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1. Introduction

Advantages offered by immobilization of any component of the reacting system are rewarding all additional efforts and the cost of the support. The majority of the methods reported have been based on the principles of solid phase organic synthesis (SPOS) in which the substrate is attached to the polymer support and excesses of reactants and reagents used to drive each synthetic step to completion. Then simple filtration affords a polymer bound product. While this approach is undoubtedly effective, there are a number of drawbacks which include the requirement for additional chemical steps to attach starting material, to develop synthetic methodology for the solid phase and to cleave products. More recently, solution phase methods, which circumvent these difficulties, have been introduced as alternatives to SPOS. These allow the use of excess of reagents followed by sequestrating either the product or excess reagents and byproducts from the reaction mixture using an insoluble functionalized polymer. Isolation and purification can then be achieved by simple filtration and evaporation.

Usually polystyrene and PEG based resins are commonly used as matrixes in SPOS, but nowadays there is observed also increased application of various beaded cellulose supports [1]. These show different solvent swelling profiles relative to those exhibited by the standard organic polymers and, being biomolecules, are biodegradable. Cellulose framework is attracting growing attention due to favorable biophysical properties, biocompatibility, low immunogenicity, relatively high resistance to temperature, inertness under broad range of reaction conditions and solvents and many other unique properties. Moreover, native cellulose microfibrils are abundant within slightly diversified properties dependent on the origin within relatively low cost. These properties make cellulose very useful for biochemical and biological investigations of interactions in aqueous as well as organic media.
Solid supported reagents were found exceedingly useful in all syntheses involving excessive amounts of substrates [2]. Inexpensive cellulose is offering high loading potential but concurrently attended by the threat of side reaction of nucleophilic hydroxyl groups. Using relatively inert towards hydroxylic group under ambient conditions triazine coupling reagents it was possible to obtain monofunctional triazine condensing reagents 1 and bifunctional reagent 2 by the treatment of cellulose with 2,4-dichloro-6-methoxy-1,3,5-triazine or cyanuric chloride respectively [3]. An independent approach towards immobilization of cyanuric chloride was confirmed the general utility of this procedure [4]. The loading of the cellulose carrier has been established by determination of Cl and N contents. For the standard laboratory Whatman filter paper, typical anchoring of triazine condensing reagent gave density of loading 0.6 – 1.0*10⁻⁶ mmole/cm².

An expedient matrix for the preparation of indexed library of amides and oligopeptides has been obtained by the demarcation of the surface of the cellulose plates chess-wise by the thin lines imprinted by polysilane, allocated separated, squared area for parallel synthesis of each individual compound (flat reactors) [5]. Application of carboxylic components into compartments of the matrix afforded “superactive” [6] triazine esters 3 linked to the support. Applying of amino components afforded the indexed library of amides and oligopeptides. The extraction of the final products from the solid support gave chromatographically homogeneous amides and oligopeptides in 60-99% yield. Chromatograms of the crude extracts from the diagonal fields of the part of the 8x12 library of amides and dipeptides are presented on Figure 1. When the size of matrix compartment corresponds to the size of typical ELISA plate, the amount of product recovered by extraction from the single “square flat reactor” was sufficient for elucidation of the structure of product by ES-MS or FAB-MS, for determination of their purity by HPLC, and even for studies involving ¹H-NMR.

Increasing the dimensions of the matrix field or application of powdered cellulose enabled “bulk” (1-10 mmole) synthesis of amides and oligopeptides. The further modification of this synthetic procedure as well as the “shape” of cellulose support, opened possibility to design of tailor made system of immobilized triazine coupling reagents the best suited to the given synthetic goal.
Figure 1. Chromatograms of selected dipeptides or amides obtained by using triazine coupling reagents immobilized on cellulose matrix divided chess-wise into separate “square flat reactors” by separation lines imprinted with polysiloxane.

In the more advanced approach, chiral coupling reagents immobilized on the cellulose were prepared and then used for enantioselective activation of racemic substrates [7]. Traceless enantiodifferentiating reagents [8] were obtained by using the cellulose membrane loaded with 2,4-dichloro-6-methoxy-1,3,5-triazine.

Scheme 2. Traceless chiral coupling reagents 2a-e prepared on cellulose.
Chiral quaternary $N$-triazinylammonium derivatives $2a-e$ immobilized on the membrane were obtained in situ by treatment of $1$ with appropriate tertiary amines ($N$-methylmorpholine, column 1; strychnine, column 2, brucine column 3; quinine column 4; and sparteine, column 5). Chirality of cellulose support (column 1) was found sufficient for enantiospecific activation of $L$ enantiomer of racemic $Z$-Ala-OH with $L/D$ ratio exceeding 90/10.

Further structure modification of the immobilized triazine $1$ proceeded directly on the membrane using chiral tertiary amines yielding spatially addressed five sub-libraries of enantiodifferentiating condensing reagents (Figure 2, 1-5). In all cases enantiodifferentiating activation of rac- $Z$-Ala-OH afforded triazine “superactive” ester $3a-e$ with different enantiomeric composition. It has been found that the effect of chiral amine used as additional chiral selector predominate an effect of cellulose. Enantiomerically enriched esters $3a-e$ in reaction with $L$-Phe-OMe (S1); $D$-Phe-OMe (S2), and $H$-Gly-OMe (S3) gave a library of alanine dipeptides of divergent configuration and enantiomeric purity (not linked to the support) and side-products (still immobilized on the cellulose membrane). The method opened an access to $L$ and $D$ alanine derivatives directly from racemic substrates. The best results (ee 92-99%) in the synthesis of $L$-alanine peptides were obtained in condensations mediated by $N$-methylmorpholine (column 1) or sparteine (column 5) when matching effects of cellulose and chiral selector were cooperated. The best results in the synthesis of $D$-alanine peptides (ee 91-98%) were obtained in condensations mediated by strychnine (column 2).

The disadvantage of procedure described above is caused by limited stability of $N$-triazinylammonium chlorides $2$ prepared on cellulose. Stable immobilized triazine coupling reagents were obtained in reactions of cellulose with $N$-methylmorpholinium $p$-
toluenesulfonates in the presence of sodium bicarbonate or DIPEA. Activation of carboxylic components proceeded under conditions similar to the standard synthesis in solution yielding “superactive” esters of N-protected amino acids anchored to the support via triazine ring (see Scheme 3).

Scheme 3. Stable triazine coupling reagents immobilized on cellulose plate.

A synthetic value of triazine reagents immobilized on cellulose was confirmed by dipeptide synthesis. The reagents were found efficient in the synthesis of Z-, Boc, or Fmoc protected chromatographically homogenous dipeptides in 72-91%. Moreover, experiments involving activation of sterically demanding 2-aminoisobutyric acid (Aib) confirmed that an access to the reactive centers of immobilized reagents remains principally unrestricted, although slightly lower yield and purity of respective peptides were noticed in this case [9].

The other modification of cellulosic fibers with tri-functional triazines was applied as control release system. The compounds employed were immobilized on cellulose substituted with monochlorotriazinyl (MCT) anchor group for fixation of an active substance and tuning the reactivity to facilitate release control. While the compounds were completely stable under dry conditions, the active substances were released simply by surrounding humidity. The reagents offered intriguing perspectives for the preparation of modified cellulosic material for single-use application in fields such as healthcare, cosmetics, or personal hygiene [10].

Cellulose was found also useful support for efficient control of selectivity of chemical reactions. In the classic procedure for the nitration of phenols, use of nitric and sulfuric acid mixtures results in the formation of ortho and para products with a ratio of about 2:1. Nitration of phenols and naphthols in the presence of biodegradable cellulose-supported Ni(NO₃)₂·6H₂O/2,4,6-trichloro-1,3,5-triazine system proceeded in acetonitrile at room temperature regioselectively. Ortho-nitrated phenols were obtained within a short reaction time with good yields. The reaction conditions were mild, and the employed cellulose could be recovered several times for further use [11]. The suggested mechanism proposes that cellulose acts as a template by forming hydrogen bonds between OH groups, phenol, and nitrate anions. This complex would transform substrate into the ortho-substituted intermediate followed by regioselective rearrangement to o-nitro phenol.
2. Cellulose acylated (grafted) with amino acids or peptides

Designing of new materials based on renewable natural resources is one of the most important scientific and technological challenges. The aim of these efforts is to open an access to materials which will have to replace toxic or non-biodegradable materials derived from fossil resources, while offering similar mechanical, thermal, or optical properties. In contrast to polymer membranes, cellulose shows high thermostability up to temperatures of about 180 °C, making it possible to use cellulose for reactions at elevated temperatures [12]. To date filter papers have been mostly used as the solid support.

The classic immobilization procedure involved the use of cyanuric chloride [13] as linker for anchoring broad range of amino acids and peptides on cellulose. Lenfeld and coworkers [14] immobilized 3,5-diiodo-tyrosine (DIT) on cellulose beads activated by the reaction with 2,4,6-trichloro-1,3,5-triazine and used prepared materials as sorbents in affinity chromatography of proteases. Also glutathione-bound cellulose for use in chromatography was prepared with cyanuric chloride as linking agent [15].

The library of p-nitrophenyl esters of oligopeptides anchored with N-terminal amino-acids via triazine linkage to the cellulose were synthesized step by step and after digestion with tissue homogenate were used for colorimetric differentiation of hydrolytic activity of primary subcutaneously growing tumor of Lewis lung carcinoma (LLC) bearing mice, lung metastatic colonies of LLC, blood serum of LLC bearing mice, and appropriate tissue homogenate of the healthy mice [16].

Cellulose is a polysaccharide containing free hydroxyl groups. In the first report cellulose free OH groups were esterified with amino acids activated previously by the transformation into appropriate acid halide or anhydride, in the presence of a catalyst, such as Mg(ClO$_4$)$_2$, H$_2$SO$_4$, H$_3$PO$_4$, or ZnCl$_2$ [17]. Recently, the more convenient procedure involved the coupling method of Fmoc protected amino acids such as Fmoc-β-Ala-OH or Fmoc-Gly-OH [18] by using activating reagents such as N,N’-diisopropylcarbodiimide (DIC), 1,1’-carbonyldiimidazole (CDI) in presence of a base, e.g. N-methyl-imidazole (NMI) [19]. There are also recommendations suggesting the use of 1,1’-carbonyl-di-(1,2,4-triazole) (CDT) instead of CDI in order to reduce the risk of the deprotection of Fmoc-amino acids during the coupling reaction [20]. Since the early reports by Frank, cellulose has found widespread application as a support in the synthesis of peptides and oligonucleotides. This involved the use of cellulose in the form of sheets, membranes, disks [21] or cotton thread used as supporting material [22]. Despite these precedents, alternative cellulose supports, notably beads which can offer considerably higher loading levels than that obtained with planar supports [23]. Beaded cellulose can be easily prepared by the coagulation-regeneration technique involving the addition of a solution of a soluble cellulose derivative, commonly the xanthate [24,25] or acetate [26], to a rapidly stirred, inert, immiscible solvent. The beads, thus formed, are precipitated either by a sol-gel process or by a reduction in reaction temperature. Chemical regeneration of the hydroxyl groups and sieving produces the active beads with the desired size distribution.
Since the hydroxyl groups are moderately reactive, the process of functionalization of cellulose is often preceded with more complex modification procedures. A reactive intermediate containing isocyanate groups was prepared by treatment of cellulose with 2,4-tolylene diisocyanate. The reactions of the intermediate with amino acids and their esters gave cellulose derivatives containing amino acid residues. The isocyanate groups reacted with amino acid esters in DMSO at low temp. under nitrogen to give high conversions. The amounts of amino acid esters bound to the cellulose through urea linkage were evaluated as 0.35-1.07 mmol/g. The selective adsorption and chelation of metal ions indicated that celluloses containing lysine and cysteine residues adsorbed 0.051 and 0.056 mmol Cu^{2+}/g, respectively [27].

An essential drawback of the ester linkage applied for anchoring peptides is instability towards aqueous media of pH > 7, not uncommon for bio-assay and stripping conditions. Moreover, cellulose and cellulose membranes show only a limited acid stability. This acid sensitivity severely restricts palette of reagents and reaction conditions that can be applied, even for the most stable commercially available cellulose materials. Therefore, besides the direct esterification of cellulose membranes with amino acids, many publications describe the use of more stable ether or amide linkers.

Cellulose undergoes facile alkaline etherification which, given the availability of up to three hydroxyl groups per glucopyranose residue, offers the potential to provide very high loading supports. Several companies already offer already modified cellulose membranes. Specially prepared cellulose membranes with a stably attached aminated spacer of 8 to 12 PEG units (PEG300-500) are available, which in contrast to common cellulose membranes is stable under strong acidic and basic conditions.

The materials, on which polypeptides were immobilized on different shaped cellulose products via chemically stable ether linkage have antimicrobial or anticancer activities. These were used as wound dressings, sutures, artificial blood vessel, catheters, dialysis membranes, clothing, and stents. Thus, material, on which beetle defensin analogue Arg-Leu-Leu-Leu-Arg-Ile-Gly-Arg-Arg was immobilized on cotton fabric showed high antimicrobial activity even after repeated washing and autoclave sterilization [28]. Also other nonapeptide Arg-Leu-Tyr-Leu-Arg-Ile-Gly-Arg-Arg immobilized to amino-functionalized cotton fibers by a modification of the SPOT synthesis technique was active against S. aureus, methicillin-resistant S. aureus and mouse myeloma cells and human leukemia cells. The assays revealed that these fibers maintained inhibition activity against bacteria and cancer cells after washing and sterilization by autoclaving [29].

Cellulose with intrinsic osteoinductive property useful for the preparation of the bone substitutes was obtained by immobilization of peptides containing Arg-Gly-Asp (RGD) fragment [30]. Biomaterials from bacterial-derived cellulose modified with cell adhesion peptide became a promising material as a replacement for blood vessels in vascular surgery [31].
Application of cellulose as a support for synthesis of complex template-assembled synthetic proteins (TASP) by orthogonal assembly of small libraries of purified peptide building blocks has been reviewed [32]. In most cases the linear template precursor was prepared by standard solid phase peptide synthesis (SPPS) on synthetic resin with orthogonal protecting groups followed by head-to-tail cyclisation of the linear precursor peptide and anchoring the template structure on cellulose. The strategy involving cleavable linker allowed control of the progress of synthesis on polystyrene resin. Final assembly of peptides prepared under standard SPPS conditions proceeded by successive cleavage of orthogonal protecting groups followed by coupling of predefined peptides.

3. Proteins immobilization on cellulose

Cotton is an excellent material for immobilized enzyme active functional textiles because, like the surface of soluble proteins, it is hydrophilic and typically non-denaturing. Many methods are now available for coupling enzymes and other biologically active compounds to solid supports [33]. Several involve the preliminary preparation of carboxymethyl or p-amino-benzyl ether derivative of a general support such as cellulose. A simple process involves the use trichloro-1,3,5-triazine [34,35] or chlorotriazine derivatives with solubilizing groups such as methoxycarbonyl or methylcarbamoyl groups which make them very convenient reagents in the coupling with a cellulose carrier [36].

There are also known other proficient approaches to the covalent attachment of enzymes to cotton cellulose. Lysozyme was immobilized on glycine-bound cotton through a carbodiimide reaction. The attachment to cotton fibers was made through a single glycine and a glycine dipeptide esterified to cotton cellulose. Higher levels of lysozyme incorporation were evident in the diglycine-linked cotton cellulose samples. The antibacterial activity of the lysozyme-conjugated cotton cellulose against B. subtilis was assessed. Inhibition of B. subtilis growth was observed to be optimal within a range of 0.3 to 0.14 mM of lysozyme. This approach has also been applied to organophosphate hydrolase and human neutrophil elastase. Immobilizing the chromogenic peptide substrate of human neutrophil elastase on cellulose and studying its interaction with the elastase enzyme provided colorimetric response of human neutrophil elastase [37].

Invertase was immobilized onto the cellulose membrane activated photochemically using 1-fluoro-2-nitro-4-azidobenzene as a photolinker and used in a flow through reactor system for conversion of sucrose to glucose and fructose [38].

Over the years, several cellulose affinity ligands have been constructed based on application of noncatalytic domain of glycosidic hydrolase (CBD). This cellulose specific anchor was originally identified in Trichoderma reesei and Cellulomonas fimi. CBDs is binding on insoluble cellulose through high-affinity noncovalent interactions [39] and it is enabling the further fusion of an antibody-binding domain (i.e., protein A, protein G, protein L). Cellulose-binding domains (CBD) are ideal immobilization domains for affinity ligands because they fold independently and do not interfere with their fusion partner. [40] Coupling to cellulose
matrices orients the fusion partner away from the solid support [41] reducing steric hindrance; and their high-affinity binding to cellulose is considered nearly irreversible. [42] At present, many CBD-tagged affinity ligands are purified before attachment to their solid support matrix. [43] For large-scale applications, it could be beneficial to directly immobilize the affinity ligand at the source of production, thus avoiding the cost and time required for purification.

Horseradish peroxidase (HRP) was immobilized to cellulose with cellulose-binding domain (CBD) as a mediator, using a ligand selected from a phage-displayed random peptide library. A 15-mer random peptide library was panned on cellulose-coated plates covered with CBD in order to find a peptide that binds to CBD in its bound form. The sequence LHS, which was found to be an efficient binder of CBD, was fused to a synthetic gene of HRP as an affinity tag. The tagged enzyme (tHRP) was then immobilized on microcrystalline cellulose coated with CBD, thereby demonstrating the indirect immobilization of a protein to cellulose via three amino acids selected by phage display library and CBD [44].

As a model system, it has been developed a fusion protein, which consisted of antibody-binding proteins L and G fused to a cellulose-binding domain (LG-CBD) tethered directly onto cellulose. Direct immobilization of affinity purification ligands, such as LG-CBD, onto inexpensive support matrices such as cellulose is an effective method for the generation of functional, single-use antibody purification system. This straightforward preparation of purification reagents make antibody purification from genetically modified crop plants feasible and address one of the major bottlenecks facing commercialization of plant-derived pharmaceuticals [45].

In several cases it could be beneficial to directly immobilize the affinity ligand at the source of production, thus avoiding the cost and time required for purification. A potential use of cellulose-supported affinity ligands for purification of other bioproducts from homogenates from genetically modified plants expressing recombinant proteins is under intensive studies. To examine the potential of immobilizing affinity purification ligands onto cellulose matrices in a single step, the yeast *P. pastoris* were engineered to express and secrete a chimeric protein consisting of antibody-binding proteins L and G [45] fused to a cellulose-binding domain. A similar fusion was recently reported for cell capture in hollow-fiber bioreactors. There are reports on the direct immobilization of chimeric LG-CBD proteins onto cellulosic resins for antibody purification. Both protein L and protein G domains retained dual functionality demonstrated by the specific binding and purification of scFv and IgG antibodies from complex feed stocks of yeast supernatants and tobacco plant homogenates. This is a step towards the rapid generation of inexpensive affinity purification reagents and systems, to reduce the costs associated with downstream processing of pharmaceutical products, including antibodies, from complex production systems such as genetically modified crop plants.

Copolymers having polypeptide side chains grafted on cellulose main chain were used for adhesion of fibroblasts. The factor likely to play a key role in determining the binding ability was the balance between the hydrophilicity and hydrophobicity of the main- and side-chain components [46].
4. Protein sensors

Current research in the field of pathogen detection in food matrixes is aimed at creating fast and reliable detection platforms. Antibody engineering has allowed for the rapid generation of binding agents against virtually any antigen of interest, predominantly for therapeutic applications, development of diagnostic reagents and biosensors. By using engineered antibodies a pentavalent bispecific antibody were prepared by pentamerizing five single-domain antibodies and five cellulose-binding modules. This molecule was dually functional as it bound to cellulose-based filters as well as *S. aureus* cells. When impregnated in cellulose filters, the bispecific pentamer recognized *S. aureus* cells in a flow-through detection assay. The ability of pentamerized CBMs to bind cellulose may form the basis of an immobilization platform for multivalent display of high avidity binding reagents on cellulose filters for sensing of pathogens, biomarkers and environmental pollutants [47]. Another approach for designing protein sensor used ultrathin films of cellulose modified on surface with small engineered peptides HWRGWV or HWRGWVA as substrate for protein detection. Primary tests run with peptide HWRGWV confirmed that there was an abundant amount of protein absorbed onto the surface, particularly with lower concentration and the sensitivity of a peptide greatly affects the ability to adsorb analytes onto the surface, demonstrating that cellulose substrates can be used to immobilize peptides which can further be used to selectively bind biomolecules [48].

The sensor for human neutrophil elastase (HNE), an enzyme engaged in chronic wounds healing was prepared based on colorimetric determination of enzyme activity. For colorimetric detection of human neutrophil elastase chromogenic peptide substrate Succinyl-Ala-Ala-Pro-Ala-pNA and its analog Succinyl-Ala-Ala-Pro-Val-pNA were attached to derivatized cellulose. Cellulose was pre-treated with 3-aminopropyltriethoxysilane to form the amino-propyloxy ether of cellulose, then reacted with the HNE chromogenic para-nitroanilide peptide substrates to form a covalently linked conjugate of cellulose (Cell-AP-suc-Ala-Ala-Pro-Ala-pNA or Cell-AP-suc-Ala-Ala-Pro-Val-pNA) through amide bond between the Cell-AP amine and the succinyl carboxylate of the substrate. The colorimetric response of the cellulose-bound chromophore was assessed by monitoring release of p-nitroaniline from the derivatized cellulose probe to determine human neutrophil elastase levels from 5.0 x 10^{-3} to 6.0 units per mL [49].

5. Epitope mapping - SPOT methodology

The SPOT synthesis of peptides, developed by Ronald Frank [50], has become one of the most frequently used methods for synthesis and screening of peptides on arrays. The method is a very useful tool for screening solid-phase and solution-phase assays with the size of arrays changeable from a few peptides up to approximately 8000 peptides [51]. Several hundred papers regarding modification and application of the SPOT method have been published [52].
The method was initiated as an uncomplicated technique for the positionally addressable, parallel chemical synthesis on a membrane support. SPOT synthesis of peptides on cellulose paper is a special type of solid phase peptide synthesis (SPPS) with each spot considered as a separate reaction vessel. The general strategy for parallel peptide assembly on a cellulose membrane is shown in Figure 3.

Figure 3. SPOT technology procedure.

Plain cellulose membranes (filter paper, chromatography paper) are commonly used as a support in the SPOT synthesis. These are porous, hydrophilic, flexible and stable in the organic solvents used for peptide synthesis. Cellulose membranes are relatively inexpensive material, which makes them very useful for biochemical and biological studies in aqueous
and organic media. However, since cellulose is not stable against harsh chemical conditions, the SPOT synthesis was developed for the milder type of the two major SPPS strategies based on the Fmoc protection of amine function of the main peptide chain [53] and orthogonal protecting groups used for protection of side chains. [54].

5.1. Membrane modifications

Cellulose membranes are still the most widely used supports for SPOT peptide synthesis. The esterification of hydroxy functions of the cellulose with an Fmoc amino acid is a convenient method to introduce a spacer molecule and, after Fmoc deprotection, a free amino function for the SPPS of peptide arrays. The stability of the cellulose to organic solvents and bases allows the synthesis of peptides by utilizing the standard Fmoc methodology. Furthermore, the hydrophilic nature of cellulose offers a high compatibility with a wide variety of biological assay systems. On the other hand, however, cellulose shows only a limited acid stability. This acid sensitivity is severely restricting side chain deprotection conditions and stimulated the search of more convenient supports. Increasing resistance of peptide-cellulose membrane linkage against various types of reagents has been achieved through the development amino-functionalized ether type membranes. Ether type membranes provide stable membrane-bonding of peptides or other compound through the chemical stability of the ether bound. The first example of this type membrane was a cellulose-aminopropyl ether membrane (CAPE membrane) prepared by the treatment of cellulose filter paper with N-protected 2,3-epoxypropylamine [55].

The use of epibromohydrin as an activating reagent allowed introducing reactive bromine attached to the cellulose via an ether bond. The bromine moiety is able to react with different diamines [56] such as DAP (1,3-diaminopropane) [57] or TOTD (4,7,10-trioxa-1,13-tridecanediamine) and aminated polyethylene-3 (PEG-3) [58]. DAP modified cellulose membranes are known as N-CAPE membranes [59], while membranes modified with TOTD as a trioxa or TOTD membranes [60]. An amino type linker functionalized planar cellulose support [61] has been obtained by activation of cellulose with tosyl chloride and subsequent reaction with broad variety of diamines.

An additional advantage of CAPE membranes is an excellent signal-to noise ratio during on-support assay because of the very low background signal of this membrane [62]. Due to these properties they were applied in biological studies [63]. Table 1 has shown the characteristic of selected examples of amino-functionalized cellulose membranes.

Another approach to improve SPOT technology involves the use of linker strategies to enable cleavage of peptides from the support. An interesting linker was proposed by Frank. It is known as a Carboxy-Frank-Linker [64]. This linker allows peptide release from the solid support in aqueous solution (pH 7–8). Other linker types used in SPOT technology nowadays are the p-hydroxymethylbenzoic acid (HMB) linker, the Rink-amide linker [65], photolabile linker [66], the Wang linker [67], thioether moieties [68] or 4-hydroxymethylphenoxy acetic acid (HMPA) and 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) linkers [69]. An attractive approach based on the use of the C-terminal amino acid
as a linker moiety was reported by Ay and co-workers [70]. Proposed solution was applied to sorting peptides according to their C termini using modified membranes with the corresponding C-terminal amino acids anchored either spot- or surface-wise.

Scheme 4. Different methods of amino functionalization of cellulose membranes: I) preparation of ester type membrane with amino acid; II) functionalization with epibromohydrin and subsequent reaction with TOTD or DAP providing TOTD or N-CAPE membranes; III) treatment with N-protected 2,3-epoxypropylamine giving CAPE membrane; IV) treatment with tosyl chloride and subsequent reaction with TOTD giving a TOTD like membrane.
### Table 1. Characterization of amino-modified membranes.

<table>
<thead>
<tr>
<th>Cellulose type</th>
<th>Cellulose membrane type</th>
<th>Capacity [μmol/cm²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whatman CHr1</td>
<td>Ester type: β-alanine</td>
<td>0.4-0.6</td>
</tr>
<tr>
<td></td>
<td>Amine type: TsCl, diamino-PEG-3</td>
<td>4.0-10.0</td>
</tr>
<tr>
<td></td>
<td>Amine type: TsCl, diamino-PEG-3 + linker</td>
<td>0.45-2.6</td>
</tr>
<tr>
<td>Whatman 50</td>
<td>Ester type: β-alanine</td>
<td>0.2-0.4</td>
</tr>
<tr>
<td></td>
<td>Ester type: glycine</td>
<td>0.8-1.9</td>
</tr>
<tr>
<td></td>
<td>Ester type: different amino acids</td>
<td>0.2-1.7</td>
</tr>
<tr>
<td></td>
<td>Ether type: CAE (amino-epoxy)</td>
<td>0.05-0.20</td>
</tr>
<tr>
<td></td>
<td>Ether type: N-CAPE, trioxa</td>
<td>0.2-1.2</td>
</tr>
<tr>
<td>Whatman 540</td>
<td>Ester type: β-alanine</td>
<td>0.2-0.6</td>
</tr>
<tr>
<td>AIMS</td>
<td>Amine type: amino-PEG</td>
<td>0.4-0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.0-5.0)</td>
</tr>
</tbody>
</table>

In cases of classical SPOT technology in which the peptide is coupled via an ester bond using β-alanine of glycine spacer, peptide can be released from the cellulose by hydrolysis at pH>9. Numerous reagents were found suitable for this goal, e.g. aqueous solutions of ammonia, sodium hydroxide, trialkylamines or lithium carbonate (see Table 2) [71]. A broadly used method for releasing of soluble peptide amides is based on the treatment of membranes with ammonia vapor [72]. When the cleavage is carried out with nucleophils in an anhydrous environment, the substitution of the ester bond leads to amides, hydrazides and other derivatives.

### Table 2. Typical linker types bounded to cellulose supports and cleavage methods used for releasing the peptides from the membrane.

<table>
<thead>
<tr>
<th>Linker</th>
<th>Cleavage conditions</th>
<th>C-termini derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine (different amino acids)</td>
<td>gaseous ammonia</td>
<td>amide</td>
</tr>
<tr>
<td></td>
<td>hydrazine</td>
<td>hydrazide</td>
</tr>
<tr>
<td></td>
<td>hydroxyl amine</td>
<td>hydroxyl amide</td>
</tr>
<tr>
<td></td>
<td>aq. NaOH</td>
<td>free carboxylic group</td>
</tr>
<tr>
<td></td>
<td>aq. triethylamine</td>
<td>free carboxy group</td>
</tr>
<tr>
<td></td>
<td>primary alkyl/aryl amine</td>
<td>alkyl/aryl amide</td>
</tr>
<tr>
<td>Boc-Imidazol linker</td>
<td>TFA + aq. buffer</td>
<td>free carboxy group</td>
</tr>
<tr>
<td>Allyl linker</td>
<td>Palladium (0)-catalyst</td>
<td>free carboxy group</td>
</tr>
<tr>
<td>Boc-Lys-Pro</td>
<td>TFA + aq. buffer</td>
<td>diketopiperazine</td>
</tr>
<tr>
<td>HMB linker</td>
<td>gaseous ammonia</td>
<td>amide</td>
</tr>
<tr>
<td>Photo-labile linker</td>
<td>UV irradiation at 365 nm</td>
<td>amide</td>
</tr>
<tr>
<td>Rink-amide linker</td>
<td>TFA</td>
<td>amide</td>
</tr>
<tr>
<td>Thioether (thiol + coupled by amino acid)</td>
<td>gaseous ammonia</td>
<td>amide</td>
</tr>
<tr>
<td></td>
<td>NaOH/H₂O/methanol</td>
<td>free carboxy group</td>
</tr>
<tr>
<td>Halosalkyl esters</td>
<td>NaOH/H₂O/acetonitrile</td>
<td>free carboxy group</td>
</tr>
<tr>
<td>Wang linker</td>
<td>TFA vapour</td>
<td>free carboxy group</td>
</tr>
</tbody>
</table>
Different type of anchoring of the peptide chain to cellulose matrix was proposed by Kaminski [73] and co-workers. 1-Acyl-3,5-dimethyl-1,3,5-triazin-2,4,6(1H,3H,5H)-trion derivatives serve both as a spacer and linker. This isocyanuric linker has been introduced by thermal isomerization [74] of 2-acyloxy-4,6-dimethoxy-1,3,5-triazines immobilized on the cellulose support or isomerization catalysed by the presence of acids. Synthetic procedure leading to peptides anchored to cellulose by 1-acyl-3,5-dimethyl-1,3,5-triazin-2,4,6(1H,3H,5H)-trion (iso-MT) is shown in Scheme 5. In the first step chloro-triazine a immobilized on cellulose was treated with N-methylmorpholine yielding N-triazinylammonium chloride b. Then compound b activated carboxylic function of Fmoc-protected amino acid to superactive ester c [75], which finally in refluxing toluene rearranges to stable isocyanuric derivative d.

Scheme 5. Synthesis of peptides with free N-termini anchored by iso-MT linker.
<table>
<thead>
<tr>
<th>Epitope</th>
<th>Peptide sequence</th>
<th>Reaction</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>UB-33</td>
<td>H₂N-Cys-His-Leu-Asp-Lys-Ser-Ile-Lys-Glu-Asp-Val-Gln-Phe-Ala-Asp-Ser-Arg-Ile-COO-cellulose</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>H₂N-Cys-His-Leu-Asp-Lys-Ser-Ile-Lys-Glu-Asp-Val-Gln-Phe-Ala-Asp-Ser-Arg-Ile-β-Ala-COO-cellulose</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H₂N-Cys-His-Leu-Asp-Lys-Ser-Ile-Lys-Glu-Asp-Val-Gln-Phe-Ala-Asp-Ser-Arg-Ile-β-Ala-β-Ala-COO-cellulose</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CSF114</td>
<td>H₂N-Thr-Pro-Arg-Val-Glu-Arg-Asn(Glc)-Gly-His-Ser-Val-Phe-Leu-Ala-Pro-Tyr-Gly-Trp-Met-Val-Lys-COO-cellulose</td>
<td>+, m</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td>H₂N-Thr-Pro-Arg-Val-Glu-Arg-Asn(Glc(OAac)₄)-Gly-His-Ser-Val-Phe-Leu-Ala-Pro-Tyr-Gly-Trp-Met-Val-Lys-COO-cellulose</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H₂N-Thr-Pro-Arg-Val-Glu-Arg-Asp-Gly-His-Ser-Val-Phe-Leu-Ala-Pro-Tyr-Gly-Trp-Met-Val-Lys-COO-cellulose</td>
<td>+, m</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H₂N-Thr-Pro-Arg-Val-Glu-Arg-Asn(Glc)-Gly-His-Ser-Val-Phe-Leu-Ala-Pro-Tyr-Gly-Trp-Met-Val-Lys-β-Ala-β-Ala-β-Ala-COO-cellulose</td>
<td>+, m</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H₂N-Cys-His-Leu-Asp-Lys-Ser-Ile-Lys-Glu-Asp-Val-Gln-Phe-Ala-Asp-Ser-Arg-Ile-β-Ala-iso-MT-cellulose</td>
<td>+, m</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H₂N-Cys-His-Leu-Asp-Lys-Ser-Ile-Lys-Glu-Asp-Val-Gln-Phe-Ala-Asp-Ser-Arg-Ile-iso-MT-cellulose</td>
<td>+, s</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>H₂N-Ser-Ile-Lys-Glu-Asp-Val-Gln-Phe-β-Ala-iso-MT-cellulose</td>
<td>+, s</td>
<td></td>
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</tr>
<tr>
<td>F-8</td>
<td>H₂N-Thr-Pro-Arg-Val-Glu-Arg-Asn(Glc)-Gly-His-Ser-Val-Phe-Leu-Ala-Pro-Tyr-Gly-Trp-Met-Val-Lys-iso-MT-cellulose</td>
<td>+, m</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H₂N-Thr-Pro-Arg-Val-Glu-Arg-Asn(Glca)-Gly-His-Ser-Val-Phe-Leu-Ala-Pro-Tyr-Gly-Trp-Met-Val-Lys-iso-MT-cellulose</td>
<td>+/-</td>
<td>70%</td>
</tr>
<tr>
<td>CSF114</td>
<td>H₂N-Thr-Pro-Arg-Val-Glu-Arg-Asp-Gly-His-Ser-Val-Phe-Leu-Ala-Pro-Tyr-Gly-Trp-Met-Val-Lys-iso-MT-cellulose</td>
<td>+/-</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Interaction of peptidic epitopes with free N-termini anchored on cellulose with antibodies.

Further stages of the synthesis included the standard SPPS conditions: deprotections of Fmoc group and subsequent condensation with Fmoc/tBu-protected amino acids by using DMT/NMM/BF₄ as a coupling reagent [76]. The data summarized in Table 3 shown that in
the several cases for the same antigen the strengths of reaction with antibody depends on the anchoring method. Moreover, for isocyanuric linker interactions with antibodies were found more selective [77].

5.2. Cellulose membrane-bound peptides with free C-termini

Unfortunately, cellulose is not suitable for classic SPOT peptides synthesis with free C-termini, due to engagement of C-terminal fragment of peptide for fixation to support. One potential solution to this problem is to synthesize peptides in a nontraditional manner (that is, from the N- towards the C-termini) using amino acid ethyl esters [78]. One major drawback with this approach is the increased risks of epimerization [79] at all coupling stages due to repeated solid support-bound carboxyl activation. There have been done numerous efforts to develop effective ISPPS strategies (inverse solid-phase peptide synthesis). One of the first reports on ISPPS described the use of amino acid hydrazides [80]. More recently, amino acid 9-fluorenylemethy (Fm) esters [81], and amino acid allyl esters [82] have been used for ISPPS. However, few if any of these amino acid derivatives are currently commercially available. The Fm ester approach looks attractive considering its similarity to standard Fmoc-based C-towards-N SPPS, but Fm esters are not as stable as Fmoc amino acids, and Fm ester-based inverse peptide synthesis apparently suffers from this limitation. The Fm ester approach also suffers from significant racemization during coupling reactions. The allyl ester-based approach is practicable and appears currently to be the method most competitive with the t-butyl ester-based ISPPS method described below. However, allyl esters are also not readily available commercially, and moreover, their deprotection requires the use of 20 mol% of Pd(PPh₃)₄, an expensive, heavy metal-based reagent. These strategies for ISPPS, therefore, appear not to be ideal, especially since suitable amino acid substrates are not easy available.

A method for solid-phase peptide synthesis on cellulose in the N- to C-direction that delivers good coupling yields and a relatively low degree of epimerization was reported by Hallberg [83] and co-workers. The optimized method involves the coupling, without preactivation, of the solid support-bound C-terminal amino acid with excess amounts of amino acid tri-tert-butoxyxysilyl (Sil) esters, using HATU or TBTU as coupling reagent and 2,4,6-trimethylpyridine (TMP, collidine) as a base. For the amino acids investigated, the degree of epimerization was typically 5%, except for Ser(t-Bu) which was more easily epimerized (ca. 20%). Efficiency of proposed methodology was confirmed on the synthesis of five tripeptides: Asp-Leu-Glu, Leu-Ala-Phe, Glu-Asp-Val, Asp-Ser-Ile, and Asp-D-Glu-Leu. The study used different combinations of HATU and TBTU as activating agents, N,N-diisopropylethylamine (DIEA) and TMP as bases, DMF and dichloromethane as solvents, and cupric chloride as an epimerization suppressant. Experiments indicated that the observed suppressing effect of cupric chloride on epimerization in the present system merely seemed to be a result of a base-induced cleavage of the oxazolone system, the key intermediate in the epimerization process. Proposed methodology can provide an attractive alternative for the solid-phase synthesis of short (six residues or less) C-terminally modified peptides, e.g., in library format. On the other hand amino acid silyl esters are difficult to prepare, unstable to store, and unstable under peptide coupling conditions.
The alternative strategy for ISPPS based on amino acid t-buty1 esters was proposed by Gutheil [84] and co-workers. Favorable features of this approach are that amino acid t-buty1 esters are stable, a large selection of them are commercially available, and the synthesis of commercially unavailable monomers is relatively straightforward. The t-buty1 ester strategy also has the benefit that this approach is exactly the inverse of the well-developed Boc strategy for normal C-to-N peptide synthesis, and the extensive knowledge of side chain protection strategies and other chemical details can therefore be transferred from Boc chemistry to t-buty1 ester chemistry. The effectiveness of the proposed solution has been demonstrated in the synthesis of tripeptides: Tyr-Ala-Phe, Tyr-Gly-Orn, Tyr-Ala-Val, Asn-D-Val-Leu, Asn-Leu-Glu, Gly-Ile-Thr, Phe-Ala-Gly. The consecutive incorporation of amino acids was performed in the presence of HATU as a coupling reagent using an excess of AA-OtBu*HCl. The observed racemization of individual amino acids was <2%.

Scheme 7. Synthesis of inverted peptides on cellulose membranes.
Another approach to the synthesis of peptides attached to the cellulose matrix within N-terminus and presenting free C-terminus [85] was based on the utilization of 1,3,5-triazine derivative as an anchoring group. The peptides anchored via N-terminal moiety to the cellulose plate, were synthesized in accord to step-by-step methodology by means of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMT/NMM/BF4) as a coupling [86]. 2-Chloro-1,3,5-triazine fragment, used as an anchoring group, was introduced by the treatment of cellulose with 2,4-dichloro-6-methoxy-1,3,5-triazine (DCMT) [87]. The first amino acid was attached to the triazine ring by the nucleophilic substitution reaction involving amine group. The oligopeptide chain was elongated in accord to step-by-step methodology in the sequence of standard reactions involving: activation of carboxylic function, coupling with the ester of appropriate amino acid, washing, capping, hydrolysis of ester moiety, and washing (Scheme 6).

An amount of natural, all-L diastereomer was sufficiently abundant for selective reaction with sera of patients with medically confirmed atherosclerosis even in the case of long epitope.

A more sophisticated approaches are based on inversion of the peptide chain following conventional (C → N) synthesis and then modification of the C-terminus. Examples of inverting solid support-bound peptides [88] and methods for the generation of liberated C-terminally modified peptides [89] via a cyclization/cleavage protocols are known. The first example of application of the synthesis of inverting cellulose support-bound peptides according SPOT-methodology with free C-termini via prepared by successive cyclization and re-linearization was described by Hoffmüller and Volkmer-Engert [90].

Aminopropyl ether cellulose (CAPE-membrane) (a) was used as the matrix. β-Alanine serves both as a spacer and to residue directly engaged in the rearrangement. Dmab-glutamic acid was coupled as a bivalent linker followed by introducing hydroxymethylbenzoic acid (HMB) as a base-labile cleavable site (→ b). The intended C-terminal amino acid was coupled through an ester bond (→ c). The Fmoc and Dmab protecting groups on the N-terminus and the side chains of the glutamic acids were cleaved off and then construct cyclized (→ e). Removal of the side chain protecting groups followed by hydrolysis of the ester bond linearizes the construct and generated free C terminus (→ f). Even if presented above method allowed successful synthesis of inverted peptides arrays, the obtained yields were low and procedure were found troublesome and time-consuming.

Therefore Volkmer-Engert [91] and co-workers developed a more robust and efficient protocol for the preparation of cellulose membrane-bound inverted peptide arrays that could be used for widespread mapping different epitopes anchored on solid support and presenting C-termini peptides.

Synthesis of inverted peptides was performed on a cellulose membrane carrying a stable N-functionalized anchor (N-modified cellulose- amino-hydroxypropyl ether membrane - N-CAPE), which retained the inverted peptides (i). The inverted and N terminally fixed
peptides (i) display a free C terminus resulting from reversal of the peptide orientation by successive thioether-cyclization/ester cleavage transformations. Key intermediates in the synthesis are the 3-bromopropyl esters of Fmoc-amino acid (Fmoc-Aaa-OPBr) (d), the membrane-bound mercaptopropionyl cysteine adduct (e), the matrix-bound amino acid ester derivative (f), and the cyclic peptide (h). Critical reaction steps are the formation of both the cleavable ester bond and the cyclic peptide.

Scheme 8. Synthesis of inverted peptides on cellulose membranes allows further modification of side chains (phosphorylation).
5.3. Application of SPOT technology

Cellulose was found to be the support of choice in the SPOT synthesis. The main area of application of SPOT technology is for epitope mapping:

1. **Physiology** - Antibodies can identify the structural fragments which allow molecules to interact between themselves or with their specific receptor. They can also be useful in understanding the structure-function relationships.

2. **Pathology** - Understanding the mechanism by which an immune-mediated pathology develops by a precise identification of both B- and T-cell epitopes on the antigen. Antibodies can, therefore, be useful in analyzing the specificity of antibodies spontaneously formed in a number of diseases in which an immune response is an important parameter. Mapping of epitopes is also essential when one wishes to unravel the mechanisms by which immune tolerance is established and/or broken.

3. **Preclinical evaluation of drugs or blood product derivatives** - Most drugs act as haptens, that is to say that they are too small for being immunogenic. However, after combination with plasma or tissue proteins, they can become immunogenic.

4. **Vaccinations** - The identification of both B- and T-cell epitopes on a micro-organism or bacterial derived products such as toxins or enzymes may have a crucial influence on the design of vaccines. This includes not only an increase efficiency of vaccines, but potentially the design of vaccines that could stimulate humoral or the cellular immune response.

5. **Diagnosis and subtyping of micro-organisms** - Antibodies of defined specificity are currently used to distinguish between micro-organisms that belong to the same strain or to render diagnostic test more specific. The identification of shared antigenic determinants between proteins pertaining to different families can also has an important impact on the understanding of cross-reactions.

6. **Mechanism of drug action** - an emerging field of interest concerning the use of antibodies to study the mechanism of action of drugs [92].

Today, experiments to identify and characterize linear antibody epitopes using peptide scans, amino acids scans, substitutional analyses, truncation libraries, deletion libraries, cyclization scans, all types of combinatorial libraries and randomly generated libraries of single peptides are standard techniques widely applied even in non-specialized laboratories [93].

The synthesis of non-peptidic compounds or peptides with non-peptidic elements has been carried out on cellulose as well as polypropylene membranes. Using the SPOT technique, one of the most frequently synthesized non-peptidic compounds are a peptoids [94]. These compounds are synthesized pure or as hybrids with peptides, so-called peptomers [95]. Zimmermann et al. [96] investigated the possibility of replacing natural amino acids by peptoidic elements. Screening of an array of 8000 hexapeptoids and peptomers was carried out by Heine et al [97]. Hoffmann et al. [98] described the transformation of a biologically active peptide into peptoid analogues while retaining biological activity. Another application of the SPOT method is the synthesis of chimeric oligomers of peptide nucleic
Weiler et al. [100] described the synthesis of a PNA oligomer library, with coupling yields of >97%. The synthesis of small organic compounds is another broad field for the application of SPOT synthesis [101].

6. Supramolecular structures formed by self-organization of N-lipidated peptides anchored to cellulose

Cellulose is a polysaccharide with two different types of hydroxyl groups i.e. primary and secondary. The primary hydroxyl groups are significantly more reactive than the secondary. Since the chains of polyanhydroglucose interacts with each other in the precisely defined way these functional groups are positioned within the reasonably regular fashion on the surface of cellulose. In the crystalline region of cellulose [102] the every second primary hydroxyl groups are exposed and accessible for interaction with reagents making after the transformation relatively regular pattern of anchored molecules separated by the distance of one anhydroglucose residue. Due to this advantageous feature of the cellulose the space available in between molecules anchored on the cellulose surface is sufficient for docking another molecules. Based on this assumption Kaminski and co-workers proposed entirely new approach for designing artificial receptors. According to the proposed concept, appropriate structure of molecules anchored on cellulose creates precisely defined and functionalized space for trapping ligands as presented on Figure 4.

The relatively weak bonding forces and conformational flexibility of both partners make docking of ligands to receptors difficult to study, to categorize by any kind of empirical rules, or to predict based on molecular modeling. Even in the case of interactions between relatively simple molecules, the possible bonding and repulsive forces of mutual host-guest interactions are multifaceted, very numerous, and difficult in terms of molecular modeling [103]. For the more advanced models involving flexible ligands and complex flexible receptor structures the rational construction plan of the host structure still exceeds our capabilities [104]. Thus, design of the molecular trap was done intuitively by mimicking structural features occurring in natural receptors, synthesis of the library of them by methods of combinatorial chemistry and selection of the most efficient representatives.

Strong, yet reversible binding force for the most of potential guest molecules were achieved by introducing into binding pockets most of the structural attributes responsible for weak intermolecular interactions [105]. These include hydrogen-bond donors and acceptors, lipophilic and hydrophilic fragments supplemented with \( \pi \)-donors and \( \pi \)-acceptors as depicted on Figure 5.

All these elements were allocated inside the linear structure forming the matrix of podands in such a way as to separate the flexible \( N \)-lipopeptide fragment from the solid support by relatively rigid, aromatic rings. Thus, a bonding “pocket” was composed from the tethered fragments of “walls” constructed from aromatic rings, expanded with a diversity of
interactions offered by flexible peptide fragments, and finally closed with a “zipper” of hydrophobic chains of lipidic fragments. Due to the conformational flexibility of interacting partners, the relative direction of the functional groups of a ligand as well as that of the binding pockets could be readjusted to the most energetically favored orientation of both counterparts [106]. Thus structures immobilized on cellulose support via triazine linker created the mosaic of binding holes, mimicking the behavior of receptor formed from the neighboring, identical lipidated peptides.

Figure 4. “Molecular traps” formed by podands regularly positioned on the support.

In the absence of some elements (Figure 6. 1-4) binding process was substantially deteriorated compared to the binding ability of complete receptor structure (Figure 6, 5).

Thus, the fully serviceable monolayer immobilized on cellulose was prepared in the stepwise process involving functionalization of cellulose with 1,3,5-triazine derivative followed by reaction with m-fenylendiamine, attachment of N-Fmoc amino acids,
deprotection of N-terminus and completing the synthetic procedure by binding of carboxylic acid (Scheme 8). In the synthesis of the peptide fragment, DMT/NMM/BF₄⁻ was used as a coupling reagent. For the final acylation of immobilized tripeptides with carboxylic acid more lipophilic DMT/NMM/TosO⁻ was found more suitable as a coupling reagent [107].

Figure 5. The concept of binding pockets with most of the structural attributes responsible for weak intermolecular interactions.

Figure 6. Influence of the structure of podands on binding of antocyan dyes from Rubus laciniatus and Beta vulgaris extracts.
Scheme 9. Synthesis of N-lipidated peptides (amino acids) immobilized on cellulose via aromatic linker.

Loading of cellulose support was calculated on the basis of N and Cl content determined by elemental analysis 9-10 μmol/cm² with the anticipated ratio of molecular fragment triazine/m-phenylenediamine/amino acid/carboxylic acid.

The studies of water permeability through the monolayer achieved with 28-element library of N-acylated aminoacid prepared from Ala, Phe, Ser and Arg, and cinnamic, 10-undeceniacid, elaicid, oleic, erucic, palmitic, and ricinic acids confirmed that penetration of water is possible only in the presence of hydrophilic functional groups incorporated into the monolayer. In their absence the podands were allocated sufficiently dense on cellulose fibers to inhibit penetration of water. This means that lipidic “zip” separate the interior of binding pocket, but the lipidic barrier remains still penetrable at least to the small, highly polar molecules of water.
Process of binding triphenylmethane dyes with an array of N-lipidated dipeptides peptides immobilized on cellulose according to the manner described above was not dependent on initial concentration of ligand reaching equilibrium within 20-30 min. The selectivity and rate of binding depends on the structure of the peptide fragment as well as N-lipidic moiety. Even tiny structural changes in guest molecules were detected by monitoring the alteration of the binding pattern [108]. Measurements of fluorescence of fluorescein docked inside the receptor pocket revealed difference in $\lambda_{\text{max}}$, curvature and intensity of fluorescence depended on the structure of the peptide motif and lipidic fragment of binding pocket. This strongly suggests an alternation of charge distribution inside the receptor pocket [109].

Binding of colorless ligand was monitored by replacement of the reporter dye due to competitiveness of process (Scheme 10).

**Figure 7.** Dependence of water permeability through N-lipidated amino acids layer.

**Figure 8.** The library of N-lipidated dipeptides immobilized on cellulose disks treated with 10 mM/L solution of bromochlorophenol blue (1) (left); the same disks after subsequent treatment with 20 mM/L solution of S-(+)-naproxen (right).
Docking colorless N-phenylpiperazines pairs of analogues with or without a fluorine atom in the phenyl ring [110] revealed that using an array of artificial receptors it is possible to verify the presence of such ligand modification.

Analysis of the binding pattern of N-phenylpiperazine derivatives showed two characteristic binding patterns dependent on the structure of amino acid residues interacting with ligands. For most amino acid residues weaker binding of fluorinated analogues and stronger binding of native phenyl substituted analogues was observed with an exception of the receptors bearing tryptophane residue inside the binding pocket [111].
An array of N-lipidated peptides immobilized on cellulose was also used in studies of tissue homogenates for early diagnosing thyroid gland cancer [112], which is the most common malignancy of the endocrine system. There were found different binding patterns of healthy and cancer tissue for the various types of cancer.

By incorporation into the peptide fragment of receptor amino acid residues characteristic of catalytic triade of the hydrolytic enzymes the binding pockets demonstrated catalytic activity [113]. These were able to catalyse hydrolysis of esters bond [114]. All members of the library of 36 structures formed by permutations of Ser, Glu, His acylated with 6 long chain carboxylic acids were active as esterase and effectively catalyzed hydrolysis of p-nitrophenyl ester of Z-L-Leu-L-Leu-OH at pH 7-7.5 and temp not exceeding 20°C. The postulated mechanism of catalytic activity of N-lipidated oligopeptides is presented on Figure 9.

![Chemical structure diagram](image)

**Figure 9.** The postulated mechanism of catalytic activity of N-lipidated tripeptides of catalytic triade.

In this case the progress of hydrolysis was so fast that under the conditions of the experiment it was difficult to identify the most catalytically active structure. The extinction at 405 nm increased from the initial value of 0.300 for the substrate to more than 0.800 before the first cycle of measurements was completed. The rate of reaction diversified enough for identification of the most active catalytic structures was afforded by using significantly less reactive, sterically hindered substrate Z-Aib-Aib-ONp.

As the final effect of catalytic activity is the transformation of relatively non-polar organic molecule into ionic species, one can expect application of this phenomena for the construction of sensors [115]. There are also many other interesting area of application of catalytically active N-lipidated peptides immobilized on cellulose. Most expected are effects stereochemical results of such transformations, expecting open access to essentially
unlimited access to configurational arrangement of stereogenic centers of polypeptide fragment [116].

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