule immobilization rely on non-covalent random absorption of the analyte on the sensor surface based on electrostatic and hydrophobic forces or, alternatively, on aspecific covalent binding [3–6]. In the latter case, covalent linkages are formed between bioprobe functional groups, mainly amine groups from lysine residues and sulfhydryl groups from cysteine side chains, and respectively cross-reactive groups present at the sensor surface. While covalent immobilization is less susceptible to

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those environment-related factors (pH, ionic strength) whose variation may compromise the stability of the probe attachment, as in the case of non-covalent interactions, the chemical conditions used during immobilization may be not always compatible with bioprobe structural and functional integrity [3].

In recent years, due to the growing awareness that peptide molecules play a key role in modulating a wide range of biological functions, along with their relative ease of synthesis and manipulation, peptides have progressively experienced an increasing application in the development of biosensors [7–9]. Nonspecific immobilization of peptide ligands on sensor surfaces potentially leads to heterogeneous presentation of the bioprobe (Fig. 1a) and, as a consequence, inherently mines the performance of the analytical assay, particularly in those cases where an excellent signal-to-noise ratio (SNR) is essential to lowering the influence of background signal levels. Spatially oriented immobilization strategies are therefore appealing to enable optimal exposure of the peptidic probe in order to guarantee the retainment of its full functionality with respect to the ligand-target interaction (Fig. 1b). To this end, a set of chemoselective strategies to specifically immobilize probe-surface linkage, suitable even in the context of highly functionalized molecule such as peptides, have been developed.

2 Bioorthogonal "Click" Reactions

Site-specific conjugation requires uniquely reactive functional groups. To meet the requirement of bioorthogonality, such reactive chemical handles should be non-native and non-perturbing and should give rise to selective reactions even in a complex biological context. Moreover, ideal bioprobe immobilization strategy should be high yielding and cost effective. The so-called "click" reactions well match these criteria. Indeed, the philosophy of "click chemistry" encompasses a wide range of chemical transformations (e.g., cycloadditions, nucleophilic substitutions, additions to carbon multiple bonds) mainly characterized by high conversion efficiency and selectivity, broad applicability, and biologically benign reaction conditions [10]. Given the often exquisite chemoselectivity they display toward common functional groups, click reactions have not surprisingly found extensive application in the realization of peptide bioconjugates and to build peptide-functionalized biomaterials [11]. Moreover, the fast reaction kinetics that commonly characterize this class of reactions well address the need for fast immobilization, particularly relevant to overcome intrinsically slow reactivity at the solution-solid support interface and thereby to reduce the bioprobe denaturation risks associated to prolonged reaction times. The most popular click reactions suitable for peptide-specific immobilization are herein presented and discussed.

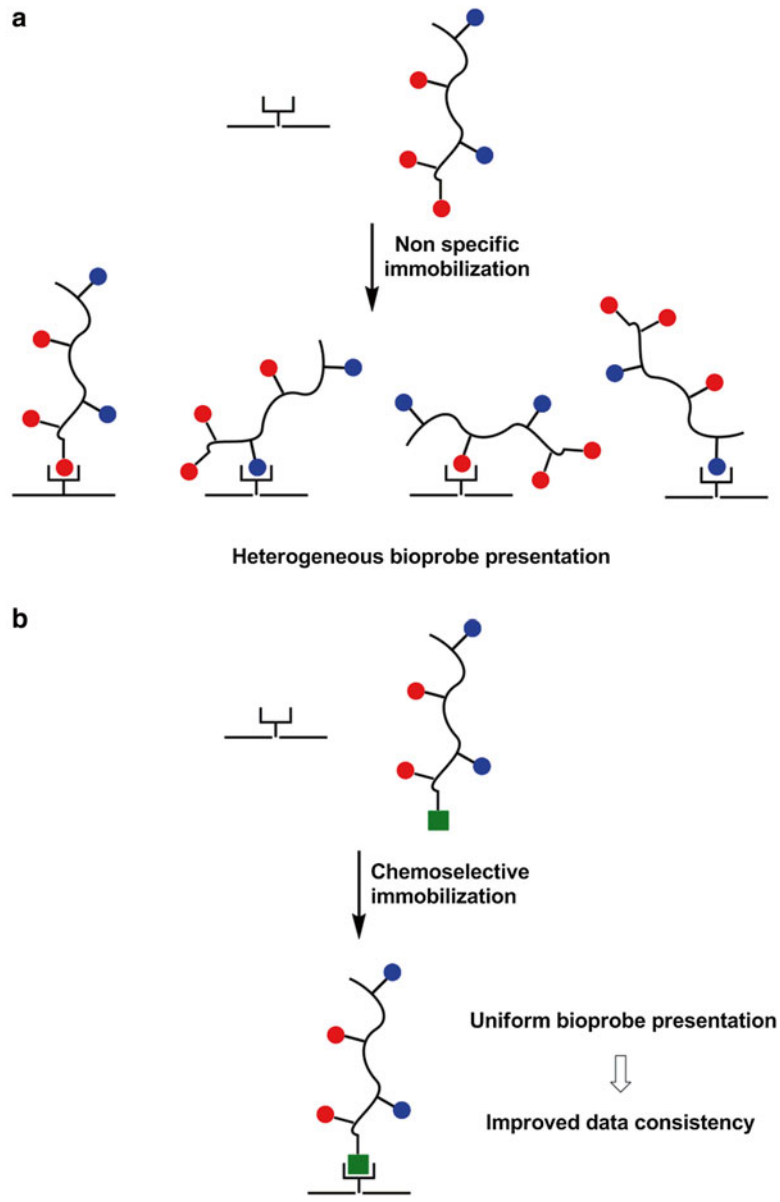


Fig. 1 Nonspecific versus chemoselective presentation. Nonspecific presentation may result in multiple forms of bioprobe display (panel **a**), potentially affecting optimal interaction with candidate target. In contrast, chemoselective immobilization results in univocal bioprobe presentation (panel **b**), which ensures ideal probe-target interaction and, thereby, reproducible data analysis

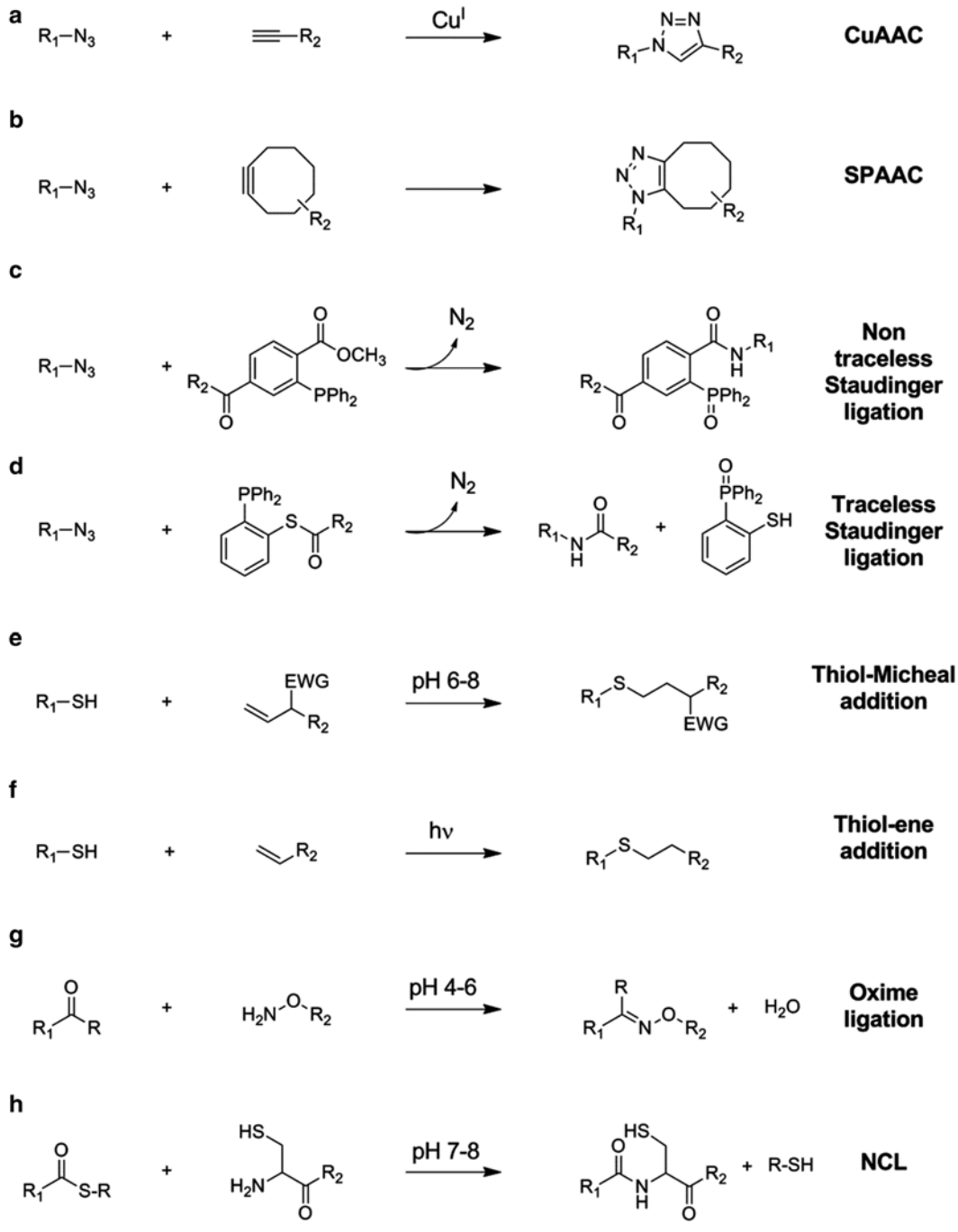
2.1 Azide-Alkyne Cycloaddition

Huisgen 1,3-dipolar cycloaddition of azides to alkynes is a well-known process, whose first reports date back to the early 1960s. However, this reaction gained an unprecedented popularity only after the introduction of the copper-catalyzed variant (CuAAC, Scheme 1a) [12, 13]. The efficiency and selectivity of this reaction combined with its wide scope, spanning from organic synthesis to

peptidomimetics and bioconjugation techniques, have represented the basis for such a great success. CuAAC is indeed generally characterized by almost quantitative yields, fast to instantaneous reaction kinetics, wide tolerance to different reaction conditions, and, notably, nearly full orthogonality with common peptide and protein functional groups. Taken together, these features allow azido- or alkynyl-functionalized peptides to undergo selective and controlled conjugation, either to other functionalized biomolecules or solid supports. Additionally, the triazole moiety which is generated upon cycloaddition is remarkably inert under standard biological conditions [14], which guarantees the stability of the immobilization. It is then not surprising that CuAAC has been thoroughly exploited to obtain functionalized surfaces for analytical purposes.

Noteworthy, Lin and coworkers elegantly showed the impact of a site-specific bioconjugation strategy by selectively immobilizing the maltose-binding protein (MBP) to a glass surface through CuAAC and by evaluating its binding activity in comparison to randomly linked MBP. Results clearly evidenced that the specifically immobilized MBP preserved considerably higher binding activity than the randomly coupled MBP [15]. Further support to oriented immobilization advantages was recently reported by Zilio et al. in the functionalization of a Si-SiO_2 substrate using a clickable polymeric coating to enable the attachment of azido-modified peptides. Correct orientation of peptidic probes was found significantly favorable for optimal ligand-target antibodies interaction [16]. CuAAC can also serve the purpose to produce peptide-functionalized surfaces with tunable ligand concentration or patterned topology to quantitatively ascertain complex molecular interactions [17–20]. In a seminal report, the Becker group produced a functionalized surface where the peptide ligand density was tuned by means of a gradient concentration of alkyne functional groups. Following immobilization of azido-modified RGD peptides via CuAAC, the effect of peptide concentration on cell adhesion could be quantitatively evaluated on a single slide [20]. Intriguingly, the generation of ligands gradient can arise from the local generation of the Cu(I) catalyst, as demonstrated by Larsen an collaborators [19].

The only potential limitations associated to CuAAC arise from residual copper catalyst which, depending on the system, can lead to ligand or target denaturation and/or interference with analytical signal detection. To partially overcome this issue, while also improving reaction performance, Cu(I)-binding ligands have been developed [21]. Moreover, in recent years, catalyst-free strain-promoted azide-alkyne cycloaddition (SPAAC) has also emerged as a gold-standard technique for conjugation of peptide-based probes, particularly for in vivo applications (Scheme 1b) [22]. In this case, the driving force of the reaction is uniquely given by the



Scheme 1 Reaction schemes for common click-type reactions (**a**) Copper catalyzed azide-alkyne-cycloaddition (CuAAC) (**b**) Strain promoted azide-alkyne-cycloaddition (SPAAC) (**c**) Non-traceless Staudinger reaction ligation (**d**) Traceless Staudinger ligation (**e**) Thiol-Micheal addition (**f**) Thiol-ene addition (**g**) Oxime ligation (**h**) Native chemical ligation (NCL)

intramolecular strain of the cyclooctyne used as the azido counterpart; thus, no metal catalyst is required. However, the high reactivity of the cyclooctyne makes it more feasible to cross-react with other functional groups, such as cysteine thiols, leading to aspecific binding. Also, this technique is limited by the costs and the difficulties in synthesizing the cyclooctyne building blocks to be incorporated in the peptide ligand. Nevertheless, many cyclooctyne-based probes have been developed, whose reactivity and properties are modulated by electronic and steric effects [23].

For instance, the azadibenzocyclooctyne (ADIBO) shows a good compromise between reactivity and synthetic accessibility and, interestingly, was claimed to outperform conventional CuAAC in the immobilization of acetylene-functionalized glass slides with azido-functionalized peptides [24]. Pfeifer and collaborators reported on the use of ADIBO-activated slide surfaces for the preparation of high-density fluorescently labeled peptide microarrays [24]. Excellent immobilization kinetics, good spot homogeneities, and reproducible signal intensities were obtained. Also, interestingly, the specific immobilization of bovine serum albumin (BSA) and dextran via SPAAC led to reduced nonspecific binding of fluorescently labeled IgG to the microarray surface in comparison with other techniques. SPAAC-mediated microarray surface functionalization with peptides was also exploited by Chaikof and coworkers [25]. Immobilization occurred in a fast (<15 min), selective, and tunable fashion. Notably, the generation of a physiologically stable linker methodology also allowed the authors to perform peptide decoration of mammalian cells without compromising their integrity.

2.2 Staudinger Ligation

Azides are chemically inert and relatively stable functional groups under standard biological conditions and only rarely appear in natural biological environments. Not surprisingly, azides have been used in several conjugation strategies, including the Staudinger ligation, which occurs between an azide and a phosphine compound to yield a native amide bond [11, 26]. Staudinger ligation can be divided into two subclasses, traceless and non-traceless, depending on whether the phosphine oxide generated during the reaction is contained or not in the ligated product (Scheme 1c, d). Noteworthy, this reaction can be performed in aqueous buffer with no need for metal catalyst. The reaction kinetics, as like as the final yields, are mainly determined by the structure of the phosphine taking part in the reaction. However, generally, reaction kinetics are considerably slower than CuAAC. The reaction is also amenable to some side reactions, mainly oxidation of the phosphine, which may result in lowered yields. However, the bioorthogonal nature of both the azide and the phosphine functions has resulted in the Staudinger ligation finding numerous applications in various immobilization strategies [27, 28].

For example, Kohn and coworkers provided new insights on the substrate specificity of protein tyrosine phosphatases (PTPs) through the generation of a phosphotyrosine (pTyr)-peptide microarray on a phosphane-modified glass slide obtained by peptide ligand immobilization via non-traceless Staudinger ligation [29]. Similarly, azide-tagged N-Ras proteins were selectively immobilized onto phosphane-modified glass surfaces, and protein functional activity was retained [30, 31]. Likewise, the Raines group reported on the immobilization of the S-protein with good efficiency and almost fully intact functionality [32]. Several others reported on the use of Staudinger ligation for selective immobilization methods, highlighting the considerable potential of this reaction for bioconjugation purposes.

2.3 Thiol-Michael Addition

The Michael addition involves the addition of a nucleophile, also called a "Michael donor," to an activated electrophilic olefin, the "Michael acceptor," resulting in a "Michael adduct" (Scheme 1e). While typical Michael donors refer to enolates, a wide range of chemical functionalities possess sufficient nucleophilicity to perform as Michael donors [33]. In the biological context, both amines and thiols are exploitable as non-enolate nucleophiles. Cysteine thiols are usually more nucleophilic than amines, and the attack of the free sulfhydryl group to the activated alkene to afford thioethers generally proceeds with fast reaction kinetics and high conversion. Moreover, smooth reaction conditions, i.e., weakly basic aqueous buffer at room temperature, are optimal for bioconjugation purposes and enable a fair selectivity with respect to the potentially competing free amine groups. Due to the plethora of electron-withdrawing activating groups that enable the thiol-Michael addition, the reaction kinetics are highly dependent on the nature of the Michael acceptor [33]. Recently, acceptor reactivity has been ranked, with maleimide, vinyl sulfone, and acrylates performing the best [34]. Accordingly, bioprobes conjugation mediated by these functionalities has been extensively exploited to generate functionalized sensor surfaces [35–37]. A case example is given by the work reported by Fu et al. [38]. An array of β-galactosidase (β-Gal)-anchoring peptides was generated by means of simple modification of aminated microwells with bifunctional linker SMCC, followed by rapid and covalent immobilization of peptides through thiol-maleimide addition. Subsequent β-Gal anchoring afforded an immobilized enzyme that exhibited considerably higher activity with respect to otherwise immobilized β-Gal. In addition, peptide-modified surfaces were found to positively affect the thermal stability of the bound enzyme, like the stability under storage conditions. Taken all together, authors estimated a 20-fold increase in enzyme activity for β-Gal on peptide-modified surface, highlighting how deep is the impact an appropriate selection of the immobilization strategy may have in obtained results.

2.4 Thiol-ene Addition

Since the introduction of the archetypal concept of click chemistry by Sharpless and coworkers, many fast and chemoselective transformations have emerged as attractive click-type processes. Among these, the thermal- or UV-initiated addition of a thiol to an alkene through a radical mechanism (Scheme 1f), commonly termed thiol-ene reaction, has earned the click status in view of its favorable peculiarities [39]. High efficiency, broad orthogonality, and compatibility with physiologic conditions make thiol-ene addition a feasible candidate for selective conjugation. Additionally, the robust thioether linkage which is formed upon thiol-ene addition displays remarkable stability in a wide range of environmental conditions. However, careful tuning of the reaction parameters is required to limit undesired side reactions, e.g., alkene polymerization, and to ensure excellent reaction yields [11]. Intriguingly, the modular nature of the reaction in virtue of the photoinitiation feature allows spatial and temporal control of supports functionalization. For example, a landmark contribution to photochemical surface patterning has been reported from the Waldmann group [40]. Photochemical coupling of a set of olefin-tagged biomolecules to a thiol-modified surface allowed the fabrication of a microarray with precise control on protein immobilization even in the sub-micrometer scale. Functional integrity of immobilized molecules was also assessed and found to be comparable to that one in solution phase. Hawker and collaborators exploited thiol-ene chemistry to produce multifunctional microarrays embedded at the surface of poly(ethylene glycol)-based hydrogels [41]. Mild reaction conditions (UV irradiation at 365 nm for 2 min) allowed the incorporation of a wide range of orthogonal chemical handles exploitable for click-mediated selective conjugation of biomolecules, including functional peptides to direct cell adhesion. The combination of polymer direct functionalization with orthogonal postfunctionalization allowed the generation of different platforms for multiple display of functionally distinct biomolecules for different investigation purposes.

2.5 Oxime Ligation

The generation of an oxime bond between two biomolecules through the condensation of a carbonyl group (aldehyde or ketone) with an aminooxy group is commonly referred as oxime ligation (Scheme 1g). This reaction is particularly attractive due to its true click character: high conversion efficiency, chemoselectivity toward other functional groups, and mild reaction conditions in aqueous media are indeed distinctive features of the oxime ligation [42]. In addition, water is the only side product formed in the process, and no metal catalyst is required. Despite this, oxime ligation has not benefitted from the same broad popularity of other click strategies such as CuAAC. This is likely due to the traditional limitations associated with the synthesis of aldehyde- and aminooxy-functionalized biomolecules. However, recent

advances have enabled oxime ligation to gain a progressively leading role for bioconjugation purposes [11, 43, 44]. One appealing feature of oxime ligation lies in its reversibility. Indeed, unlike other covalent linkages, the oxime bond is reversible as a function of pH. Conveniently, the operational range of pH 4–8 largely avoids undesired hydrolysis of the oxime bond and guarantees the stability of the conjugate under standard biological conditions. Oxime bond hydrolysis can instead be triggered at lower or higher pH values. Notably, the pH responsiveness of the oxime bond enables the generation of dynamic biomaterials which can be adopted for controlled capture-and-release strategies. Patterned peptide-presenting surfaces are essential tools in the investigation of cell behavior in biomaterials and tissue engineering. Functionalized surfaces with patterned topography were realized by capping either the aminooxy or aldehyde group with a photolabile group. Upon unmasking of the reactive groups with site-specific UV irradiation, ligand immobilization can be performed via oxime ligation to functionalize the surface with controlled spatial resolution. This approach found application in the Dumy, Yousaf, and Barner-Kowollik groups [45–47]. Maynard and collaborators have exploited a combination of oxime ligation and CuAAC to immobilize different proteins on a functionalized surface. The same authors reported a different strategy to surface patterned functionalization by electron-beam lithography. Aminooxy groups on the surface were then coupled to ketone-functionalized RGD peptides, which were shown to retain functional integrity [48].

3 Native Chemical Ligation

Native chemical ligation consists in the condensation of two free peptide fragments to yield a new construct linked by a native amide bond (Scheme 1h). The reaction occurs between a peptide thioester and another peptide fragment bearing an N-terminal cysteine and proceeds through an initial (reversible) transthioesterification step followed by an irreversible S,N-acyl shift which originates the new native amide bond. Although not always regarded as a prototypic click reaction, NCL chemoselectivity and efficiency, along with mild operational conditions, are surely appealing features in the peptide chemistry arena. Remarkably, NCL likely represents the gold-standard technique for the total chemical synthesis of proteins [49]. To overcome synthetic limitations associated with the synthesis of peptide thioesters, new strategies entailing thioester surrogates and precursors have been developed, like unnatural thiolated amino acids which have been synthesized to expand the potential of NCL [50]. NCL reaction kinetics are highly dependent on the substrates participating in the reaction; however, the time

required for nearly quantitative product formation can be considerably short (<1 h). Despite NCL has recently experienced a growing application in the preparation of peptide bioconjugates, its potential for the functionalization of surfaces for analytical applications remains widely unexplored. Only few examples have indeed been reported to date. Among these, Helms et al. realized a cysteine-functionalized biosensor surface for conjugation with peptide thioesters [7]. Specific and complete immobilization of a decapeptide occurred with fast kinetics, and the peptide ability to engage in specific binding to its target was preserved. Interestingly, a peculiar feature of cysteine-functionalized sensors is that functional groups maintain their reactivity for extended periods of time. As a consequence, ligand density can be tuned stepwise at any time in sensor's life by using short ligation pulses, and the same functionalized surface can be used for separate experiments. Conversely, peptides bearing an N-terminal cysteine can be chemoselectively immobilized onto thioester-functionalized slides, as, for example, reported by the Yao group [51]. More recently, Dendane et al. exploited this kind of approach for the site-specific and chemoselective immobilization of peptides on hydrogen-terminated silicon nanowires [52].

4 Conclusions and Future Perspectives

Microarray-based screening is progressively gaining central importance in the dissection of a number of biological processes. The development of innovative analytical platforms strongly relies on new synthetic methods to allow controlled, oriented, and robust bioprobe immobilization on sensor surfaces. In this context, the last decade has experienced a growing application of the so-called click reactions for the sophisticated functionalization of sensors. Among these, some of the most popular have been discussed in the present work. Notwithstanding, the click repertoire already includes a more extensive range of chemoselective transformations, such as Diels-Alder reactions, which have been exploited for the generation of peptide and protein bioconjugates. Additional reactions that match the click-type criteria are likely to be discovered in the near future. Moreover, the potential of already existing click-type reactions is presumably going to be fully unrevealed thanks to new synthetic breakthroughs that may overcome current limitations. The combination of different and orthogonal strategies will, also, contribute to widen the available analytical toolbox for new devices development. Overall, a growing synthetic flexibility, a more and more refined control on immobilization parameters, and an increasing range of possible applications are likely to characterize the microarray field over the upcoming years.

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