**Immobilization Strategies for Peptide Microarrays**

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# Abstract

Peptide microarrays have been developed over the last decade into a technology that can profile biomolecules. It has proven to be a versatile tool for epitope-mapping, substrate profiling and probing antigen-antibody, protein-protein and protein-ligand interactions, which all can lead or have led to the discovery of new drugs and drug targets. Microarrays are convenient to use because only miniscule quantities are needed for screening a high number of substances. Peptides used in peptide microarrays are synthesized in such a way that they can bind via either a covalent or non-covalent bond to the microarray surface. These peptides often need a special functionality, but there are also strategies that make use of the occurring moieties in the peptide to achieve immobilization. This thesis gives a short summary of the development of peptide microarrays and discusses the strategies that have been developed for the immobilization of peptides on glass microscopic slides.

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# List of Abbreviation

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| |  |  | | --- | --- | | Ahx | Aminohexanoic acid | | Ala | Alanine | | APTS | 3-Aminopropyltriethoxysilane | | Arg | Arginine | | Asn | Asparagine | | Asp | Aspartic acid | | Boc | *Tert*-Butyloxycarbonyl | | BOP | Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate | | BSA | Bovine serum albumin | | CDI | 1,1’-Carbonyl-diimidazole | | Cys | Cysteine | | DIC | *N,N'*-Diisopropylcarbodiimide | | D*i*PEA | *N*,*N*-Diisopropylethylamine | | DMF | Dimethylformamide | | DNA | Deoxyribonucleic acid | | *e*.*g*. | Exampli gratia | | EDCI | 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide | | Fmoc | Fluorenylmethyloxycarbonyl | | GAPTS | 3-Glycidoxypropyltrimethoxysilane | | Gln | Glutamine | | Glu | Glutamic acid | | Gly | Glycine | | GST | Glutathione S-transferase | | His | Histidine | | HOBt | Hydroxybenzotriazole | | *i*.*e*. | Id est | | IgG | Immunoglobine G | | Ile | Isoleucine | | IPT | Isopropylidene tartrate | | Leu | Leucine | | Lys | Lysine | | MeNPOC | [R,S]-1-[3,4-[methylene-dioxy]-6-nitrophenyl]ethyl chloroformate) | | Met | Methionine | | MMA | Micromirrorarray | | MPAA | 4-mercaptophenylacetic acid | | MSA | Methylsulfonic acid | | NBD | 7-Nitrobenz-2-oxa-1,3-diazole | | NHS | *N*-Hydroxysuccinimide | | NMI | *N*-methylimidazole | | PAMAM | polyamidoamine | | PBS | Phosphate Buffered Saline | | PDMS | Polydimethylsiloxane | | PEG | Poly(ethyleneglycol) | | PGA | Photo generated acid | | PGA-P | Photo generated acid Precursor | | PGR | Photo generated reagent | | Phe | Phenylalanine | | PMMA | Polymethylmetacrylate | | PMPI | *p*-Maleimidophenyl isocyanate | | Pro | Proline | | PTP | Protein tyrosine phosphatase | | PyBOB | Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate | | SADP | N-succinimidyl-(4-azidophenyl)1,3-dithiopropionate | | SAM | Self-assembled monolayers | | SATA | Succinimidyl Acetylthioacetate | | SDPD | N-Succinimidyl-3-(2-PyridylDithio)-Propionate | | Ser | Serine | | SIAB | N-succinimidyl[4-iodoacetyl]aminobenzoate | | SMCC | Succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate | | SPPS | Solid Phase Peptide Synthesis | | SPR | Surface plasmon resonance | | TBTA | Tris(benzyltriazolylmethyl)amine | | TBTU | *O*-benzothiazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate | | TCEP | Tris(2-carboxyethyl)phosphine | | TFA | Trifluoroacetec acid | | TfOH | Trifluoromethanesulfonic acid | | THF | Tetrahydrofuran | | Thr | Threonine | | Trp | Tryptophan | | Ttds | 4,7,10-Trioxa-1,13-tridecanediamine succinimic acid | | Tyr | Tyrosine | | Val | Valine | |

# Introduction

Since the human genome project, researchers have been driven to develop new technologies for the identification of new genes targets. Genomics and its accompanied technologies led to the initiation of additional “-omics” fields, such as proteomics. Array technologies have become a powerful tool for rapidly analyzing and characterizing the entire proteome. This technology can potentially reveal protein functions and protein interactions in an organism and, moreover, can reveal the protein-drug interactions, which can largely contribute to drug discovery research.

The identification and function of the protein can be based on several types of arrays including protein microarrays, peptide microarrays, small molecule arrays and cell arrays. [1](#_ENREF_1) Compared to DNA microarrays, the development of the other types of arrays is more challenging. Enzyme profiling experiments, for example, have to meet up with several criteria such as proper folding or orientation of the enzyme or substrate, cofactors, pH and temperatures. Additionally, one has to take into account that the natural activity of the enzyme can be influenced by the immobilization on the surface. Enzyme identification and functionality studies can be performed with peptide microarrays, instead with protein microarrays. Peptides are known to be biologically active and can be easily synthesized in the laboratory. Compared to proteins, peptides are not always dependant on the folding to be active and are also in general more resistant against temperature and pH. The application of peptides on microarrays thus gives a large variety of molecules for robust screening assays. [2](#_ENREF_2)

Microarrays, also referred as (bio)chips, have attracted the interest of researchers due to the use in high throughput screening. Libraries containing large amounts of different molecules can be spatially and addressable immobilized on a surface and can be simultaneously analyzed with one or more samples, which are used to probe the array. It also allows of rapid data acquisition, parallel sample comparison, automated read out and integrated data analysis. Furthermore, because of the miniaturization in this method only a small sample volume is needed and miniscule quantities of expensive reagents. [3](#_ENREF_3) Peptides libraries used in microarrays are synthesized in various ways of peptide chemistry, *i.e.* split and mix, parallel or solution.

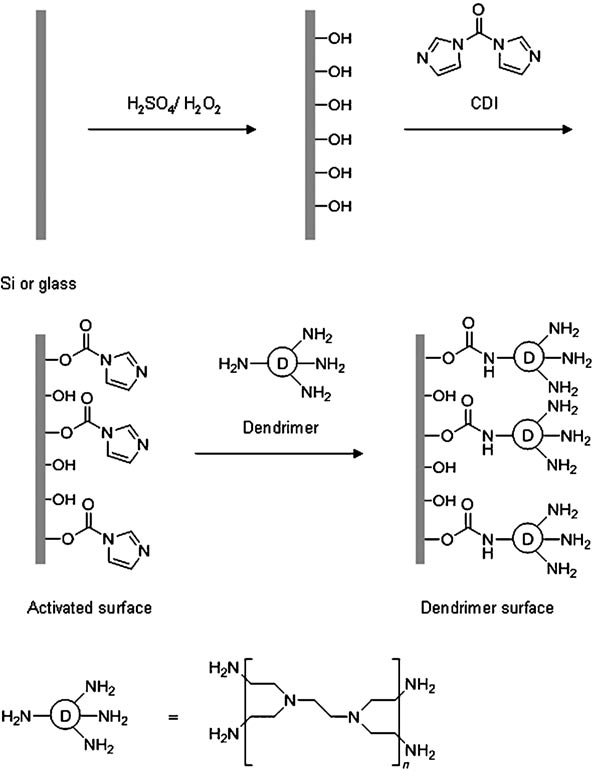
Since its discovery, peptide microarrays have evolved greatly and different techniques have been developed to optimize the immobilization of peptides to the surface of a microarray. These immobilization strategies will be discussed in this thesis.

# 1. The microarray surface

Before peptides can be immobilized to a microarray surface, both peptide and surface have to be functionalized. The first step in a microarray experiment is the fabrication and design of the support material. There are various surface materials that are suitable for peptide microarrays, which can be divided in flexible, porous planar supports (three dimensional supports) and rigid non-porous planar materials (two dimensional supports). Some examples of the porous materials are cellulose, cotton, membranes and polymeric films such as polyurethane and polyethylene. Rigid non-porous planar supports are for example glass, silicon, gold-coated surfaces, titanium and aluminium oxide, but also polymers such as polymethylmethacrylate, polypropylene, polystyrene, polypyrrole. In most cases amino acids or peptides cannot directly couple to the support, which means that the microarray surface has to be functionalized with appropriate reactive groups. The quality of the peptide microarray is determined by the surface properties such as homogeneity, roughness, hydrophobicity, density of functional groups, spacing between surface and biologically active components and the amenability to interact with proteins and enzymes. [4](#_ENREF_6) Although developments for the synthesis of the solid supports of the microarray are still ongoing, most of the supports are commercially available. Due to expensive and special equipment needed for the functionalization, at the moment it is most convenient to make use of these commercial available slides. The analytical methods that can be applied after the array experiment are for a large part dependent on the microarray surface that has been used, due to background signals. Usually radioactive or fluorescent tags are coupled to the peptides, but also label-free methods have been developed. The latter is favored, because tagging of the molecule creates extra steps and can cause possible side reactions. The label-free method is still undergoing a lot of development and the equipment needed is not standard. [5](#_ENREF_7) All of these variables have to be considered when choosing a microarray setup. In this chapter the most common used surfaces will be discussed.

1.1. Spacer

The direct attachment of a peptide to a support can cause steric constraint of the peptide’s reactivity or interaction capability compared to the peptide or protein in solution. Multiple contacts with the surface can decrease the activity of the peptide. By introducing a spacer between the peptide and the reactive group on the surface, these effects can be minimized. The spacer can also improve the efficiency of the peptide-ligand interaction and increase the density of immobilized peptides on the surface. To improve loading and homogeneity on surfaces, coupling with dendritic molecules and subsequent crosslinking can be performed, as shown in Figure 1.

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**Figure 1 Production of dendrimer-functionalized chips using poly(propylene imine) dendrimers. After treatment with piranha solution, the glass or silicon surface can be activated with carbonyldiimidazole (CDI).**[**6**](#_ENREF_8)

Dendrimers are compounds with branched structures that can carry a range of chemically reactive groups at their periphery. The branched structures have been applied for surface derivatization to create a larger functional surface area. The dendrimers are usually generated by direct surface modification with a presynthesized branched structure, such as polyamidoamine (PAMAM). [7](#_ENREF_9) phosphine [8](#_ENREF_11), or poly(propylene imine) (PPI) [6](#_ENREF_8). In Figure 2 a general structure of a PAMAM dendrimers is shown. Depending on the branching a subsequent generation (G) is reached.

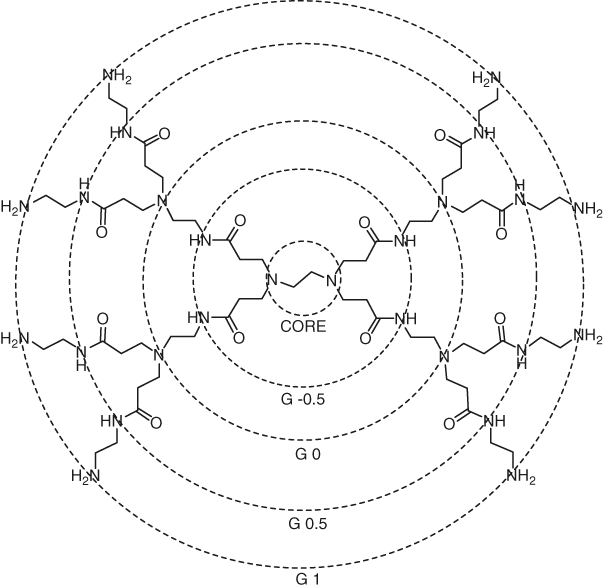
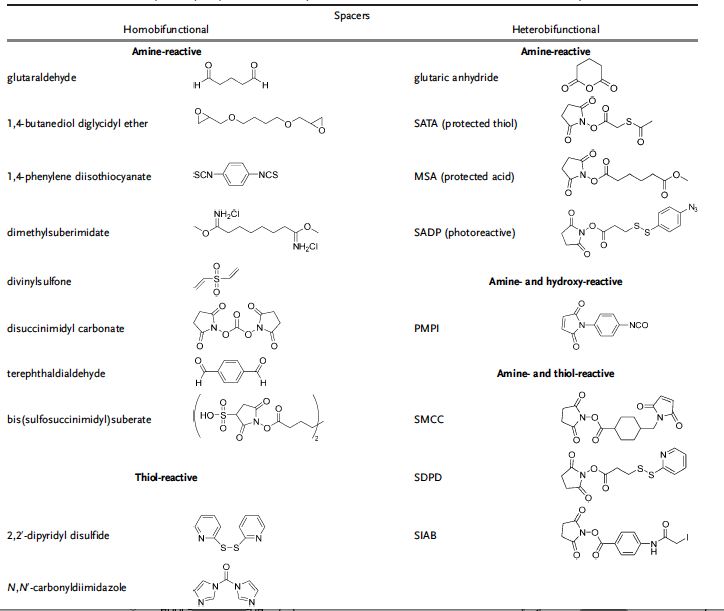


Figure 2 General structure of a polyamidoamine (PAMAM). G indicates the generation of the branching.

Spacers can posses a variety of chemical characteristics, but are often bifunctional, consisting of either two similar (homobifunctional) or two different (heterobifunctional) groups. Commonly used examples are listed in Table 1. Heterobifunctional linkers are often used instead of homobifunctional linkers to avoid the spacer connecting to two neighboring groups on the support or on the peptide. [9](#_ENREF_12) Poly(ethyleneglycol) (PEG) spacers are used very often as it prevents non-specific binding of peptides and proteins. [10](#_ENREF_13) Commonly used spacers are often commercially available and several companies supply them, in the paper of Jonkheijm and coworkers [9](#_ENREF_12) an overview of these spacers can be found.

Table 1 Commonly used spacer in microarrays. [9](#_ENREF_12" \o "Jonkheijm, 2008 #308)



1.2. Glass support

Glass slides are the most commonly used form of the rigid non-porous planar materials. They are impermeable and therefore diffusion of peptides and solvents into pores cannot take place, which accelerates the binding process and improves reproducibility. Glass slides are furthermore chemically inert, can sustain high temperatures and harsh washings steps, have a low intrinsic background fluorescence that do not interfere during imaging and scanning processes, which are all desirable properties for microarrays. Via commonly used organosilanization chemistry, glass slides can be easily modified. [9](#_ENREF_12), [11](#_ENREF_15) Functionalized glass microscopic slides are nowadays easy to produce, inexpensive and commercially available. The most available slides are functionalized with aldehyde, amino, epoxy, *N*-hydroxysuccinimide (NHS), biotin or maleimide reactive moieties. However, the functionalization of the glass slide is still performed in-house especially when a specific spacer is required or when the functionality is very reactive and cannot be stored well*, e.g*. isocyanate-functionalized glass slides.

Silanol groups can be generated on the glass slide by pretreatment of the glass surface with piranha solution (H2O2/H2SO4) or oxygen plasma. Organofunctional silanes of the general structure (RO)3Si(CH2)nX or trichlorosilanes are then used to introduce new functional groups as a monolayer on the surface (Figure 3). A large variety of silane reagents are available with amine, thiol, carboxy, epoxide, maleimide, hydroquinone and other functional groups for subsequent modification steps. [9](#_ENREF_12)

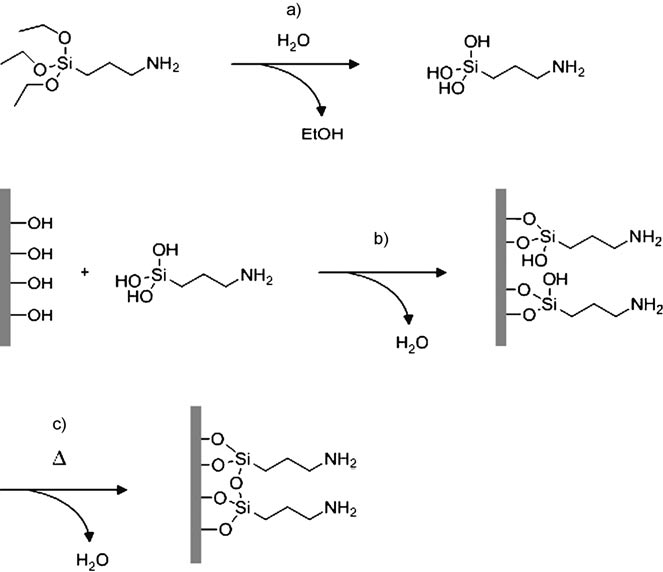


Figure 3 Mechanism for the silanization with 3-aminopropyltriethoxysilane (APTS). (a) Hydrolysis of the reactive siloxanes (b) Condensation with surface silanol groups (c) Cross-linking with thermal heating. [9](#_ENREF_12)

1.3. Silicon support

Next to glass, silicon has also been used as a surface material for the fabrication of microarrays. Silicon substrates are used, because they possess high electric conductivity, great chemical resistance to solvents, good mechanical stability, and low intrinsic fluorescence. Silicon surfaces, however, can oxidize spontaneously in air into an amorphous silica layer. The silicon-supported microarrays therefore are fabricated with a pretreatment of HF to remove the oxidized parts on the surface, which then can further react with unsaturated **ω**-functionalized alkenes upon ultraviolet irradiation or thermal activation. [12](#_ENREF_16) This functionalization causes self-assembled monolayers (SAMs) to form. Self-assembled monolayers are highly ordered molecular assemblies, which form spontaneously by chemisorption of functionalized, surfactant-like molecules. [13](#_ENREF_17) These molecules organize themselves laterally through van der Waals interactions between long aliphatic chains. Alternatively, silicon can be oxidized with plasma [14](#_ENREF_18) and functionalized with organosilanes (Figure 3), like carried out on glass slides.

In silicon supports, monolayers are covalently bound to surface hydroxy groups through Si-O bonds. The molecules used in the formation of such monolayers are either long chain chlorodimethyl alkylsilanes, alkyltrichlorosilanes, or trialkoxy(alkyl)silanes. [15](#_ENREF_19) Often, a ‘‘baking’’ step is necessary to ensure covalent bond formation between the SAM and the surface hydroxy groups.

1.4. Polymeric support

Another type of a rigid non-porous planar support is the polymeric support. The use of polymers is attractive, because they are inexpensive in production and can be implemented in microarray equipment. However, the surface of polymers is inert so, before it can be applied in a microarray first the surface has to be functionalized. Polymer surfaces can be modified by silylation, corona treatment, γ-radiation or graft copolymerization. Such treatments can enhance the polymer’s reactivity and/or modify the hydrophobic or hydrophilic character of the surface. [16](#_ENREF_20) The chemicals used to functionalize the surface are, however, hazardous and expensive. Performing the functionalization of polymeric surfaces furthermore needs special equipment, which laboratories are not standard equipped with.

One of the polymeric surfaces that is often used is polydimethylsiloxane (PDMS), which is optically transparent and can be used for various materials, such as peptides. PDMS is functionalized via plasma oxidation and the reaction with for example 3-mercaptotrimethoxysilane to generate a thiol-terminated surface (Figure 4). [17](#_ENREF_22) Polymethylmetacrylate (PMMA) can also be used in a similar fashion. [18](#_ENREF_23) PMMA slides can also bear an animated surface; this can be accomplished with a treatment of native PMMA with diamines such as 1,6-hexanediamine and ethylenediamine. [19](#_ENREF_24)

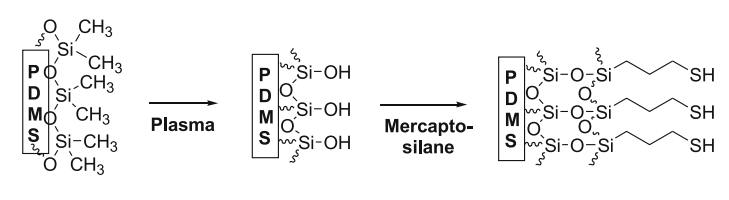


Figure 4 Representation of the PDMS surface-modification process to thiols. In the first step the PDMS surface is oxidized followed by the functionalization with a mercaptosilane. 17

1.5. Other types of support

As discussed in the introduction of this chapter other types of supports can be used for microarrays. This report will not go into detail on these supports, but two of these, gold and cellulose, are shortly discussed here.

Gold surfaces offer the advantage of being easily activated with SAMs or ω-functionalized thiols. The chemistry of the gold-thiol interface is much easier to control than organosilane chemistry. Usually, gold films are applied onto polished glass or silicon. The thermal deposition of gold onto silicon wafers is the most feasible method for the preparation of gold surfaces. However, a signiﬁcant additional cost is involved in the gold coating, which must be considered when thinking of using this method in routine applications. [9](#_ENREF_12), [14](#_ENREF_18)

Cellulose is available as filter paper, and in contrast to other screening surfaces is very inexpensive. Cellulose membranes are porous, hydrophilic, flexible and stable in the organic solvents and acids used for the peptide synthesis. These properties make cellulose membranes very useful for the biochemical and biological investigations of interactions in aqueous as well as organic media. Cellulose membranes show high thermostability up to temperatures of about 180°C, making it possible to use cellulose membranes for reactions at elevated temperatures. The loading on cellulose membranes is sufficiently lower compared to non-porous planar supports, which limits it in the use in high throughput systems. [20](#_ENREF_26)

# 2. Immobilization of peptides on a functionalized microarray surface

With peptide microarrays there are two approaches to immobilize a peptide on a planar surface: the *in situ* synthesis microarray approach and the *ex situ* synthesis microarray approach, also known as the spotting microarray approach. A schematic representation of both is shown in Figure 5. This thesis will focus on the spotting microarray approach.

There are numerous ideal features for the generation of the peptide microarrays. One of these is the use of low concentration of reagents for immobilization because the reaction of the peptide with the surface occurs rapidly and efficiently. A second one is that the synthesis of the peptides requires little to no post-synthetic modifications before immobilization, this to maximize the number of compounds that can be generated and minimize the cost of these reagents. Another desirable feature is that the reaction has controllable kinetics, which can be monitored with conventional spectroscopic methods that are also able to measure the density on the chip. [21](#_ENREF_29)

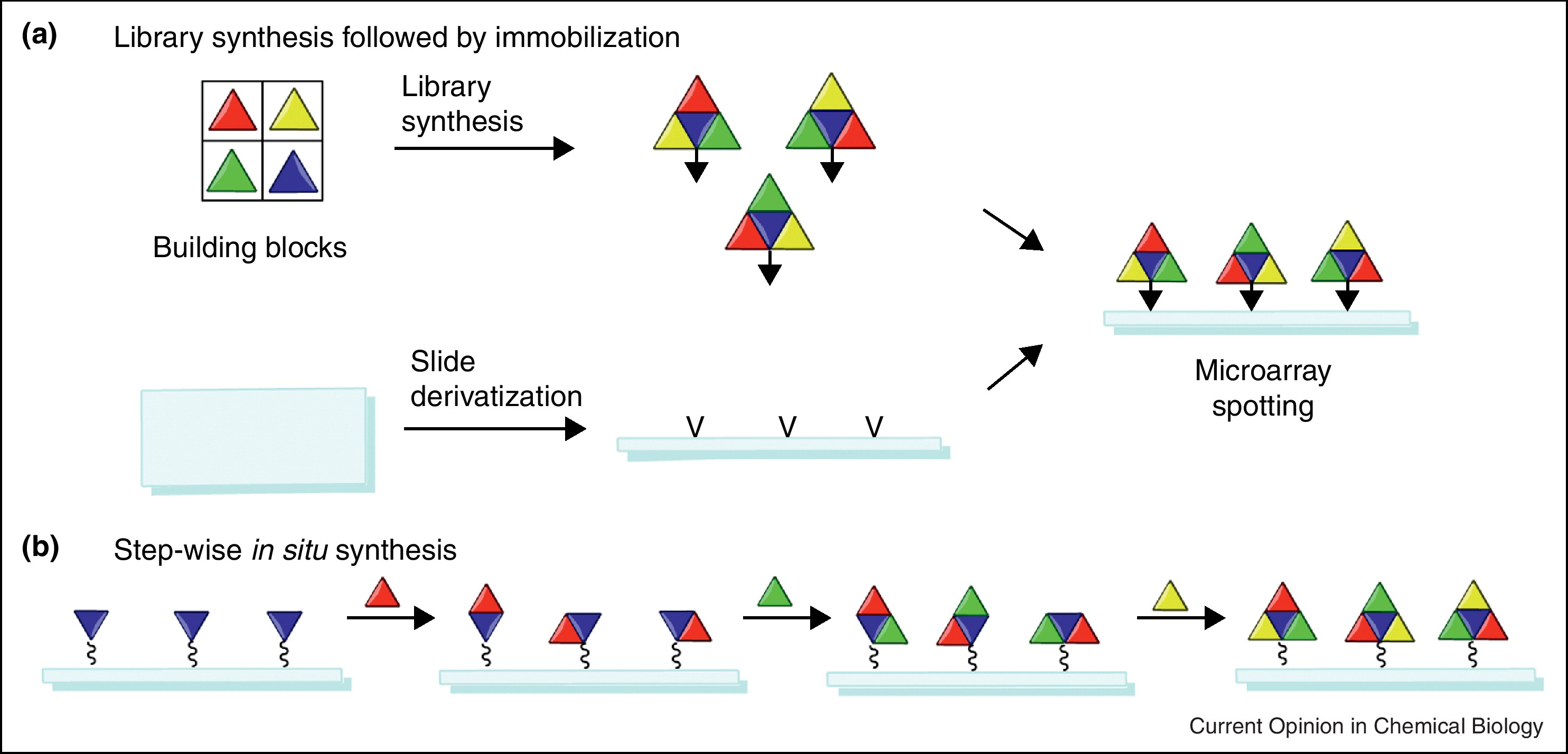


Figure 5 Strategies for the fabrication of peptide microarrays. (a) Library synthesis followed by immobilization. Libraries are synthesized using combinatorial chemistry, also incorporating a feature to facilitate immobilization on appropriately functionalized slides. (b) Step-wise *in situ* synthesis. Through iterative coupling on the solid support, libraries of compounds may be synthesized *in situ* on the slide surface. [22](#_ENREF_30)

2.1. In situ synthesis

The idea of the *in situ* synthesis is that a panel of peptide sequences on the microarray is grown layer by layer, until the desired peptide length is achieved (Figure 5b). Using this method, the entire array is built directly on the surface over which the screening will take place. Efficient coupling chemistries are required to ensure good purity of the synthesized peptides. The maximum peptide length is typically limited to no more than 40 residues. [23](#_ENREF_31) The two techniques generally used for *in situ* synthesis are the SPOT synthesis and the light directed synthesis, both of which are described below.

2.1.1. SPOT Synthesis

The basic principle of the SPOT synthesis technique is using a droplet to create a reaction vessel on a surface. A schematic overview is shown in Figure 6. [3a](#_ENREF_3) The boundaries of the droplet will function as the boundaries of the reaction vessel. A small volume of activated reagent solutions, *e*.*g*. Fmoc and Boc protected amino acid derivatives (activated by N-hydroxybenzotriazole (HOBt) esters or with commercially available pentaﬂuorophenyl esters), are spotted onto the surface. The reagents can then react as in conventional solid phase peptide synthesis. During the last step, the side chains can be deprotected. This gives the desired peptide on the surface, which can then be used for solid-phase screening of membrane-bound peptides or the peptide can be cleaved from the membrane surface for analysis and cell-based bioassays. [24](#_ENREF_32)

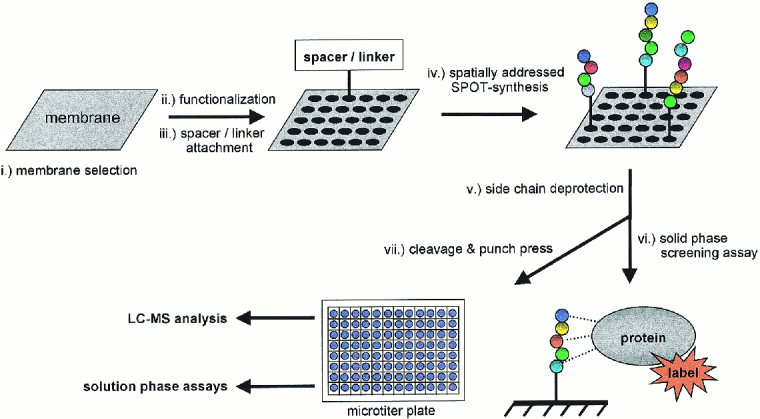


Figure 6 Schematic view of the SPOT-synthesis technique and subsequent screening and analytical processes. [24b](#_ENREF_33)

Surfaces commonly used for SPOT synthesis are cellulose, aminated polypropylene and aminopropylsilylated glass slides. Glycine ester-type cellulose membranes are suitable supports for the synthesis of soluble peptides, whereas β-alanine ester-type membranes are mostly used for binding studies performed directly on the support. [4](#_ENREF_6) Because of the size of the spots and the distance between the spots, SPOT synthesis technique results in a relatively low-density array. The typical density of the array is 25 spots per cm2, which limits its applications in high-throughput screening.

The size of the spot in an important aspect of the SPOT technique. Some parameters that influence the size of the resulting spots are the volumes dispensed, the physical properties of the membrane surface, the solvent system, the minimum distance between the spots and the number of peptides that can be synthesized per membrane area. SPOT synthesis on non-porous planar supports is rare, due to difficulties in generating addressable spots on such surfaces. [25](#_ENREF_34) Furthermore, the peptide quality in SPOT synthesis is lower, because the peptide on the chip cannot be purified, which can lead to “false positive” responses. Nevertheless, the SPOT synthesis technique is a convenient method and easy to adapt to a wide variety of assays and screening methods, especially when it is automated. [20b](#_ENREF_27), [25](#_ENREF_34) A pipetting robot is often used in SPOT synthesis, because generation of large peptide libraries is more convenient and less time consuming that way. It allows for the reliable and regular addressable distribution of the activated amino acid solutions on the membrane surface in a reasonable time scale. [25](#_ENREF_34)

Hilpert and coworkers applied the SPOT technology to analyze the modes of action and sequences requirements of host defense peptides. [26](#_ENREF_35) The SPOT technology revealed sequence requirements and an optimization strategy for short antimicrobial peptides. The cellulose membranes hydroxyl groups were functionalized to amino moieties, by treatment of an activated solution of glycine with 1,1’-carbonyl-diimidazole (CDI) and the base N-methylimidazole (NMI), as shown in Figure 7 .



Figure 7 SPOT synthesis. a) The cellulose membrane is amino-functionalized with a CDI activated N-protected glycine, which is followed by deprotection of the glycine with piperidine and b) the coupling of amino acids via standard Fmoc peptide synthesis.

The amino acids were then coupled via standard peptide synthesis and the side chains were deprotected in the last step. [20b](#_ENREF_27), [26-27](#_ENREF_35) The same research group applied the SPOT technology to synthesize two large random 9-amino-acid libraries based on the amino acid composition of the most active peptides against bacteria. The best peptides sequences were effective against a broad array of multidrug resistant bacteria. The peptide synthesis for these libraries was performed on cellulose. [28](#_ENREF_37) In both cases the researchers made use of the convenient pipetting robots.

Flitsch and coworkers used SAMs on gold supports together with SPOT immobilization. This was an approach to create a non-porous planar surface, which is especially useful for microarray technology. The research on the gold support was performed to evaluate substrate specificity of proteases and glycosyltransferases. For this a 64-well gold surface was used and self-assembled monolayers on gold surfaces were prepared by spotting a mixture of carboxylic acid-terminated and tri(ethylene glycol)-terminated alkanethiols. The terminal carboxylic acid was activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) and NHS. *N*-Fmoc diaminobutane was spotted on the gold slide and deprotected with piperidine to functionalize the slide with amino groups, this is shown in Figure 8. This amine functionality was suitable for coupling with Fmoc-protected amino acids using standard peptide synthesis conditions and final TFA-mediated side-chain deprotection. [29](#_ENREF_38)



Figure 8 SPOT synthesis on a gold support. a) After extensive cleaning, the gold slide is functionalized with carboxylic acid-terminated and tri(ethylene glycol)-terminated alkanethiols. This is followed by b) the activation of the terminal carboxyl acid with a solution of EDC and NHS c) *N*-Fmoc diaminobutane is then spotted and d) deprotected with piperidine to obtain the amino functionalized gold slide.

Carducci and coworkers demonstrated the feasibility of applying the SPOT synthesis technology (a graphical abstract is shown in Figure 6), combined with a prediction program, to the identiﬁcation of targets of peptide-binding domains in viral proteomes. This strategy facilitated the identiﬁcation of critical nodes in the human proteome that are targeted by viruses. The approach applied by Carducci and coworkers identified 114 new potential interactions between the human SH3 domains and proline-rich regions of two viral proteomes. [30](#_ENREF_39) Cellulose membrane-bound peptides were automatically synthesized according to standard SPOT synthesis protocols using an automated pipetting machine. The peptide arrays were synthesized on commercially available amino-PEG500-cellulose membranes. The peptides were synthesized using Nα-Fmoc amino acids that were preactivated with HOBt ester and *N*,*N’*-diisopropylcarbodiimide (DIC) and the side chain protecting groups were removed by treatment with TFA in the last step, a similar protocol is shown in Figure 7.

In literature many more examples can be found, where researchers apply peptide microarrays or where peptide microarrays are further developed. [31](#_ENREF_40)

The examples discussed show that the SPOT technology is still used nowadays, although miniaturization is still a challenging topic. This is mostly due the composition of membranes that are used. These membranes make it possible to produce good quality macro-arrays, but are, however, not suitable for microarrays. Research is still ongoing to improve the array on micro scale. The SPOT technique is often used for the synthesis of large peptide libraries, because of the easy synthesis method.

2.1.2. Light directed synthesis

Alongside SPOT synthesis, light directed synthesis is an *in situ* technique to create peptides on a solid surface. Light directed *in situ* synthesis of microarrays is based on the combination of solid phase peptide synthesis with a semiconductor fabrication system. [32](#_ENREF_42) A photolabile protecting group is used instead of standard Fmoc or Boc groups. These groups can be removed by irradiation with UV. Which part of the surface is irradiated can be controlled with so-called photomasks. The peptide synthesis can be precisely controlled using these photomasks in predefined configurations that allow for selective deprotection. Peptides are synthesized by repeated cycles of photodeprotection and coupling steps. The precisely controlled synthesis leads to selective coupling of different amino acids onto different peptides. A schematic representation of light directed synthesis is shown in Figure 9. [32b](#_ENREF_43)

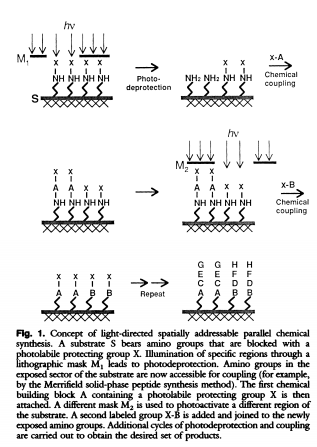


Figure 9 Concept of light directed in situ synthesis of microarrays. An amino-functionalized slide is blocked with a photolabile protecting group X. Illumination of specific reagents through a photomask (M1) leads to deprotection. Amino groups in the exposed location of the glass slide are now accessible for coupling with a next amino acid with a photolabile protecting group. [33](#_ENREF_44)

A new light directed method was developed where the expensive photomasks were replaced by a digital micromirror array (MMA). [34](#_ENREF_45) There was also a method developed with the generation of photogenerated reagents (PGR). [35](#_ENREF_46) This chemistry is referred to as PGR chemistry, which is based on light sensitive materials (precursors), which only upon irradiation are transformed into a desired reactive reagent. Photogenerated acids (PGA) are mostly used for peptide microarrays, because these can be applied as the acid needed in the deprotection of Boc protected amino acids and peptides. In is shown in Figure 10 how a PGA is generated. In Figure 11 a schematic representation of peptide synthesis with the use of PGR chemistry is shown. [35](#_ENREF_46)



Figure 10 PGA formation of an sulfonium salt upon light irradiation. SSbF6-: triarylsulfonium PGA precursor(PGA-P), R1: *S*-phenyl [35](#_ENREF_46)

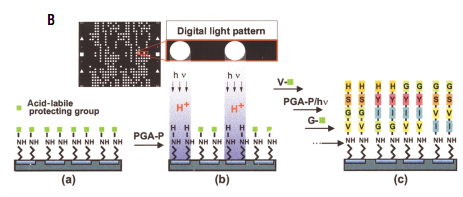


Figure 11 Schematic scheme of peptide synthesis with photolabile amino acids using PGA deprotection a) Linkers have terminal amino groups protected by acid-labile Boc groups (squares). b) The reaction sites are each filled with a PGA precursor (PGA-P). On the basis of the sequences to be synthesized, predetermined digital light patterns are generated; At irradiated reaction sites, PGA forms, and Boc groups are removed to give terminal amino groups. (c) The Boc-protected amino acid is coupled to the deprotected terminal amino groups. [36](#_ENREF_47)

It was demonstrated by Pellois and coworkers that peptide microchips were suitable for epitope-binding assays using PGR chemistry (Figure 11). [36](#_ENREF_47) The peptides were produced with solution phase peptide chemistry with removal of acid-labile protecting groups using PGR and digital light directed synthesis. These experiments have led to a better understanding of the antibody binding site at the molecular level. The microscopic glass slides used in this experiment were prepared with piranha solution and treatment with 3-aminopropyltriethoxysilane (APTS), which was coupled to Boc-aminohexanoic acid (Boc-Ahx). This was followed by coupling of Boc-βAlanine and again to Boc-βAla to obtain the spacer. The Boc deprotection step was performed with thioxanthenones as PGA precursor (PGA-P). With a digital micromirror, a predetermined light pattern was projected onto the surface of the microchip plate. The coupling of the other residues, the capping, and the side-chain deprotection reactions were carried out according to standard peptide-synthesis procedures. [36](#_ENREF_47)

Shin and coworkers demonstrated that the light directed synthesis method efficiently quantified the binding activities of protein-peptide interactions and it can be used for additional biological assay applications. [32b](#_ENREF_43) The light directed synthesis method using MMA was used on a photolabile *o*-nitroveratryloxycarbonyl (NVOC) protected glass slide. In this light directed synthesis the PGR is directly coupled to the amino acid to obtain a photo-activatable amino acid, in contrast to the example before, where the PGR is an additional reagent in the mixture. For the preparation of the peptide microarray, an automated peptide array synthesizer was built in a closed box, a computer program controlled the light and injection of the amino acids. For this microarray format, the photo-activatable amino acids were synthesized inhouse with NVOC. In Figure 12 the reaction cycle of the peptide synthesis is shown.



Figure 12 Reaction cycle of the light directed peptide synthesis on a glass slide with photolabile NVOC protected amino acids. [32b](#_ENREF_43)

The functionalization of the glass slides was performed with piranha solution and 3-glycidoxypropyltrimethoxysilane (GPTS) to obtain epoxide functionalities. The attachment of the hydrophilic polymer, chitosan (CHI), was carried out on the GPTS treated glass slide followed by the coupling of amino-PEG. It was shown that PEG-CHI-GPTS glass slides were more favorable for light directed peptide synthesis, because of the higher coupling yield. To generate the photolabile NVOC-protected surface, the surface was exposed to NVOC-PEG functionalized with succinic acid. In Figure 13 the functionalization of the glass surface with the immobilization of the NVOC spacer is shown. Standard coupling reagents were used for coupling the photo-labile peptides. [32b](#_ENREF_43)

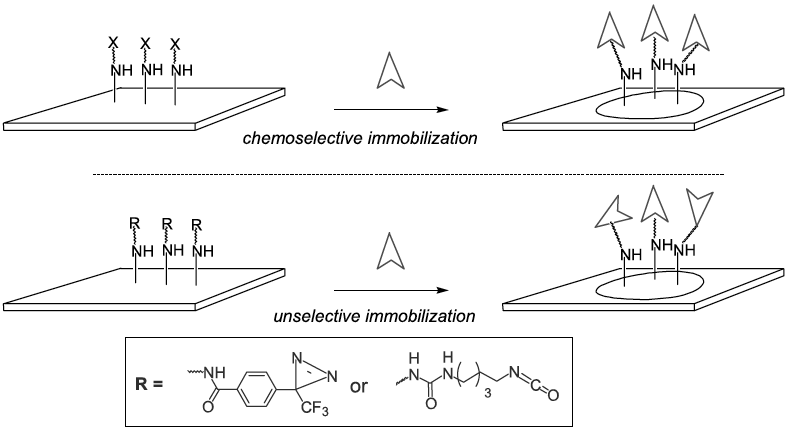


Figure 13 Coupling of NVOC spacers to the aminated glass surface. a) Aminolation of the glass slide is performed with GPTS and chitosan CHI followed by b) the coupling of the NVOC-PEG functionalized with succinic acid with couplings reagents HOBt and BOP.

In the last years, the light directed synthesis has not developed as much as expected compared to the SPOT approach and spotting approach. This is probably due to technical difficulties to construct arrays with twenty different masks for each monomer elongation or the difficult synthesis of twenty photo-activatable, and therefore light sensitive, protected amino acids. Furthermore, in this strategy all amino acids have to be equally spread over the slide for the light beam to reach them and the process has to be performed in a dark area. Although *in situ* light directed synthesis of peptides can create high density microarrays, the photochemical reaction is always an additional synthetic step. Therefore only short peptides (or peptide analogs, e.g. peptoids) may be sufficiently synthesized on an *in situ* synthesized array. Furthermore in the light directed synthesis production of photomasks or MMA is time consuming, and requires expensive equipment.

2.2. Spotting microarray approach

In the spotting microarray approach peptide libraries are presynthesized and thereafter applied (spotted) to defined addresses on the glass slides. This technique needs design considerations for both the slides and peptides to facilitate covalent immobilization. There are two types of immobilization strategies in the spotting approach: site-specific (one covalent bond) or non-specific (different orientation). In non-specific approaches, the exact orientation of the peptides is undefined, resulting in a mixed presentation of peptides on the microarrays from multiple potential interaction sites on either the slides or peptides (Figure 14). This approach is especially useful for new drug targets as it can predict new binding domains in peptides and proteins. Immobilization strategies have been developed for molecules that lack a specific common functionality to ligate it to the microarray. [37](#_ENREF_48) In contrast, site-specific immobilization often referred to as chemoselective immobilization, facilitates a uniform presentation of peptides across the array, with the interacting groups on the slides and peptides defined in advance (Figure 15). The intrinsic advantage of using the spotting microarray approach is the formation of a stable covalent bond, the controllable purity and the predictable specific orientation peptides on the microarray. The latter is advantageous, because the peptide can be designed to bind at a specific location so that its interactive part is not shielded. [4](#_ENREF_6), [23](#_ENREF_31)



**Figure 14 Varieties in immobilization. Chemoselective immobilization will yield a peptide with a defined orientation (top) whereas unselective immobilization will allow different orientations (bottom).** [**37**](#_ENREF_48)

2.2.1. Covalent immobilization methods

There are several chemical reactions that are commonly used for the spotting microarray approach (Figure 15). [37](#_ENREF_48) Here functional groups on the peptide such as amines, thiols, carboxylic acids, alcohols, and so on, react specifically in the immobilization process and bind covalently to the functionalized surface. The peptides are specifically designed and synthesized with a functionality that can ligate to an appropriately functionalized surface.

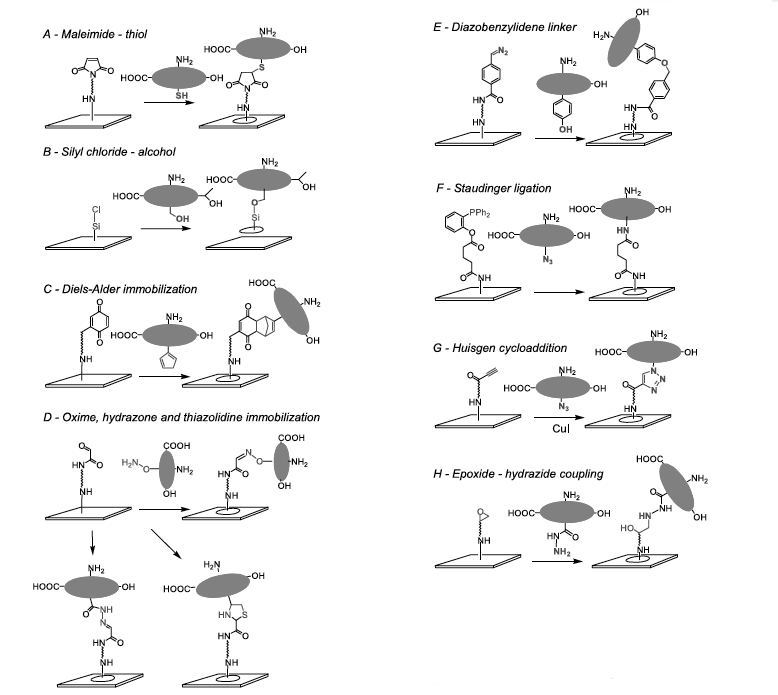


Figure 15 Common methods for ligation of a peptide with a functionalized surface via: A) maleimide linker, B) silyl linker, C) Diels-Alder ligation, D) glyoxylyl linker, E) diazobenzylidene linker, F) Staudinger ligation, G) Huisgen clycloaddtion, H) hydrazide linker. [37](#_ENREF_48)

Depending on the desired chemistry, characteristics of the molecules, experience, etcetera researchers prefer one or the other strategy to immobilize peptides on the microarray surface. In this paragraph the chemistry of the reactions is explained and the application as an immobilization technique for peptides is discussed.

2.2.1.1. Maleimide-Thiol

The thiol side chain of a cysteine containing peptide can be ligated to a maleimide via the Michael addition (Figure 16). Thiol-containing compounds readily attach to the surface upon printing, via the thioether linkage, a nucleophilic reaction between a thiol and a maleimide. *C*- or *N*-terminal cysteine containing peptides can be synthesized via standard solid phase peptide synthesis protocols.



Figure 16 Reaction of a maleimide functionalized glass slide with a thiol containing peptide. After silanization of the glass slide with APTS a) the heterobifunctional linker sulfo-SMCC is added in presence of D*i*PEA, which is followed by b) the reaction with the thiol bearing peptide.

The peptide is usually terminated with a glycine-serine-cysteine sequence to facilitate the binding with the slide. There are several protocols to obtain a maleimide functionalized slide. Usually, the slide is derivatized to maleimide via a heterobifunctionl linker as described by Schreiber and coworkers shown in Table 1, page 9. [38](#_ENREF_49) A drawback of this method is that a lack of site selective binding to the slide surface is observed in aqueous buffer, which is attributed to the hydrophilicity of the maleimide functionality. The ligation method, using the maleimide-thiol linkage, additionally is not site-specific when multiple thiols are present in the peptide. [38](#_ENREF_49)

Svarovsky and coworkers synthesized 10.000 random 20-mer peptides to screen their binding with O-antigenic glycans of bacterial lipopolysaccharides, which are involved in inflammatory response and septic shock. [39](#_ENREF_50) The discovered peptide substrates could be used as anti-endotoxic and antimicrobial lead compounds and as diagnostic and affinity reagents. In this research the peptides were synthesized with a *C*-terminal linker of glycine-serine-cysteine without other cysteines in the chain. The peptides were immobilized on maleimide functionalized glass slide. The glass slides were obtained by treatment with APTS followed by functionalization with sulfo-SMCC, as shown in Figure 16, to create the maleimide-activated surface. [39](#_ENREF_50)

The maleimide-thiol immobilization method has been exploited by Kodadek and coworkers in peptoid (oligo-*N*-substituted glycines) microarrays. [40](#_ENREF_51) They discovered a unique protein fingerprint generated for ubiquitin and glutathione S-transferase (GST) across the peptoid microarray. The array also revealed that the unique fingerprint of GST could be recognized when it is arrayed in the presence of a large excess of other bacterial proteins. The peptoids were synthesized with *C*-terminal cysteines, excluding other cysteines in the sequence, and immobilized on maleimide functionalized glass slides. [40](#_ENREF_51) More recently, Kodadek and coworkers were able to identify specific Immunoglobine G (IgG) biomarkers from serum that were characteristic only to Alzheimer diseased patients using a similar peptoid microarray format. [41](#_ENREF_52)

Johnson and coworkers made used of the specific reaction between a maleimide and a thiol to explore the feasibility of using a random peptide microarray to map antibody epitopes, useful for antibody therapeutic development and vaccine design. [42](#_ENREF_53) Here peptides were generated with a *C*-terminal glycine-serine-cysteine linker, excluding other cysteine residues, and spotted on maleimide functionalized glass slide. These slides were synthesized via APTS glass slides and functionalized to maleimide with the reaction of the amine and the sulfo-SMCC linker. [42](#_ENREF_53)

Simultaneous measurement of enzyme activity and binding to peptide substrates was investigated on a peptide microarray by Woodbury and coworkers. [43](#_ENREF_54) In this experiment, it was possible to identify peptides that bound to enzymes, *i.e.* horseradish peroxidase, alkaline phosphatase and β-galactosidase, and to measure the altered enzyme activity. This application of peptide microarrays shows how they can be used as an easy and quick method to find enzyme inhibitors or stabilizers. In this research, peptides were synthesized with a glycine-serine-cysteine linker on the *C*-terminus, without other cysteines in the sequence. The sulfhydryl was incorporated in the peptide for conjugating to maleimide functionalized glass slide. The glass slides were prepared via APTS surfaces functionalized with the sulfo-SMCC linker. [43](#_ENREF_54)

Time-resolved Surface Plasmon Resonance (SPR) measurements in an imaging format have been used to measure real-time interaction between peptides with proteins. The thiol-maleimide ligation method was applied for this measurement. For the SPR measurement, a class of maleimide-terminated self-assembled monolayers of alkanethiolates on gold was developed for the immobilization of the peptides ligands. Using SAMs on a gold support gives a good control over the density of maleimide over the film and therefore is well suited for defining the density of ligands on the substrate. Although this research was very promising for kinetic studies and control over the density, the required analytical equipment and materials are expensive. Functionalization of the glass slides is a time consuming and an additional surface feature, which makes it a less suitable support. [21](#_ENREF_29)

2.2.1.2. Silyl chloride-Alcohol

Alcohol functionalities in a peptide (serine and threonine residues) can be used for ligation via a silyl chloride surface. This ligation method does not have a linker between the surface and the peptide, which increases the steric interaction between the peptide and the surface, as shown in Figure 17.



Figure 17 Reaction of a silyl chloride functionalized glass slide with a primary alcohol containing peptide. After functionalization of the glass slide with silanol a) thionyl chloride is added to obtain the chlorosilane functionalization, which is followed b) by the reaction with the alcohol bearing peptide.

Despite the lack of the linker, [44](#_ENREF_55) successful binding assays have been performed, for example for analyzing glucose signaling pathways. [45](#_ENREF_56) Chlorosilanes are known to react readily with primary alcohols due to the oxophilicity of the silicon moiety. This is convenient, because natural occurring products often contain primary hydroxyls groups and research by Schreiber and coworkers showed the primary alcohols were strongly preferred over of secondary, phenolic, and methylether alcohols. [46](#_ENREF_57) The silylether bonds are also preferable to silylester bonds, mainly due to the base liability of the silylester. Additionally, short peptides with phenolic alcohol cannot be immobilized effectively on the glass surface. To address this problem, an immobilization strategy with diazobenzylidene functionalized surfaces has been developed (see paragraph 2.2.1.3). [47](#_ENREF_58) Glass slides for the silyl chloride-alcohol ligation are prepared by treatment of the slide with piranha solution and the reaction with SOCl2 in THF using catalytic amount of DMF, which transforms the silanols on the surface into chlorosilanes. [44-45](#_ENREF_55)

In the group of Schreiber the silyl chloride-alcohol immobilization technique has been applied and with this method they discovered a ligand: Ure2P, a central repressor of genes involved in yeast nitrogen metabolism. [45](#_ENREF_56) Using the same microarray format Schreiber and coworkers investigated a subunit of a yeast transcription factor complex. After screening the complex they were able to find an inhibitor for the complex. [46](#_ENREF_57) The ligation in this research was, however, between a silyl chloride and an alcohol containing small molecule and not an alcohol containing peptide. In literature, so far, this immobilization technique has not been reported outside the Schreiber group.

2.2.1.3. Diazobenzylidene linkers

Diazobenzylidene linkers were developed to broaden the immobilization strategies to functional groups in peptide compared to the silyl chloride-alcohol technique, as mentioned in the previous paragraph. Diazobenzylidenes are known to react with phenols, carboxylic acids, sulfonamides and other heteroatoms bearing acidic protons. Initial transfer of a proton from the heteroatom to the methylidyne carbon atom of the diazobenzylidene is followed by nucleophilic displacement of N2 by the heteroatom. The expulsion of N2 is a strong driving force of the reaction: an overview is shown in Figure 18. [48](#_ENREF_59)



Figure 18 The reaction of a diazobenzylide functionalized glass slide with a small molecule bearing an acidic proton. After functionalization of the glass slide with APTS a) toluenesulfonylhydrazone is coupled with PyBOP, which is followed by elimination of tosyl with sodium methoxide and c) the reaction with the phenol bearing small molecule.

Glass slides derivatized with a diazobenzylidene moiety are prepared by coupling the toluenesulfonylhydrazone to APTS slides through formation of an amide bond. Subsequent base-induced elimination yields the diazobenzylidene functionalized glass slides. The activated slides are simple and inexpensive to generate in large quantities and can be stored. [48](#_ENREF_59)

Schreiber and coworkers evaluated the above mentioned immobilization method with a series of FKPB12 and biotin derivatives, demonstrating that the diazobenzylidene functionality reacts only with heteroatoms having a proton with a pKa<11. [48](#_ENREF_59) The effectiveness of this strategy for ligand discovery was conﬁrmed with the discovery of new calmodulin ligands from a library of more than 6000 immobilized phenols. So far, this immobilization technique has not been applied outside the Schreiber group and, furthermore, has only been used with small molecules, not peptides. [48](#_ENREF_59)

2.2.1.4. Diels-Alder ligation

Cyclopentadiene-containing peptides can be ligated via the bio-orthogonal Diels-Alder [4+2] cycloaddition to dienophile functionalized surfaces. This ligation method can result in a rapid and specific immobilization of peptides on the glass slides and can proceed in water and at room temperature with a good rate and selectivity in organic solvents. [9](#_ENREF_12) In the Diels-Alder cycloaddition an electrocyclic reaction takes place with the 4 π-electrons of the cyclodiene and the 2 π-electrons of the dienophile, which is driven by the formation to new more stable σ-bonds. An overview of this rection on a microarray surface is shown in Figure 19. However, due to the unnatural cyclopentadiene moiety in the peptide, the synthesis of these kinds of peptides is challenging and not easily accessible. [49](#_ENREF_60)



Figure 19 The Diels-Alder reaction of a quinone gold slide, derivatized from a hydroquinone, with a cyclopentadiene ligand. After functionalization of the gold slide with hydroquinone (reactions not shown in the figure) a) the benzoquinone is yielded by oxidation with a saturated solution of benzoquinone. This is followed by b) the Diels-Alder reaction of the quinone with the cyclopentadiene ligand.

Quinone functionalized glass can be synthesized via hydroquinone functionalized alkanethiolates, with a PEG spacer, on gold which are activated via chemical or electrochemical oxidation to the benzoquinone. [50](#_ENREF_61) Besides the unnatural cyclopentadiene functionality necessary in the peptide, the functionalization of the glass slides is time consuming, expensive and analysis with expensive equipment is needed. For this reason, this method has not been applied often and only a few reports can be found in literature, which are described below.

The Diels-Alder ligation method described above was used by Mrksich and coworkers. [49](#_ENREF_60) This group developed a peptide chip for the rapid and quantitative evaluation of peptide phosphorylation. [49](#_ENREF_60) Due to the regular homogeneous environment for immobilized peptide ligands, it makes them well suitable for quantitative assays.

Kodadek and coworkers applied the Diels-Alder immobilization to develop a rapid identification method for protein ligands with large peptide libraries. In the research they actually wanted to make use of the maleimide-thiol immobilization method, however, this caused side reactions with a methionine in the peptide sequence. A furan containing peptoid residue was therefore incorporated in the peptide ligand, which could also undergo cycloaddition with the double bond of the maleimide on the glass slide. [51](#_ENREF_62)

2.2.1.5. Oxime, Hydrazone and Thiazolidine Ring Formation

Glyoxylyl functional groups on glass slides react selectively with oxyamine to form an oxime, with hydrazine to form a hydrazone and with 1,2-aminothiol to form a thiazolidine ring. Glyoxylyl functionalized slides were shown to react site-specific on the glass slide with hydroxylamine or hydrazine labeled peptides or a *N*-terminal cysteine residue of a biomolecule. [52](#_ENREF_63) The oxime and thiazolidine ring formation are, however, relative instable. The thiazolidine ring has, additionally, a restricted orientation, which hinders the free interaction of the peptides with target proteins. [53](#_ENREF_65) These difficulties could make the application of this ligation method unfavorable. Despite these difficulties, the use of this ligation method has been reported often. Glyoxylyl-functionalized glass slides are commonly obtained by derivatizing APTS glass slides via coupling of Fmoc-Serine-OH followed by Fmoc-deprotection with piperidine and oxidation with NaIO4. Another method is by coupling of the APTS glass slides with protected glyoxylic acid and final deprotection with HCl. [52b](#_ENREF_64) An overview of the method using Fmoc-Serine-OH for the functionalization is shown in Figure 20.



Figure 20 The reaction of a glyoxylyl functionalized glass slide with an oxyamine containing peptide. After APTS functionalization of the glass slide a) Fmoc-Ser-OH is coupled using coupling reagents DIC and HOBt, which is followed by the b) Fmoc-deprotection with piperidine and c) oxidation with NaIO4 to form the glyoxylyl functionalized glass slide. This is followed by d) the reaction with the oxyamine bearing peptide dissolved in NaCl and NaOAc at pH 5.2.

Novel methods for the oxime ligation are being developed, using catalysts, however these are not yet generally applied, since specific (expensive) equipment and additional materials are needed. [54](#_ENREF_66)

In the group of Lam and coworkers pre-synthesized peptides were regio-specific immobilized on glyoxylic acid functionalized slides via oxime bond formation or thiazolidine ring formation. [52b](#_ENREF_64) Using this ligation method Lam and coworkers could rapidly analyze the functional properties of numerous ligands and were the first persons using peptide microarrays to study peptide-cell interaction. Peptide ligands, derivatized at the *C*-terminus with 4,7,10-trioxa-1,13-tridecanediamine succinimic acid (Ttds) hydrophilic linker coupled to an amino-oxy or 1,2-aminothiol group, were synthesized and glyoxylyl functionalized slides were produced as describe above with APTS glass slides and deprotection of the glyoxylic acid. The linker was incorporated to increase the accessibility. [52b](#_ENREF_64)

Schneider-Mergener and coworkers could detect autophosphorylation sites, prime phosphorylation events and find potential activation loops of human kinases using peptide microarrays with aldehyde functionalized slides and amino-oxy-acetyl functionalized peptides. [55](#_ENREF_67) For the ligation of the peptides to the slide, an amino-oxy-acetyl moiety was coupled to the *N*-terminus of the peptides. The aldehyde modified glass slide that was used was commercially available. The hydrophilic linker 1-amino-4,7,10-trioxa-13-tridecanamine succinic acid was inserted between the amino-oxy-acetyl and the peptide sequence to make the peptides more accessible for the enzymes. [56](#_ENREF_68) In the same group casein kinase II substrate specificity was analyzed and potential *in vivo* substrates were detected using the same peptide microarray format. [55](#_ENREF_67)

Ellmann and coworkers synthesized fluorgenic 7-amino-4-carbamoylmethyl coumarin (ACC) peptides, which are effective substrates for determining the *N*-terminal substrate specificity of serine and cysteine proteases. The peptide microarray showed selective cleavage of preferred substrates with trypsin, thormbin and granzyme B. It also showed the ACC substrate specificity of thrombin. In this research ACC peptides were first printed on commercially available aldehyde-functionalized slides, but were inefficiently cleaved by trypsin. Therefore BSA-functionalized glass slides were covered with 4-carboxybenzaldehyde, using standard carbodiimide coupling conditions, to increase the accessibility. [57](#_ENREF_69) Oxime bond formation occurred between the aldehyde functionalized slide and the inhouse synthesized alkoxylamine fluorogenic peptide substrate. [58](#_ENREF_70) A disadvantage of this type of ligation is that he ACC bond to a peptide is usually an anilide. This moiety can negatively influence the natural substrate activity with proteases because it is not a native peptide. Furthermore, ACC peptides can only be used to determine the *N*-terminal substrate specificity because the coumarin is active upon cleavage at the *C*-terminus and special resin beads are necessary for the synthesis of the fluorgenic peptides. [57](#_ENREF_69)

Melnyk and coworkers made use of the same immobilization strategy only here the peptides were tagged with glyoxylyl and the glass slide were functionalized with semicarbazides forming α-oxo semicarbazone site-specific ligation (Figure 21). In this research it was discovered that the microarray strategy gives high sensitivity and specificity for antibody detection in complex biological samples such as human sera. [59](#_ENREF_71) Glyoxylyl peptides were easily generated by periodate oxidation of an *N*-terminal seryl precursor in solution. This was performed via an automated peptide synthesizer with an isopropylidene tartrate (IPT) based linker to effectively cleave to glyoxylyl peptide from the resin. Glass slides covered with a semicarbazide layer were prepared by silanization of the glass with APTS and subsequently, coupling NHNH2 to install the semicarbazide functions. [59-60](#_ENREF_71) The stability of semicarbazide glass slides was tested and it was found to be less sensitive to air pollution than amine surfaces and chemically stable. [61](#_ENREF_74)



Figure 21 Immobilization via α-oxo semicarbazone site-specific ligation. After APTS functionalization of the glass slide a) the isocyanate is obtain via the trisphogene, which is followed by b) coupling of Fmoc-NHNH2 and c) Fmoc-deprotection with piperidine and DBU. This is followed by d) the reaction with the glyoxylyl bearing peptide in a saline sodium citrate solution. [61](#_ENREF_74)

Recently, Meijer and coworkers made use of the oxime ligation with aldehyde or ketone functionalized peptides on an amino-oxy-functionalized glass slide, using aniline as a catalyst for the ligation. *N*-terminal aldehydes or ketones were introduced into peptides either by oxidation of *N*-terminal amino acids with pyridoxal 5′-phosphate or by oxidation of *N*-terminal serine or threonine residues. [54](#_ENREF_66) The amino-oxy functionalized slides were prepared by modifying commercially available carboxylate-functionalized SPR slides with alkoxy-amine derivatives. The peptides were synthesized with an *N*-terminal serine/threonine and oxidized with periodateto an *N*-terminal glyoxyl group with a complete conversion to the aldehyde. [54](#_ENREF_66) Although this method was successful and promising, the additional element in the reaction, here the aniline catalyst, is not desired.

2.2.1.6. “Click” Chemistry

A widely known type of cycloaddition is the Cu(I) catalyzed azide-alkyne cycloaddtion (CuAAC) also known as the “click” reaction. This reaction generally makes use of the coupling between an azide-functionalized peptide and a terminal alkyne to from a triazole ring. [62](#_ENREF_75) The copper in this reaction coordinates with the alkyne moiety. The “click” reaction is known to be orthogonal to most other functional groups and has been applied as immobilization strategy in microarrays, especially in protein microarrays. [63](#_ENREF_76) It was reported that the immobilization of alkyne-modified proteins onto azide-coated surfaces proceeds more efficiently. [64](#_ENREF_78) This effect could be because the alkyne function coordinates Cu(I) in solution more efficiently than on the surface, which could improve the immobilization reaction.[64a](#_ENREF_78), [65](#_ENREF_80) Various Cu(I) sources can be used directly, but the catalyst is better if it is prepared *in situ* by reduction of Cu(II) salts, such as CuSO4.5H2O. Among the different reducing agents that can be used, TCEP.HCl is one of the most competent reagents for the in situ reduction of Cu(II) and has been shown to react only very slowly with aliphatic azides. [66](#_ENREF_81) In recent years, a number of copper-free variants of the original “click” reaction have been developed to avoid the cytotoxicity of the Cu(I) catalyst. These reactions use various functionalized cyclooctynes where the reaction is promoted by strain relief within the eight-membered ring. [67](#_ENREF_83) Azide functionalized slides are generally prepared through commercial available amine functional glass slides, which are derivatized to azido-coated surfaces via reaction with disuccinimidyl glutaric dicarboxylate and D*i*PEA. The NHS-activated slides are then immersed in 3-azidopropylamine and D*i*PEA. The click reaction can proceeded with alkyne modified peptides in PBS (pH 8.0), TCEP, tri(triazolyl)amine or sodium ascorbate as reducing agents and CuSO4 (Figure 22).[64a](#_ENREF_78)



Figure 22 The reaction of an azide functionalized glass slide with an alkyne containing peptide. After APTS functionalization of the glass slide a) Disuccinimidyl glutaric dicarboxylate and D*i*PEA are added to create the NHS-activated slides, which is followed by b) the addition of 3-azidopropylamine and D*i*PEA and finally c) the click reaction with the alkyne bearing peptide in presence of the Cu catalyst and TCEP.

Recently, Liao and coworkers developed a peptide microarray by conjugation with alkyne-containing peptide using “click” chemistry, which could be used for profiling protease activity. This reaction proceeded at low temperature and in aqueous solution. [68](#_ENREF_85) In this research the hetero-bifunctional tetra(ethyleneglycol) linker was synthesized to prepare the azide derived glass surface in one step. A highly efficient microarray could be prepared with the bifunctional linker. The azidosilane linker could be coupled to glass slides using standard silanization protocols. Peptides with a *N*-terminal alkyne and on the *C*-terminus a fluorophore functionality were commercially produced. The click reaction could proceed in a solution of the peptides in PBS (pH 8), CuSO4, and sodium ascorbate. [68](#_ENREF_85) Using the same ligation strategy, Liao and coworkers could monitor the eﬃciency of peptide conjugation. Fluorescent 7-nitrobenz-2-oxa-1,3-diazole (NBD) amino acids were developed and could be incorporated into peptides at any position, making direct measurement of the peptide activity possible. Both non-speciﬁc and site-speciﬁc immobilizations of peptides were performed on the slide and it was shown that the active site of the alkyne-peptides conjugated to the surface was preserved. [69](#_ENREF_86)

Besides proteins and peptides, the CuAAC ligation method has also been applied in peptoid microarrays. A peptoid library was designed and screened for binding to the *C. Albicans* group I ribozome. Numerous peptoids were found that bound the RNA of *C. Albicans* and could be tested for their abilities to inhibit self-splicing. With the preformed peptoid microarray, Disney and coworkers were able to develop a molecule with a six fold more potent inhibitor than Pentamidine, a drug used clinically to treat *Pneumocystis carinii*infections. This developed molecule has also been shown to inhibit self-splicing of the *C. albicans*intron *in vitro*and *in vivo*. In this research they made used of amine-functionalized slides coated with agarose, which was oxidized and functionalized to alkyne by submerging with propagylamine. The click reaction proceeded in presence of Tris-Cl, CuSO4, the reducing agent ascorbic acid and stabilizing agent tris(benzyltriazolylmethyl)amine (TBTA). [28](#_ENREF_37)

Another cycloaddtion type is the “click” sulfonamide reaction (CSR). Waldmann and coworkers developed alkyne-modiﬁed biomolecules that could be immobilized site-selectively on sulfonylazide slides under very mild conditions. [70](#_ENREF_87) The CSR-based immobilization was applied with the Ras binding domain (RBD), which was modified with an alkyne on its *C*-terminal, on sulfonazide slides. Screens with Ras, showed that the alkyne-RBD could differentiate between active and inactive Ras, meaning that the RBD kept is activity after immobilization. Commercial available COOH-terminated PAMAM dendrimer glass slides were functionalized in D*i*PEA and amine deprotected taurylsulfonyl azide. The “click” reaction proceeded rapidly in presence of Cu(CH3CN)4PF6, TBTA, sodium ascorbate and NaHCO3. Immobilizations with peptides however have not been reported using the click sulfonamide reaction.

2.2.1.7. Staudinger ligation

The Staudinger ligation is the reaction between an azide and a substituted phosphine which forms a stable amide bond, as shown in Figure 23. The reaction proceeds through the nucleophilic attack of a phosphine on an azide to give an azaylide intermediate, which is subsequently trapped in an intramolecular fashion by a methoxycarbonyl group on one of the aryl rings of the phosphine to give an amide-linked phosphine oxide after hydrolysis. [71](#_ENREF_88) Raines and coworkers designed a traceless Staudinger ligation, to obtain a native amide bond as the final product without having the phosphine oxide still attached (Figure 23 II), which can be achieved via phosphothioester and phosphoesters. [72](#_ENREF_89) Compared to the Huisgen cycloaddition this method does not require catalysts such as Cu(II) or the use of ligand such as triazolyl, which can result in the denaturation of proteins during the reaction.

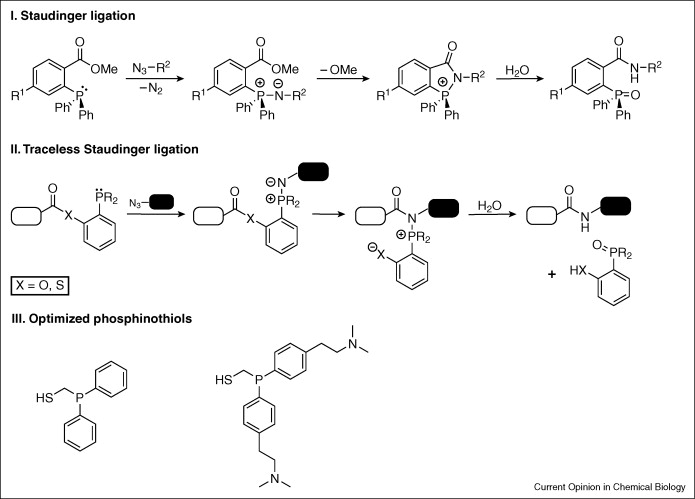
**

Figure 23 Non-traceless (I) and traceless (II) Staudinger ligation. (I) Via the attack of the phosphine on the azide, an azaylide intermediate is formed. This is followed by intermolecular reaction on the aryl phosphine and hydrolysis gives the resulting in the amide-linked phosphine (II) Trans(thio)esterfication of the (thio)ester results in the is activated ester, which can react with the azide to iminophospharane. This is followed by an intermolecular rearrangement to the amidophosphonium and after hydrolysis to the amide and phosphine oxide. [71](#_ENREF_88)

The microarray surface for the Staudinger ligation is generally prepared via an amine-functionalized glass slide with PAMAM dendrimer, which is derivatized with glutaric anhydride to introduce terminal –COOH groups. Subsequently, these are esterified with *o*-(diphenylphosphine)phenol, resulting in azide reactive phosphine groups functionalized slides. [72a](#_ENREF_89), [73](#_ENREF_91) Liskamp and coworkers found that *o*-(diphenylphosphine)phenol ester could react with an azide to amide, but also can undergo side reactions with the amine side chain in lysine. [74](#_ENREF_92) Therefore Bertozzi and coworkers have developed a particular phosphine, which is shown in Figure 24. [72b](#_ENREF_90), [75](#_ENREF_93) This phosphine possesses three aryl substituents to limit air oxidation. One of the aryl rings is derivatized with a methyl ester *ortho* to the phosphorus atom, providing an electrophilic trap for the nucleophilic nitrogen in the azaylide formed on reaction with azides. [75](#_ENREF_93)



Figure 24 Phospine reagent for non-traceless Staudinger ligation. [75](#_ENREF_93)

Raines and coworkers found out that immobilization via the Staudinger ligation is remarkably rapid. In 2003, they were able to immobilize the first 15 azide functionalized peptides from the ribonuclease S′ protein in less than one minute in 67% yield. [76](#_ENREF_94) APTS glass slides were derivatized with PEG disuccinimidyl propionate. To generate a surface-bound phosphinothioester, phosphinomethanethiol was added to the NHS glass slides.

Waldman and coworkers have developed an efficient method for preparing phosphopeptide microarrays by employing Staudinger ligation for immobilization. They demonstrated its application for mapping the *in vitro* substrate specificity of protein tyrosine phosphatases (PTPs). In this research a phosphotyrosine peptide library was synthesized and immobilized on a phosphine-modiﬁed glass slide following the Staudinger ligation strategy. The phosphine used, led to the ‘non-traceless’ ligation products. To amine-terminated PAMAM dendrimer coated glass slides, *N*-Fmoc-protected Ahx was coupled as the linker. This was followed by Fmoc deprotection and coupling to the phosphine reagent. To minimize interactions of the PTPs and the peptides with the surface, both the azide functionalized peptides and the phosphine-functionalized surfaces carry an Ahx linker. This strategy conﬁrmed the preferred phosphotyrosine-binding motif of the receptor-like phosphatase. In addition, with the information of the binding motif a potent inhibitor was developed. [77](#_ENREF_95) A schematic scheme of this reaction is shown in Figure 25.



Figure 25 The reaction of a phosphine functionalized glass slide with an azide containing peptide. After PAMAM functionalization of the glass slide a) the protected Ahx linker coupled with reagents DIC and HOBt, which is followed by b) Fmoc deprotection with piperidine and c) coupling to the phosphine reagent also with DIC and HOBt. This is followed d) by the traceless Staudinger reaction with an azide bearing peptide in a dioxane/H2­O solution.

Waldmann and coworkers also immobilized proteins with the Staudinger ligation. The immobilization of the Ras protein was achieved via the azide-modified *C*-terminus, which was generated by expressed protein ligation, and phosphine functionalized glass slides. This research showed that proteins could also be site-selectively immobilized tot the surface. [78](#_ENREF_97) For this experiment, although, an additional step was needed to synthesize the modified protein.

2.2.1.8. Epoxide

Epoxide linkers represents a very versatile moiety for immobilization, because it can react with nearly all possible side chain functionalities of peptides and reacts faster with hydrazines than other nucleophilic functionalities such as, hydroxyl, amine, carboxylic and thiol. These functionalities generally perform a nucleophilic attack to the least substituted carbon of the epoxide, which results in an alcohol and the linked peptide. Epoxide derivatized glass slide can be prepared from amine-coated slides in poly(ethyleneglycol) diglicidyl ether. [79](#_ENREF_98)



Figure 26 The reaction of an epoxide functionalized glass slide with an thiol containing peptide. After PAMAM functionalization of the glass slide a) poly(ethyleneglycol) diglicidyl ether is added, which is followed by b) the reaction with the thiol bearing peptide.

Due to the reactivity of epoxides with the peptide functionalities this ligation is often non-specific, which meas that different orientation can be observed when compounds bearing multiple functionalities are bound to the surface. This can cause that not all immobilized peptides are oriented suitably for subsequent screenings.

Shin and coworkers developed a strategy to immobilize hydrazine-functionalized peptides on epoxide derivatized glass slides. [79b](#_ENREF_99) In their research it was shown that the utility of this ligation method could be efficiently applied in the screening for selective peptide-protein binding. [79b](#_ENREF_99) The slides were prepared as described as above and the hydrazine peptides could be made via solid phase synthesis with hydrazine resin. [79b](#_ENREF_99)

In their research to identify HIV-1 and *M. tuberculosis* peptides for vaccine design, Maeurer and coworkers used peptide microarrays with epoxide functionalized glass slides. Peptides with a partial overlap of the HIV-1 sequence were synthesized by the SPOT technology and attached to the slide via the amino-oxy-acetylated *N*-terminal peptides. [80](#_ENREF_100) Several binding peptides were discovered which could be used is the vaccine design.

Epoxide derivatized glass slide were also applied in the epitope mapping of the immune response to a peanut allergen. [81](#_ENREF_103) In this research, peptides covering the sequence of the peanut allergen were commercially synthesized, immobilized on the epoxide functionalized glass slides and screened with sera of allergic an non-allergic patients. Several specific antibody-peptide interactions were discovered using this microarray. These peptides could be used to make easy applicable allergy tests. [81](#_ENREF_103)

2.2.1.9. Native chemical ligation

In native chemical ligation, the thiolate group of an *N*-terminal cysteine residue of an unprotected peptide attacks the *C*-terminal thioester of a second unprotected peptide in an aqueous buffer This transthioesterification step is chemoselective and regio-selective and forms a thioester intermediate. The intermediate rearranges by an intramolecular *S,N-*[*a*](http://en.wikipedia.org/w/index.php?title=Acyl_shift&action=edit&redlink=1)*cyl shift* that results in the formation of a native peptide bond at the ligation site. [82](#_ENREF_104) Forming a native peptide bond makes this ligation method a favorable strategy, because this bond is very stable and can be site-specifically applied at the immobilization of peptides. The most important advantage of this immobilization strategy is that *N*-terminal cysteine containing peptides can be synthesized very easily via standard peptide synthesis protocols and the peptides do not obtain an unnatural moiety. Additionally, the functionalization of glass slides is simple to prepare and furthermore this reaction proceeds under physiological conditions and no external reagents are needed, an example of the reaction is shown in Figure 27. [83](#_ENREF_105) Thioester functionalized slides are prepared by treatment of an epoxide functionalized glass slide with diamine-PEG linkers, followed by acidification with succinic acid. The thioesters functionalities are formed by reaction with benzylmercaptan. [53](#_ENREF_65) Anderson and coworkers recently developed a more reactive glass surface functionalized with thiophenyl esters. The slides, however, had to be synthesized in a glove box, because of the moisture sensitivity of the thioester silyl chlorides. [84](#_ENREF_106) Native chemical ligation is usually performed in sodium phosphate buffer (pH 7.5) containing TCEP and 4-mercaptophenylacetic acid (MPAA). The MPAA acts as catalyst and reverses nonproductive thioester development. In combination with TCEP, MPAA preserves the *N*-terminal cysteine in the reduced form. [84](#_ENREF_106)



Figure 27 Immobilization via native chemical ligation. After APTS functionalization of the glass slide, a) a buffered solution succinic anhydride is added b) and the glass slide is activated with a solution of TBTU, D*i*PEA and NHS. This is followed by c) the addition of the benzylmercaptan and D*i*PEA c) Native chemical ligation takes place with the reactive sulphur atom on the side-chain of a *N*-terminal cysteine peptide. It attacks a *C*-terminal thioester peptide, producing a new thioester intermediate that spontaneously rearranges to yield a peptide bond. [53](#_ENREF_65)

Yao and coworkers showed that native chemical ligation could be applied as an immobilization strategy for peptide microarrays. In this experiment *N*-terminal-cysteine-containing kinase peptide substrates were immobilized onto a thioester-functionalized slide, these were synthesized as described above. The peptide activity was probed with the corresponding kinases, followed by successful detection of the phosphate group with fluorescein-labeled antibodies of phosphotyrosine and phosphoserine. [53](#_ENREF_65)

Using the same immobilization strategy, Yao and coworkers also identified critical amino acid residues required in mediating the activity of a tyrosine kinase. The peptides could be easily synthesized by SPPS with an *N*-terminal cysteine and two glycines severing as linker. In this research they discovered that using a PEG layer was beneficial, as it can act as a hydrophilic ‘”cushion” preventing non-specific binding in binding assays. [10b](#_ENREF_14)

2.2.1.10. N-hydroxysuccinimide

By derivatization of a glass slide with an *N*-hydroxysuccimide ester (NHS), all nucleophilic groups of a peptide (-NH2, -SH, -OH, etc.) can react to give a covalent bond. This can lead to a non-specific immobilization when multiple available nucleophilic groups are present in the peptide. Furthermore, non-specific immobilization can cause a critical issue if a highly dense array and a specific orientation is needed. However, as mentioned before, this unspecific orientation strategy is very useful to predict and research new binding domains. NHS functionalized slides can be made by condensing APTS with reactive hydroxyl groups on the glass slide, which generates the aminosilylated slide. Subsequently, the surface amino groups are treated with homobifunctional linker disuccinimidyl glutarate to generate the NHS moiety. [7a](#_ENREF_9) The reaction is shown in Figure 28.



Figure 28 The reaction of an NHS functionalized glass slide with an peptide containing a nucleophilic group. After amine functionalization of the glass slide by APTS a) the linker disuccinimidyl glutarate is added , which is followed by b) the reaction of NHS and the peptide bearing a nucleophilic group.

Recently, a new, one-step method for the modification to NHS glass surfaces has been developed. In this method NHS ester functionalized dimethallylsilanes (Figure 29) react with the silanols presented on glass surfaces. The acid TfOH was found as superior catalyst in this one step reaction. [85](#_ENREF_107)



Figure 29 One of the dimethallysilanes where the silanos on the glass slide is derivatized with. [85](#_ENREF_107)

Schreiber and coworkers preformed the first functional kinase assay using the immobilization strategy of peptides on NHS functionalized glass slides. [57](#_ENREF_69) In their work, three different kinase substrates were immobilized on the NHS functionalized slides and incubated with kinase. With this array the phosphorylation of the immobilized peptides could be visualized. The slides were prepared from amine functionalized glass slides, which were first covered with BSA. The BSA was activated with *N*,*N*′-disuccinimidyl carbonate (see Table 1, page 9) in the presence of the base D*i*PEA to obtain the NHS functionality on lysine, aspartate and glutamate residues. [57](#_ENREF_69)

In the laboratory of Kunz and coworkers, a glycopeptide microarray was developed to profile serum antibodies from mice that were vaccinated with synthetic MUC1, a glycoprotein antigen found on tumour cells. [86](#_ENREF_108) The microarray identified new binding epitopes and revealed differences in the selectivity patterns for antibodies raised against different, closely related, glycopeptide antigens. In this research peptides were synthesized with a tri(ethyleneglycol) on the *N*-terminus and were immobilized on a NHS functionalized surface. [86](#_ENREF_108) This immobilization strategy is, nowadays, often used in cancer research to find biomarkers. [87](#_ENREF_109)

2.2.1.11. Photocrosslinking Immobilization

In photocrosslinking immobilization the highly reactive carbene on the glass surface reacts with peptides via photogenerated radical cross-reactions. The photoreactive surface is prepared from diazirines [88](#_ENREF_112) or aryl azide [89](#_ENREF_113), which after irradiation with UV generate a carbene and undergo non-specific insertion reactions with biomolecules. Photocrosslinking, like immobilization via NHS and isocyanate (discussed in the next paragraph), is seen as advantageous because it means that small molecules will be presented into different orientations, which reduces the chance that the attachment point to the surface interferes with binding to its target. [90](#_ENREF_114)



Figure 30 Photocrosslinking immobilization. a) After irradiation of light the diazirines forms into the reactive carbene and b) can insert into the peptide. [91](#_ENREF_116)

The reactive carbene from a diazirine can be synthesized by UV irradiation of amine-coated gold slides derivatized with a linker ending in trifluoromethyldiazirinbenzoyl, a photoaffinity reagent. This then releases nitrogen creating the carbene (Figure 30). [92](#_ENREF_117) To date, however, this immobilization strategy has not been reported often for the immobilization of peptides. This is probably because the other described non-specific methods are easier to perform and do not have to handle with such a highly reactive functionalities on the glass slide.

2.2.1.12. Isocyanates

Isocyanates are very reactive to nucleophiles and have shown to be reactive towards alcohols (primary and secondary), phenols, thiols, carboxylic acids, amines (primary and secondary) and anilines (Figure 31). [93](#_ENREF_119) Due to its broad range of reactivity the peptides can attach to an isocyanate functionalized surface with a range of orientations with various functional groups available to interact with probes. Moreover, isocyanate is so reactive that it is very unstable and can react readily with water. This makes it difficult to synthesize and store.



Figure 31 Isocyanate-coated surfaces react with a variety of nucleophilic functional groups and can be used to covalently immobilize peptides and other small molecules in a non-specific fashion. [94](#_ENREF_120)

For this instability issue a protected isocyanate functionalized glass surface has been developed. [95](#_ENREF_121) Although they are protected isocyanate-functionalized slides, they still have to be stored in inert atmosphere, because of their high reactivity. This makes the use of this particular immobilization method time consuming and expensive. The isocyanate-functionalized slides are furthermore not commercially available and have to be synthesized in-house. An isocyanate-functionalized surface is obtained via coupling of PEG spacers to amino silianized glass slides followed with activation with diisocyanatohexane. A catalytic amount of pyridine vapor is needed to immobilize the peptide. [37](#_ENREF_48), [96](#_ENREF_123)

Hecht and coworkers investigated the aggregation pathway of amyloid-β (Aβ peptide), which is involved in the beginning of Alzheimer’s disease. Here the peptide microarray was used to screen various peptides and other molecules that would interact with the Aβ peptide. The interacting peptides provided leads that could be used in drug design to cure or prevent Alzheimer disease. The isocyanate moiety in this research was separated from the surface by a special designed PEG spacer to ensure that the small molecules were presented at a distance from the surface sufficient to enable interactions with the Aβ peptide. [97](#_ENREF_124)

2.2.2. Non-covalent immobilization

Peptide immobilization involves predominantly covalent strategies, but also various non-covalent options to produce peptide microarrays have been employed. Non-covalent immobilizations involve either passive adsorption onto hydrophobic surfaces or electrostatic interaction with charged surfaces. The covalent mode is preferred especially when stringent or harsh washing conditions are necessary to minimize non-specific binding effects or to optimize signal/background ratios. [4](#_ENREF_6), [23](#_ENREF_31) Two non-covalent methods are described below.

2.2.2.1. Biotin-Avidin bonding

The protein-ligand bonding between Avidin and Biotin has been one of the most exploited non-covalent immobilization strategy for peptides onto glass surfaces. Avidin, a tetrameric protein, can interact with Biotin with a dissociation constant of 10-15 M, which is one of the strongest known protein-ligand interactions. This interaction is therefore frequently used in a wide variety of biotechnological applications. While this interaction is almost as strong as a covalent bond, the protein in this case can denaturize and disrupt the interaction with biotin. Avidin, however, is a very stable protein and is applicable for glass slide derivatization. It has shown to act as a molecular layer, which minimalizes non-specific binding of peptides and proteins to the surface. [53](#_ENREF_65) Biotinylated peptides can be easily synthesized via standard peptide protocols, where Biotin is coupled at one of the termini. The interaction between the biotinylated peptide and Avidin on the glass surface takes place almost instantaneously and efficiently, reducing the long immobilization time. Avidin functionalized glass surfaces are generally prepared by treatment of the glass surfaces with a piranha solution followed by derivatization to epoxy via reaction with glyicidoxypropyltrimethoxysilane. Reaction with a basic avidin solution results in the desired glass functionality. [53](#_ENREF_65) In Figure 32 a scheme of the Biotin-Avidin interaction is shown.



Figure 32 Schematic scheme of the interaction of an Avidin functionalized glass slide with a Biotin containing peptide. a) After epoxide functionalization of the glass slide with 3-glycidoxypropyltrimethoxisilane b) Avidin in a solution of NaHCO3 is added, which is followed by c) the interaction with the Biotin bearing peptide.

Felger and coworkers used the immobilization strategy of Avidin-Biotin for the detection of parasitic infection in human sera with peptides on a microarray. In this research, peptides were synthesized via Fmoc solid phase peptide synthesis with an aminohexanoic linker and a Biotin at the *N*-terminus. Suitable streptavidin slides were commercially available. A combination of peptides that were reactive with the infected sera could be used for the development of new drug targets. [98](#_ENREF_125)

Phosphoserine/phosphothreonine binding domains constitute a major class of signaling domains with prominent roles in regulation of mitosis, DNA damage, and apoptosis. One of the involved proteins is the so-called 14-3-3 protein. Inhibitors for these proteins are not available. In the study of Yao and coworkers a peptide microarray has been developed to screen binding to the 14-3-3 proteins. Peptides were synthesized via standard Fmoc-peptide chemistry with a double glycine linker between the *N*-terminal or *C*-terminal Biotin linker. The Avidin glass slides were prepared by treatment of glass slide with pirhana solution followed by a reaction with APTS, succinic anhydride and N-hydroxysuccinimide to obtain the NHS derivatized slides. This was then functionalized with Avidin using a solution of Avidin in NaHCO3.[99](#_ENREF_126) The research resulted in a peptide with a potent inhibiting property.

The malfunction of protein tyrosine phosphatases has been linked to major human diseases, including cancer. One of the possibilities of the malfunction could be its reaction to certain substrates. Yao and coworkers synthesized phosphopeptides substrates to investigate their interaction with the phosphatase activity. The peptides were synthesized via the Fmoc-peptide chemistry, biotinylated at the *N*-terminus and immobilized on avidin glass slides as described by Yao and coworkers. [99](#_ENREF_126) Screening of the array against protein tyrosine phosphatases provided a substrate fingerprint against each phosphatase and new candidate peptide substrates that could be used in the design of new protein tyrosine phosphatases inhibitors. [100](#_ENREF_128) In the same laboratory they constructed a peptide microarray with the ability to discriminate between various infection stages of the malaria parasite. For these stages, the cysteine proteases were profiled with *N*-terminal biotin and *C*-terminal aldehyde peptide substrates. The peptide substrates were synthesized via standard solid-phase peptide synthesis with Fmoc chemistry and the avidin coated slides were prepared as described before. [99](#_ENREF_126), [101](#_ENREF_132) With the results of this experiment they could develop stage specific inhibitors.

2.2.2.2. Fluorous - Fluorous interactions

The interaction between fluorine atoms in perfluorated carbons is besides strong also very selective and it has been applied in hydrophilic carbohydrate microarrays. [102](#_ENREF_133) Recently, it has also been exploited as a site-specific immobilization strategy of peptides on the glass surfaces (Figure 33). Schreiber and coworkers showed that more hydrophobic molecules, like peptides, could also be screened. [103](#_ENREF_134)



Figure 33 The interaction of a fluorous functionalized glass slide with a fluorous tagged peptide. After functionalization of the glass slide with Rf8-ethyl-SiCl3, the fluorous tagged peptide can interact with the fluorous functionalized glass slide.

Fluorocarbons are relatively unreactive and are immiscible in water and organic solvents. This latter characteristic is useful if separation of the fluorinated peptides from the slide is desired. Although it is a very promising method, in literature only one example for fluorous functionalized peptides can be found.

Phol and coworkers used fluorous coated slides for probing serine protease substrates. In this research serine proteases were compared in their ability to cleave peptide substrates while these were immobilized on the fluorous surface. [104](#_ENREF_135) This non-covalent attachment strategy allowed for the detection of the hydrolysis with the spotting of peptides at almost 10-30 fold lower concentrations than the previously reported covalent peptide microarray strategy. [58](#_ENREF_70) In this research it also became clear that fluorous atoms did not significantly inactivate the enzymes. The fluorinated glass slides used by Phol were commercially available and fluorinated peptides were synthesized via standard Fmoc peptide chemistry. The fluorocarbon tag C8F17 was synthesized in-house and was coupled to the peptide sequence.

Except that the fluor tag has to be synthesized in-house and cannot be easily coupled to the peptide, this non-covalent strategy is very new and is still being further developed.

# Discussion & Outlook

The development of peptide microarrays has evolved over the last years. Peptides libraries can be more easily and conveniently synthesized in large scale and unnatural functionalities can be incorporated into the peptides. There are various possibilities for the surface materials where the peptides are immobilized on. Choosing the best surface depends on the peptide functionalities or the desired analysis methods. In this thesis multiple aspects of the design and synthesis of peptides, functionalized slides and the immobilization strategy have been mentioned and discussed.

At the moment, the best and cheapest way to design and produce microarray surface materials is using microscopic glass slides. One of the main reasons for this is that the glass slides do not require a special pretreatment before or after functionalization. In contrast, silicon slides require a pretreatment of HF and often needs a “baking” step to ensure covalent bond between the monolayer and functional groups of the slide. Polymeric slides have the disadvantage that functionalization requires expensive, hazardous and distinctive equipment in the laboratory. Gold-coated slides are made on glass or silicon slide, so has an extra step at the production, and is expensive. Porous non-planar support, like cellulose, is less sufficient due to the lower loading possibilities on the surface. Due to the inexpensive synthesis of glass microscopic slides, chemicals and other equipment, glass slides are most convenient to apply. Furthermore, functionalized microscopic glass slides are nowadays commercially available in large quantities and good quality. The commercially available slides are therefore regularly used in laboratories and do not have to be produced in-house. A list of companies, which provide simple functionalized slides are listed in the review of Reineke *et al.* [4](#_ENREF_6)These commercially available glass slides often require further functionalization to obtain better characteristics for the peptide immobilization. This further functionalization is usually performed in-house. An example is the addition of a PEG linker or dendrimers, the latter to optimize the surface loading and the former to preserve the activity of the peptide or to prevent non-specific binding of peptides.

For peptide microarrays, peptide libraries can be made using combinatorial chemistry. Depending on the slide surface, different analytical methods can be used. On glass slides usually fluorescent measurements are made due to its low intrinsic background fluorescence. However, for these kinds of measurements the peptides need a fluorescent label. This labeling requires extra steps and can cause side reactions.

The *in situ* and *ex situ* synthesis are the two approaches for the immobilization of peptides on a microarray surface. In the *in situ* approach peptides are synthesized on the slide surface and there is growing panel of a peptide sequence until the desired peptide length is achieved. Using the *in situ* synthesis approach, the entire array is built and organized directly on the surface over which the screening will take place. The two *in situ* synthesis approaches are the SPOT synthesis technique and the light directed synthesis. The SPOT synthesis technique is used often for peptide arrays. It is a convenient method and easy to adapt to a wide screen of assays and screening methods. It is, however, most suitable for macro- and not micro-scale. The SPOT synthesis is currently being developed to be applicable on micro-scale. Light directed synthesis has not been developed and applied compared to the other methods, because of the technical difficulties that occur during peptide synthesis. It requires very expensive equipment and is time consuming. The *ex situ* approach is generally referred to as the spotting microarray approach. In this approach peptide libraries are pre-synthesized and thereafter spotted to defined locations on glass slides. The peptides for the spotting microarray approach are designed and synthesized with a functionality that can ligate to an appropriately functionalized surface. There are different ligations strategies for attachment of the peptides to the surface, either covalent of non-covalent. Due to the passive adsorption onto hydrophobic surfaces or electrostatic interaction with charged surfaces, covalent attachment is predominantly used to immobilize peptides on the microarray.

As mentioned in the introduction of Chapter 2, there are some ideal features for the immobilization of peptides on the microarrays surface. The covalent immobilization strategy on glass microscopic slides is, as mentioned above, the preferred method for peptide microarrays. There are however various choices in chemistry to immobilize the peptides covalently to the slide surface. All possibilities discussed in this thesis were successful, however, choosing the right immobilization strategy for a specific situation is challenging. Each method has it advantages and drawbacks.

Depending on whether natural occurring peptides or synthetic modified are immobilized, the peptides can be ligated site-specific, non-specific to the slide surface. Ligation to the surface via maleimide, silyl chloride, diazobenzylidene, epoxide, NHS, carbene and isocyanate functionalized slide are often non-specific, because they can reacted with multiple functionalities in peptides. However, peptides can be synthesized or modified to contain only one reactive moiety to fit the slide. The slides are usually bought commercially and further functionalized to the desired reactive moiety with an appropriate linker. Most of the slides for these immobilization strategies are synthesized via standard procedures. The preparation of the highly reactive carbene and isocyanate slides, however, require additional steps and are difficult to store. Carbene functionalized slides need UV irradiation to be activated and immobilization of peptides on isocyanate slides requires pyridine vapor. Therefore immobilization via these two strategies is less favorable. The most promising non-specific immobilization strategy seems to be via maleimide functionalized slides. This reaction has shown to be only selective towards thiols and when non-specific immobilization is desired the peptide only needs to be tagged with a natural cysteine residue.

The advantages of immobilization via Diels-Alder ligation, an oxime, a hydrazone, a thiazolidine ring, “click” chemistry and native chemical ligation is that these strategies yield a site-specific covalent bond and therefore the chemistry and orientation is more controlled. The peptides for the mentioned site-specific strategies all need to be coupled to a tag prior to immobilization, except for native chemical ligation. The tag in the peptide creates an unnatural moiety in the sequence, which is sometimes not beneficial for the peptides activity. These tags are not always commercially available and have to be synthesized in-house. The functionalized slides for site-specific immobilization are usually not readily available, however can be produced in the laboratory via known protocols with the appropriate linker. Due to the synthesis of the tag, the coupling of the tag to peptide and specific reaction conditions, it is understandable that more drawbacks occur in site-specific immobilization strategies. Although immobilization via Diels-Alder reaction has been successful, slide derivatization is time consuming and the unstable cyclopentadiene moiety makes it difficult to use. This problem is also apparent with the unstable thiazolidine ring, oxime, hydrazone and phosphine tag in the Staudinger ligation. Cytotoxicity due to the copper catalyst, in “click” chemistry can raise a problem, however the catalyst is used in negligible quantities to cause that issue. Furthermore, a copper free reaction [105](#_ENREF_136) has also been developed, however has not been incorporated yet in the microarray field of chemistry. The most promising site-specific immobilization strategy appears to be native chemical ligation. In this strategy a natural strong amide bond is formed and furthermore no unnatural moieties are required.

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