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Review

Veni, Vidi, Vici: Immobilized Peptide-Based Conjugates as Tools for Capture, Analysis, and Transformation

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Abstract: Analysis of peptide biomarkers of pathological states of the organism is often a serious challenge, due to a very complex composition of the cell and insufficient sensitivity of the current analytical methods (including mass spectrometry). One of the possible ways to overcome this problem is sample enrichment by capturing the selected components using a specific solid support. Another option is increasing the detectability of the desired compound by its selective tagging. Appropriately modified and immobilized peptides can be used for these purposes. In addition, they find application in studying the specificity and activity of proteolytic enzymes. Immobilized heterocyclic peptide conjugates may serve as metal ligands, to form complexes used as catalysts or analytical markers. In this review, we describe various applications of immobilized peptides, including selective capturing of cysteine-containing peptides, tagging of the carbonyl compounds to increase the sensitivity of their detection, enrichment of biological samples in deoxyfructosylated peptides, and fishing out of tyrosine–containing peptides by the formation of azo bond. Moreover, the use of the one-bead-one-compound peptide library for the analysis of substrate specificity and activity of caspases is described. Furthermore, the evolution of immobilization from the solid support used in peptide synthesis to nanocarriers is presented. Taken together, the examples presented here demonstrate immobilized peptides as a multifunctional tool, which can be successfully used to solve multiple analytical problems.

Keywords: immobilized peptides; peptide conjugates; sample enrichment; receptors; mass spectrometry; capturing; enzyme specificity

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1. Introduction

A chemist needs a whole toolbox of various methods and procedures to perform the synthetic and analytical tasks they may be called upon to provide. There are many ways to perform the majority of tasks and many tools are available which can be utilized to solve analytical and synthetic problems. Peptides are usually associated with the search for biological activity and drug design. However, due to the variety of functional groups, as well as the relative ease of synthesis, peptides actually serve many purposes. In this review, we will show immobilized peptides as a Swiss-army-knife-type tool, which can be successfully used for many applications related to the capture, analysis, and transformation of other compounds.

The example of non-typical role of peptides could be found in the field of proteomics, in which the goal is to identify changes in the proteome that are indicative of pathological states, thus enabling easier and faster diagnosis of various diseases [1]. This is particularly important with respect to cancer and similar diseases, which can progress for a long time

without obvious symptoms, yet there is a large difference in treatment possibilities and outcomes depending on the stage at which the illness was detected.

A number of peptides and proteins has been found to act as biomarkers of specific states of the organism [2,3]. However, the high complexity of the cell and tissue proteome is a serious limitation in the analysis of peptide biomarkers of pathological states of the organism. Currently, the methods of enriching the sample in specific peptides by immobilizing them are intensively investigated. Examples include various ways of capturing cysteine-containing peptides [4], but can be also tailored for enriching samples in post-translationally modified peptides, such as phosphorylated [5], citrullinated [6] or glycated [7] species. In some cases, a simple enrichment is not enough, as the analyzed biomarker is poorly ionizable in mass spectrometry—examples include steroids, such as cholesterol. However, peptides can be used to increase the ionizability of the molecules in question. Attaching the peptide enrichment tag itself can increase the ionizability, since peptides often provide several easily ionizable functional groups. However, this tag can also be tailored to provide a specific analytical enhancer, for example, in the form of a quaternary ammonium salt (QAS) [8].

Other applications of immobilized peptides include utilizing them to test the specificity of proteolytic enzymes. To perform a screening test in which many peptides are analyzed in parallel, combinatorial chemistry methods are used. While not all of them require immobilization, the unique feature of immobilized peptides is the ability to use the one-bead-one-compound (OBOC) strategy. The benefit of this strategy lies in facile identification of the peptide which undergoes proteolytic reaction, usually by the means of mass spectrometry [9].

Peptides can also be used to catalyze chemical reactions. It is possible not only due to a large variety of functional groups, but also to the possibility of forming conjugates of peptides and various other moieties. The examples include attachment of heterocyclic systems, usually involved in the coordination of metal ions, exhibiting catalytic activity [10].

In that way, irrespective of the application, when a peptide is synthesized and immobilized (*veni*), and introduced to the reaction environment (*vidi*), it can perform the necessary job swiftly and efficiently (*vici*).

2. Immobilized Peptides as Multifunctional Tools

2.1. Capturing Cysteine-Containing Peptides

The research on proteomic biomarkers is often limited due to the high complexity of the proteome of studied tissues. To facilitate the analysis, methods of sample enrichment, based on specific peptide functional groups, including the cysteine thiol group, can be developed, to reduce the complexity of the analyzed sample and increase the proteome coverage [4].

Cysteine is a sulfur-containing amino acid. In proteins, this residue can easily be oxidized to form a dimer containing a disulfide bridge between two cysteines, strengthening the tertiary and quaternary structures of the protein and making it more resistant to thermal denaturation [11]. Furthermore, the sulfhydryl side chain of cysteine is a strong metal binder responsible for holding metals in place. It was assessed that 91% of the known human proteins contain at least one cysteine residue. Furthermore, the cysteine residue was present in more than 24% of the predicted tryptic peptides [4]. The ubiquitous cysteines are attractive targets for the chemoselective capture. The functional group of cysteine is redox-active and has highly nucleophilic properties, due to the large atomic radius of the sulfur atom and the low dissociation energy of the thiol S-H bond [12]. This makes the cysteine residue an easily modified target for electrophilic reagents and thiol-disulfide reagents [4].

Different strategies for cysteine-containing peptide capture, including the application of thiopropyl sepharose [13] or thio-Michael addition to the maleimide derivatives [14] have been developed and applied in the immobilization of biomolecules on stationary phases [15,16]. In addition, a tag that selectively alkylates reduced cysteine thiols and

enhances chromatographic separation was applied to capture cysteine-containing peptides [17]. Another strategy was based on the derivatization of cysteine residue by a quaternary ammonium tag, followed by enzymatic digestion and the use of strong cation exchange chromatography to enrich cysteine-containing peptides [18].

The method of capturing cysteine-containing peptides using thiopropyl sepharose [13] involved the digestion of a protein sample by trypsin and labeling tryptic peptides with ^{16}O or ^{18}O isotopes, followed by the selective capturing by thioprophyl speharose resin. In the last stage of the procedure, the resin-bound samples were treated with dithiotreitol, leading to the cleavage of cysteine-containing peptides (Figure 1). The analysis and quantification were carried out using an accurate mass and time (AMT) tag approach. The proposed method was found to be highly efficient, with no side reactions during the reversible capture and release of thiopeptides. Furthermore, the post-digestion ^{18}O labeling strategy incorporates two atoms of ^{18}O in tryptic peptides, allowing for accurate quantification.

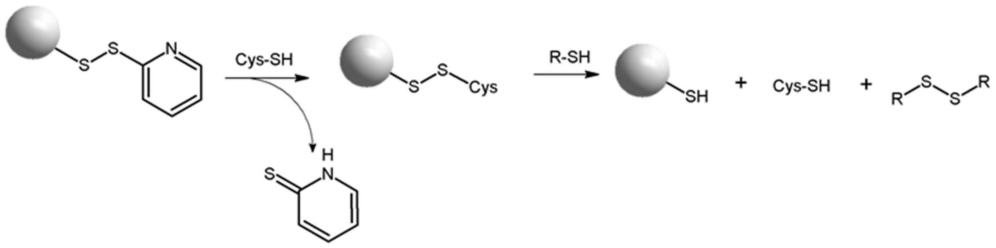


Figure 1. Schematic presentation of cysteine-containing peptide enrichment by Thiopropyl Sepharose 6B. R-SH represents a low molecular weight thiol, such as 1,2-dithiothreitol.

Maleimides have been applied in the modification of the thiol group due to their specificity, lack of side products, and stability of the desired thioether addition product. In addition, it has been reported that succinimide thioethers can readily undergo a reversible addition reaction in solution. Moreover, under physiological conditions, the crossover with other disulfides or thiol groups could occur [14]. Maleimide moieties have been applied for the biomolecular immobilization of proteins and peptides containing a thiol group. However, there were no reports that the commercially available resins used in peptide synthesis, modified with maleimide groups, could be applied in thiopeptide capture. In our recent work, we presented the method of chemoselective fractionation of cysteine-containing peptides using a solid support enrichment. The commercially available TentaGel R RAM resin was modified with 9-aza-3,6,12,15-tetraoxa-10-on-heptadecanoic acid acting as a linker that accommodates the trans-*N*-succinimidyl 4-(maleimidomethyl)cyclohexane-1-carboxylate, resulting in the formation of tio-Michael active site [19]. Subsequently, a model peptide that was a fragment of podocin (a protein with the potential to be a biomarker of preeclampsia) was incubated with a thiol-specific maleimide-functionalized resin.

The reaction was carried out in 0.1 M TEAB (triethylammonium bicarbonate) buffer at room temperature for 1, 3, and 24 h. In the first experiment, the modified resin was incubated with a model peptide, and the results were assessed by mass spectrometry after cleaving the whole construct from the support under acidic conditions (95% TFA) (Figure 2). However, the analysis by tandem mass spectrometry did not allow for the confirmation of the chemical structure of the obtained product due to the low intensity of the signal. To increase the ionization efficiency, a quaternary ammonium tag was used—the 2,4,6-triphenylpyrylium salt (TPP). The captured resin-bound peptide containing lysine with the reactive ε-amino group was tagged with TPP, according to the method described previously [20]. The modification allowed for a 100-fold increase in the intensity of the signals corresponding to the final product.

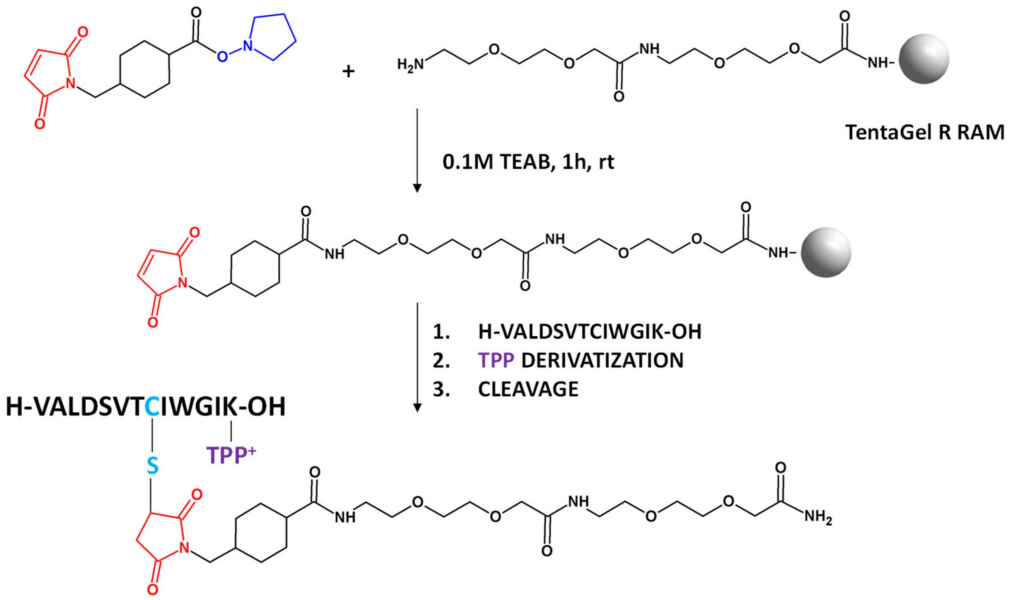


Figure 2. Schematic presentation of the TentaGel R RAM resin preparation, capturing cysteine-containing peptides, and their derivatization by a fixed charge tag in the form of TPP.

The sensitivity of the proposed method was tested using the captured peptide obtained from a single resin bead. Mass spectrometry analysis revealed the expected TPP-peptide with an attached linker, with no traces of other products. The proposed procedure was successfully applied to the more complex samples containing the tryptic digest of podocytes [19].

2.2. Capturing Carbonylated Compounds

The carbonyl moiety can be found in a wide variety of organic compounds. In living organisms, it is present in the form of ketone bodies—compounds primarily derived from fatty acids [21], steroids, including important steroid hormones [22], and α,β-unsaturated aldehydes and γ-ketoaldehydes, with strong neurotoxic properties [23]. A few new psychoactive substances, especially cathinone derivatives, also contain carbonyl. However, their frequently unknown chemical structure, as well as their low concentration in biological matrices pose an analytical challenge [24].

The direct analysis of biological samples to identify carbonyl compounds is extremely difficult due to the presence of the matrix which dominates the samples, while the analytes are present in very small amounts. The solution to this problem is to perform sample derivatization based on the unique property of the carbonyl groups to form a Schiff base. In the literature, the analysis of carbonyl compounds involves phenylhydrazine derivatives [25], biotin hydrazides [26], hydrazides containing quaternary ammonium salts (Girard's reagent) [27], as well as affinity chromatography based on phenylboronic acid derivatives [28]. Undoubtedly, the search for new derivatization reagents and functionalized materials allowing for the selective isolation of specific carbonyl group-containing analytes from the mixture is important.

To facilitate the analysis of carbonyl compounds, including steroids, we have recently developed two protocols of selective sample enrichment.

In our first approach, we merged the unique properties of hydroxylamine derivatives and the advantages of immobilizing reagents on a solid support, creating the functionalized resin derivatized with an aminooxy group that is ready for selective detection of carbonyl unit in peptides or steroids (testosterone derivatives, exemestane, methandrostenolone or methasterone) [29], which combined the enrichment and derivatization procedure of steroids into a single step protocol. For this purpose, we developed a method of synthesis of a functionalized resin (Fmoc-AOA-GRG-CMRR) containing (i) aminooxyacetic acid (AOA),

selectively reacting with compounds containing a carbonyl group, and (ii) an arginine residue to increase the ionization efficiency of the compound during mass spectrometry analysis. The chemoselective selectivity towards testosterone and carbonylated peptides was tested on the hydrolysate of human and bovine serum albumins. The capture efficiency of the functionalized resin with and without the matrix was comparable (70% for carbonyl peptide, 90% for testosterone). The utility of developed functionalized resin was demonstrated for qualitative and quantitative analysis of free testosterone contained in a urine sample using the LC-MS/MRM method. The proposed strategy opens new possibilities for the simple and quick detection and analysis of various ketones, including research on oxidative stress biomarkers and even illegal biological compounds.

The first approach proved the chemoselective reactivity of the designed functionalized resin towards compounds containing carbonyl groups and improved the ionization efficiency during the LC-MS/MS analysis. However, better ionization enhancers, such as quaternary ammonium salts (QAS) are described in the literature [30–34]. One of the methods uses 2,4,6-triphenylpyrylium salt to introduce a permanent positive charge in the form of 2,4,6-triphenylpyridinium salt. Unfortunately, the dissociation of the N-O bond in the hydroxylamine derivative was observed in the presence of a quaternary ammonium group introduced into the linker, which prevents the application of a selective enrichment protocol on this functionalized resin. Therefore, a different method of carbonyl compound derivatization has to be developed that would contain both QAS and a moiety to react with the carbonyl group.

Here, we have designed and prepared an immobilized glutamic acid-related hydrazine reagent, containing QAS, and tested its efficiency on the derivatization of benzaldehyde as a model carbonyl compound. The prepared reagent reacts with benzaldehyde, producing the corresponding hydrazone derivative (Figure 3) [8]. Our current work focuses on testing this reagent on a group of carbonyl compounds and complex environmental matrices.

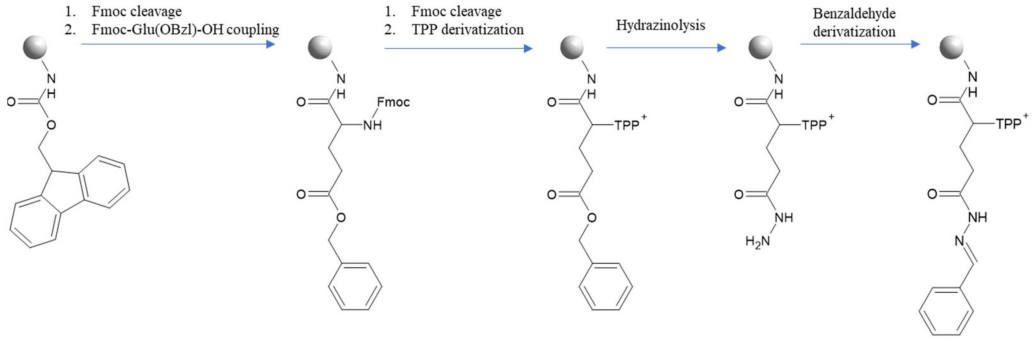


Figure 3. Immobilized glutamic acid-related hydrazine reagent, containing the QAS reaction scheme.

The ability of the hydrazine derivatives to bind to the carbonyl moiety may be used not only for the derivatization of carbonyl compounds. The literature describes the use of hydrazone linker derivatives as drug carriers for controlled release drug delivery systems. The pH-responsive system can lead to the development of a controlled and site-specific drug delivery method, leading to the increased effectiveness and safety of the therapy, while administering the appropriate dose at the target site. The change of the pH at the target site leads to the release of an active compound via the hydrolysis reaction, resulting in a reduction of side effects. The carriers include amino acid hydrazine derivatives, as well as functionalized nanoparticles [35].

In conclusion, the hydrazone-based reagents can be used not only to increase the sensitivity of carbonyl compounds detection, but also in modern drug delivery methods.

2.3. Boronate Affinity Separation for Glycoconjugates Capture

Glucoconjugates are a large family of compounds that can be classified depending on the sugar moiety attached to the amino acid side chains. Glycosylation is an enzyme-

directed site-specific process, as opposed to the non-enzymatic chemical reaction of glycation. On the contrary, glycans are predominantly N-linked to asparagine or O-linked to serine and threonine [36]. In addition, early glycation products are formed as a result of the rearrangement of Amadori products, where the deoxyfructosyl moiety is attached to the lysine side chain (N-linkage) [37]. Protein glycosylation plays an important role in many cellular processes, such as cell adhesion, receptor activation, and signal transduction [38], while the glycated compounds are correlated with diabetes mellitus [39], ageing [40] or neurodegenerative diseases [38]. Therefore, the analysis of the proteins' glycostructures and concentration levels is important in diagnosis and proteomics [41–43]. Despite the significant development of mass spectrometry coupled with liquid chromatography (LC-MS) and bioinformatics methods, the determination and characterization of glycoproteins is severely affected by the presence of non-glycoproteins, due to the inherent low abundance of the former in complex biological samples. Therefore, efficient methods for isolation, enrichment, and recognition of glycoproteins are indispensable. The implementation of this task requires the design and synthesis of support that can selectively interact with the selected compounds, as well as an optimization of the detection procedure, in order to eliminate the matrix effect (interference effect), hampering the analysis of substances in the presence of other chemical compounds. The use of sample enrichment methods increases the chance of identifying trace amounts of new compounds that may enable early identification of pathological changes by detecting molecular biomarkers of specific pathological states of the organism [42].

Glycoproteins can be captured in a variety of ways, such as lectin affinity chromatography [44], solid-phase hydrazide capture [45], and hydrophilic interaction chromatography (HILIC) [46]. In recent years, boronate affinity materials (BAMs) have been studied and optimized to enable various promising applications for the selective enrichment of compounds containing *cis*-diols [47,48]. The binding to BAMs is pH-controlled, covalent, and reversible, making this method perfect for the capture and recognition, as well as the labeling and assays of *cis*-diol containing molecules, including catechols [49], nucleosides, nucleotides [50], and glycoproteins [45,51,52] (Figure 4). The *cis*-diols interact with boronate ions in alkaline conditions, which may be inconvenient in the case of labile compounds. Therefore, there was a need to develop ligands capable of binding *cis*-diols at lower pH [53] containing, for example, electron-withdrawing groups [54], Wulff-type boronic acids with intramolecular B-N or B-O bonds [55], and heterocyclic rings [56].

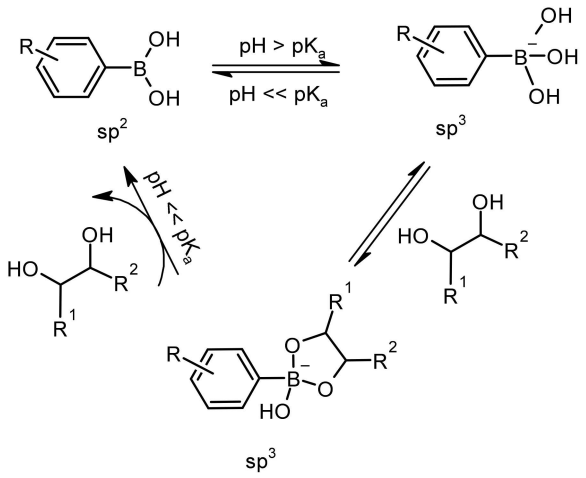


Figure 4. Interactions between the phenylboronic acid derivative and compounds containing cis-diol groups (scheme based on the work of Liu et al. [47]).

Many ligands with boronic acid groups immobilized on monoliths [56,57], magnetic particles [58], mesoporous silica [59], and gold nanoparticles [60] have been investigated for enrichment of the glycosylated peptides flexible materials with multiple attachment

points, for example, dendrimers and polyethyleneimines (PEIs) have been used to improve the binding capacity of BAMs [61–63]. The 3-carboxybenzoboroxole (3-CBB) functionalized monolithic columns [64] and MNPs-dendrimers [63] were reported as efficient glycopeptide enrichment tools for the analysis of tryptic HRP digest [64] and yeast, human cells, and mouse brain tissues [63], with sensitivity towards both *N*-glycopeptides and *O*-glycopeptides. Moreover, BAMs based on metal organic frameworks (MOFs) [65] and covalent organic frameworks (COFs) [66] have also been used for glycopeptides enrichment.

While new methods for the preparation of samples of glycosylated peptides are extensively studied, in the case of glycated peptides the commercial *m*-aminophenylboronic acid-Agarose [45,51] is most frequently used, even though this reagent was developed for capturing nucleotides [67] and adopted for affinity towards glycation products. The qualitative and quantitative analysis of glycated peptides in plasma samples was employed in the search for biomarkers that may allow for the early diagnosis of type 2 diabetes mellitus [45]. In our recent paper, we reported the affinity and selective binding of glycated peptides in complex peptide mixtures, obtained from hydrolysis of human and bovine serum albumin by a novel ChemMatrix® Rink resin functionalized with two phenylboronate (PhB) moieties attached to the *N*-α and *N*-ε amino groups of a lysine residue (Figure 5) [7]. The innovative approach was based on one solid support to both synthesize a linker containing phenylboronic acid derivatives and carry out an efficient procedure for the enrichment of a biological sample. Moreover, the use of the ChemMatrix® resin as solid support allows for the selective enrichment procedure both in aqueous and organic solvents, and the release of the linker from the solid support according to the standard procedure and its characterization by various analytical methods, in order to determine the loading of the functionalized resin.

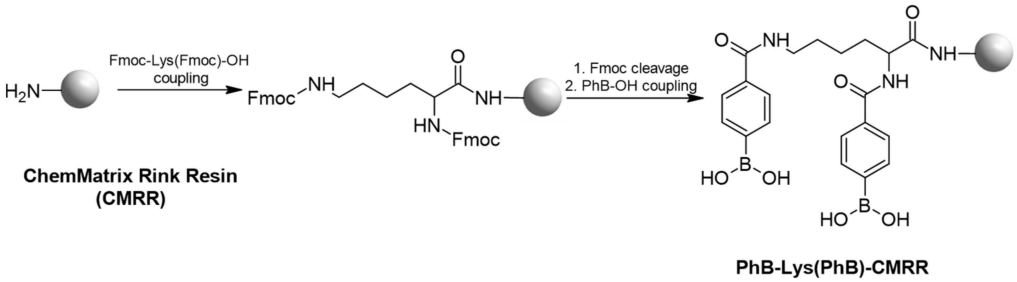


Figure 5. Synthesis of the functionalized resin PhB-Lys(PhB)-ChemMatrix® Rink (scheme based on the work of Kijewska et al. [7]).

Without a doubt, enrichment methods based on affinity or other specific interactions are fundamental phenomena constantly inspiring novel ideas that may eventually lead to the essential breakthrough in the search for disease biomarkers.

2.4. Capturing Tyrosine-Containing Peptides by Diazonium Salts

Coupling of diazonium derivatives is a technique that has been exploited for more than 100 years [68]. It is an electrophilic substitution reaction between aryldiazonium cations (acting as electrophiles) and strongly activated aromatic systems (phenols or anilines). The products of coupling reaction are colorful azo compounds (R_1–N=N–R_2), which are widely used as azo dyes and pigments in the textile, food, and cosmetics industries, and as therapeutic agents [69–71]. Currently, researchers are screening the aromatic azo compounds for their potential biomedical use, including cancer diagnosis and therapy [72]. The high reactivity of these reagents makes them good candidates for protein labeling, especially in the case of peptides containing a tyrosine residue. Azo compounds can be introduced into peptides and act as a linker [73], may form the azo bridge for the stabilization of biologically active conformation [74] or may coat an active peptide [75].

Tyrosine is a particularly interesting amino acid for the chemoselective modification of peptides and proteins due to its low content, accounting for only 3% of the total amino

acid content in proteins [76]. The modification of peptides containing tyrosine residue has been the subject of numerous studies. One of the methods of selective bioconjugation for the directed modification of tyrosine residues is the azo-coupling chemistry with aryldiazonium salts [77]. This kind of azo coupling found preliminary applications in protein modifications and the development of peptide diagnostics [78,79]. The significant disadvantage of this method is its poor selectivity observed in conjugation reactions. Therefore, despite numerous studies, the design of azo structures with controllable and predetermined biological and chemical properties is an important research target.

We investigated a new approach to peptide modification using the azo compounds, obtained by standard solid phase synthesis. The immobilized peptide linker modified with a diazonium salt (electrophile) was coupled with a model phenol. The reaction was performed on solid support, and the diazotization and coupling steps were carried out in an aqueous medium (Figure 6). The choice of resin was crucial, since according to the standard solid phase synthesis protocols, peptides are synthesized in organic solvent. Therefore, it was necessary to use a resin compatible with aqueous and organic solvents, and we decided on TentaGel R Ram-Resin [80].

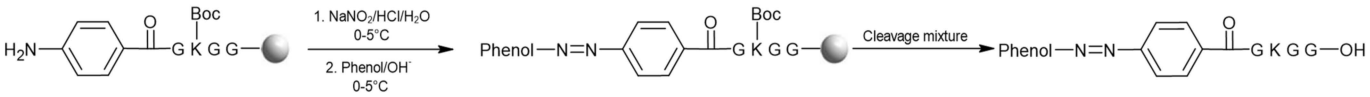


Figure 6. Scheme of the synthesis of azo derivatives used for reactions with phenols.

We have obtained the derivatives shown in Figure 7. Our preliminary studies show that the method may be an effective method for immobilizing tyrosine and for the selection and determination of peptides containing tyrosine residues. The colored products formed as a result of the coupling reaction make it possible to quickly assess the presence of phenols/tyrosine in the sample.

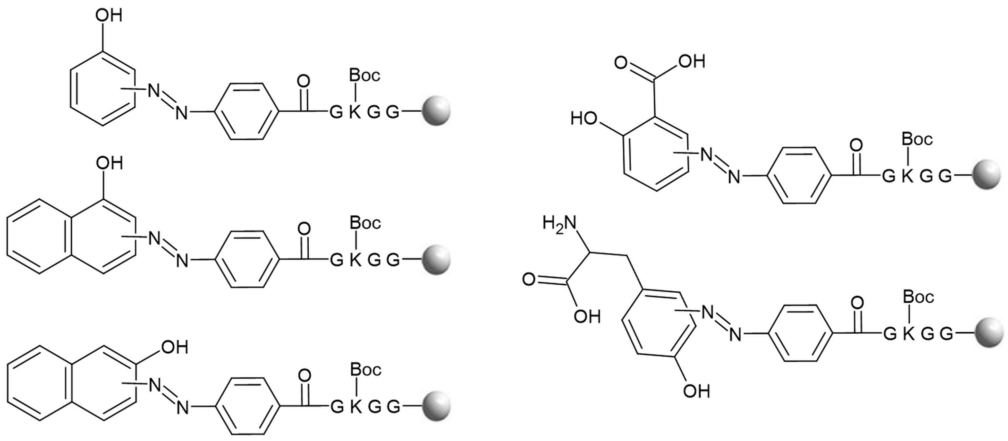


Figure 7. Structures of azo derivatives obtained during the synthesis on a solid support.

We decided to use lysine in the peptide linker to test the proteolytic stability of solid-supported and free azo derivatives against trypsin. The obtained results showed that azo compounds are characterized by high resistance to enzymatic degradation. In the case of immobilized compounds, we did not observe any degradation products even after 2 weeks of incubation with the enzyme. Moreover, the colored products obtained after cleavage from resin were characterized by high stability in aqueous solutions—incubation at 40 °C for 30 days did not cause their decomposition. The obtained results correspond to literature reports [81].

Azo compounds are extremely stable, and their resistance to degradation is a significant problem from a biological point of view. The metabolism of azo compounds by

intestinal bacteria is one of the most extensively studied bacterial metabolic processes [82]. This is particularly important since it can be the key to designing prodrugs, which, when released, may act locally in the colon or be available for possible absorption into the blood.

In conclusion, our preliminary study shows that the method may be used to detect peptides containing tyrosine residue or in drug delivery methods.

2.5. Enzyme Specificity and Activity Analysis

Proteolytic enzymes play a key role in signalling and metabolic pathways, with infection, inflammation, and apoptosis, as typical examples. The interference in the proteolytic degradation of the peptides and proteins may result in the maladaptation [83]. Therefore, the activity profile of selected proteases may serve as a specific biomarker for various health disorders. The investigation of these activities requires specific substrates and inhibitors for the analysis of enzyme kinetics [84,85]. Among the analytical methods, used in proteolytic enzyme activity studies, the fluorescence-based solutions are usually used, including the application of 7-amino-4-carbamoylmethylcoumarin (ACC). This fluorogenic agent is compatible with the classical solid-support peptide synthesis and could be introduced into peptide as the *C*-terminal residue [86]. When the amide bond between the last regular amino acid residue and ACC is hydrolyzed, the release of free ACC causes a steep ~900-fold increase in fluorescence intensity. The ACC-containing combinatorial peptide libraries were successfully applied in the analysis of substrate specificity of different endopeptidases, including caspases, neutrophil serine proteases, matrix metalloproteinases (MMPs), and cysteine cathepsins [87–89]. The Förster resonance energy transfer (FRET) substrates and internally quenched fluorescent (IQF) peptide substrates form powerful tools for the efficient analysis of endopeptidases [90]. The most commonly used IQF/FRET substrate pairs (donor/quencher) include Edans-Dabcyl, ABz-Tyr(NO_2) [91], ABz-EDDNP [92], Trp-Dansyl [93], and 7-methoxy-coumarin-4-yl acetic acid-2,4-dinitrophenyl-lysine (MCA-Lys(DNP)) [94]. Recently, Drag et al. developed the new IQF pair composed of ACC and Lys(DNP), for the substrate specificity analysis of caspases, elastase, legumain, MMP-2 and MMP-9, and trypsin endopeptidases [95]. Representative examples of protease sensors are presented in Table 1.

Table 1. Peptide-based protease sensors. ICE: Interleukin 1 beta converting enzyme; DNS: 5-(Dimethylamino) naphthalenesulfonamide (DANSYL); DABCYL: 4-(4-*N*,*N*-dimethylaminophenyl)azobenzoic acid; FITC: Fluorescein isothiocyanate; 1,5AEDANS: 5-((2-Amino-ehyl)amino)-naphthalene-1-sulfonic acid; PPE: Poly(phenyleneethynylene); DNPED: *N*-2,4-dinitrophenyl ethylenediamine; SWCNHs: Single-walled carbonnanohorns; DMOAP: *N*,*N*-dimethyl-n-octadecyl-3-aminopropyltrimethoxysilyl chloride.

Analyte	Interaction Mode	Signal Output	Strategy	Signal Marker 1	Signal Marker 2	Reference
Trypsin Chymotrypsin	Cleavage	Mass spectrometry	Lanthanide-Code	Lanthanide ions		[96]
Papain Cathepsin B	Cleavage	Fluorescence	Energy transfer	Benzoxazol-5-yl-alanine derivatives	Tyr NO_2	[97]
AspN Endoproteinase Chymotrypsin	Cleavage	Fluorescence	Self-quenching	Cy-5 or Fluorescein		[98]
MMP-2 and MMP-9	Cleavage	Fluorescence	Self-quenching	Dye Ls276		[99]
Aspartyl Proteinases	Cleavage	Fluorescence	Quenching	*o*-aminobenzoyl	DNPED	[100]
Renin	Cleavage	Fluorescence	Quenching	1,5AEDANS	DABCYL	[101]
ICE	Cleavage	Fluorescence	Quenching	1,5AEDANS	DABCYL	[102]
Cytomegalovirus protease	Cleavage	Fluorescence	Quenching	EDANS	DABCYL	[103]
Neprilysin and insulin-degrading enzyme	Cleavage	Fluorescence	Quenching	Alexa-350	DABCYL	[104]
Secretase and caspases	Cleavage	Fluorescence	Quenching	PPE	QSY-7	[105]
Trypsin, chymotrypsin, proteinase K, and thermolys	Cleavage	Fluorescence	Quenching	FITC	Gold nanoparticles	[106]
PSA	Cleavage	Fluorescence	Quenching	FITC	Gold nanoparticles	[107]
Chymotrypsin Trypsin Thrombine Human neutrophil elastase Granzyme B	Cleavage	Fluorescence	Quenching	ACC		[41,86]
Thermolysine Chymotrypsin	Cleavage	Fluorescence		DANS		[108]
Lipases Esterases	Cleavage	Fluorescene	Quenching	AMC		[109]
Synchronous detection of trypsin and chymotrypsin	Cleavage	Fluorescence	Quenching	DEAC Rhodamine B		[110]
Trypsin Chymotrypsin	Cleavage	Fluorescence	Quenching	DMOAP-coated glass slides		[111]
Caspase-3	Cleavage	Fluorescence	Quenching	FAM	Graphene oxide	[112]
Chymotrypsin and MMP-2	Cleavage	Fluorescence	Quenching	Metalloprotoporp hyrins, QXL $_{570}$	Graphene oxide	[113]
Thrombin	Cleavage	Fluorescence	Quenching	FAM	SWCNHs	[114]

Table 1. *Cont.*

Analyte	Interaction Mode	Signal Output	Strategy	Signal Marker 1	Signal Marker 2	Reference
Trypsin, chymotrypsin, V8 protease, plasmin, thrombin, and pepsin	Cleavage	Fluorescence	FRET	Mca-fluorophore	Dinitrophenyl	[113]
Prokaryotic enzyme	Cleavage	Fluorescence	FRET	FITC	Dabcyl (Dbc)	[115]
MMP-1 and MMP-9	Cleavage	Fluorescence	FRET	5FAM	Cy5	[116]
HIV protease	Cleavage	Fluorescence	Quenching	EDANS	DABCYL	[117]
MMP-7	Cleavage	NIR fluorescence	FRET	Cy5.5	NIR8Q20	[118]
Thrombin	Cleavage	NIR fluorescence	Self-quenching	Cy 5.5		[119]
Tumor protease	Cleavage	NIR fluorescence	Self-quenching	Cy 5.5		[120]
PSA	Cleavage	Current	Electrochemical	Ferrocene		[121]
Trypsin and α-Thrombin	Cleavage	Current	Electrochemical	Ferrocene		[122]
MMP-7	Cleavage	Color density	Silver enhancement on Au particle	Au particle		[123]
PSA	Cleavage	Luminescence	Electrogenerated luminescence	Ru (II) chelate		[124]
PSA	Cleavage	Luminescence	Electrogenerated luminescence	Ru (II) chelate	Ferrocene	[125]
Trypsin Chymotrypsin Caspases	Cleavage	Mass spectrometry	Fixed-charge tag-coded	Fixed-charge tagged ions		[126–129]

However, all of the aforementioned developments described the substrate specificity or enzyme activity analysis using a peptide or protein in the solution, while the commonly used one-bead-one-compound (OBOC) peptide combinatorial libraries include the on-bead enzymatic digestion. This approach makes it possible for the fast and efficient screening of large sets of candidate compounds for the expected biological activity [128,129]. Nowadays, electrospray ionization mass spectrometry (ESI-MS) is preferred for the analysis of compounds obtained from single beads of the resin. However, the main limitation of this method is the trace amount of compound cleaved from a single resin bead (femtomolar level), which is usually not sufficient for the reliable peptide sequencing. Recently, we developed the method of fixed charge tag derivatization of peptides by quaternary ammonium (QA) group allowing ultrasensitive ESI-MS/MS peptide analysis even from the single resin bead of OBOC library [126,127]. The described method is based on the application of both linear and bicyclic quaternary ammonium groups as ionization enhancers, located at a properly designed linker connecting the peptide with the resin. The applicability of the proposed method in OBOC chemistry was confirmed with the model peptide library of α-chymotrypsin substrates.

The application of a fixed charge tag results in a considerable increase in the ionizability of the analysed compounds in ESI-MS experiments, making it possible for the identification of the compounds at attomolar level [9,130], despite the fact that the identification of peptide fragment released to the supernatant after hydrolysis of the peptide bond that occurs on the resin is generally characterized by a low yield [131]. The strategy was examined in the investigation of caspase activity using model peptides with sequences DEV**D**/G, DEV**E**/G, and DEV**A**/G, prepared on the TentaGel HL-NH_2 solid support. The linker designed for this study consisted of methionine residue, allowing the cleavage of the peptide by CNBr, a spacer in the form of βAla residue, and Gly, which is known as the most recognized residue for caspases at position P1. It was found that both DEV**D**/G and DEV**E**/G sequences are recognized and cleaved by caspase 3 and 7. However, the DEV**E**/G sequence is hydrolyzed very poorly [95]. Therefore, this sequence was used to analyze the sensitivity of the on-bead monitoring of the activity of caspases.

The synthesized model peptides were derivatized at the *N*-terminus by the bicyclic quaternary ammonium group, which served as an ionization enhancer. In addition, the resulting peptidyl resin was applied in the analysis of the specificity and activity of caspases 3 and 7. The proteolysis of the amide bond on the resin, occurring even with low efficiency, results in the release of the peptide fragment modified with a fixed charge tag. Ultrasensitive LC-ESI-MS/MS analysis of the supernatant produces the desired sequence even after the enzymatic digestion is performed on a single peptidyl resin bead (Figure 8). In the presented case, we found that the progress of proteolysis of the resin bound QA-DEV**D**/G model peptide after 5 min was sufficient for its identification in the supernatant, whereas the QA-DEV**E**/G model peptide required a longer hydrolysis time (15 min). The DEV**E**/G sequence was previously reported to be poorly recognized by caspases 3 and 7, which is the reason for the presence of a very small amount of proteolysis product in the supernatant. Additionally, the signal corresponding to the QA-DEV**A**/G sequence hydrolysis product was not observed, as expected. The developed strategy was applied to the model substrates of caspase 3 and 7. The obtained results confirm substrate specificity of executioner caspases, which suggests the applicability of the proposed methodology in the analysis of on-bead substrate specificity and enzyme activity.

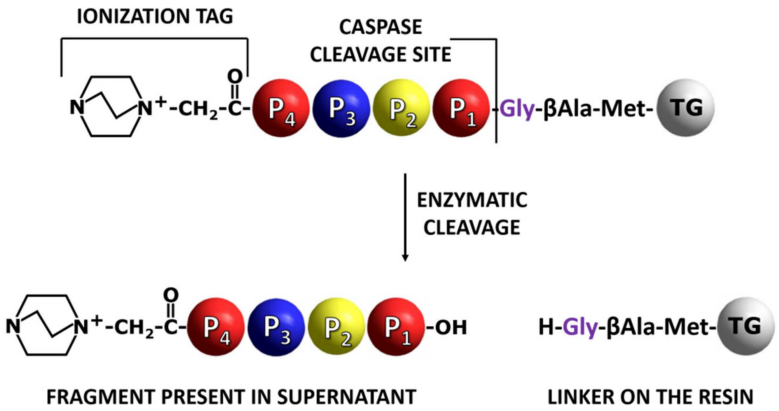


Figure 8. Schematic representation of the on-bead peptide proteolysis analysis using a resin-bound peptide modified by the ionization tag. TG: TentaGel resin.

2.6. Immobilized Heterocyclic Peptide Conjugates as Ligands

The affinity of peptides towards metal ions is a well-known and broadly studied phenomenon [132]. The complexation of metal ions may involve peptide-based ligands formed directly from unmodified peptide sequences [133–135] resembling metalloenzyme active centers [136] or structural element-like zinc fingers [137], peptides containing nonproteinaceous amino acids or modified by the attachment of high affinity motifs to functional groups in peptide side chains [138]. The organization of peptide primary and secondary structures offers a unique opportunity to arrange and orient coordination spheres for metal ions [139].

The solid-supported synthesis of peptides allows not only for a quick and efficient assembly of peptide chains, there are also multiple procedures for the on-resin modification of peptides directed at the formation of heterocyclic structures (Figure 9A,B) [140,141]. The relatively easy method for producing novel arrangements of structural features was explored in the design and synthesis of combinatorial libraries aimed at optimizing the complex stability [142–144]. The distribution of functional groups in peptide scaffold allows not only for the *N*-terminal and *C*-terminal tethering of the ligand moiety, but also for the attachment to specific side chains using the existing functionalities or the application of nonproteinaceous amino acids, including the formation of pincer structures [145–148].

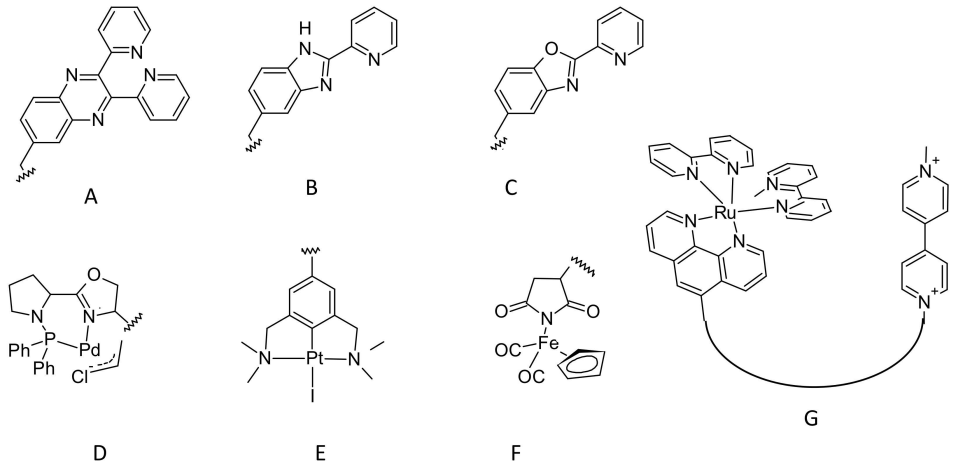


Figure 9. Selected examples of structures discussed in the text. Nonproteinaceous amino acid heterocyclic side chains: Substituted quinoxaline (**A**), benzimidazole (**B**), and benzoxazole (**C**). Coordination motives: Phosphine-oxazoline palladium complex (**D**), terdentate, monoanionic pincer platinum(II) complex (**E**), cyclopentadienyl iron dicarbonyl unit (**F**). Fluorophore ruthenium(II) bis-bipyridine-phenanthroline and methyl viologen quencher set (**G**).

The available functional groups, combined with specific linkers and reagents, offer different levels of accessibility and stability of conjugation. The arrangement of amino acids in peptide chain could be used to investigate the effect of spacing. A structurally well-defined environment of oligoproline was used in a study on chromophore and electron transfer donor assemblies for light harvesting [149] or water oxidation [150].

Although solid phase peptide synthesis (SPPS) and solid phase organic chemistry (SPOC) are methods of choice for the preparation of peptides and peptide conjugates, in most of the cases, the formation of coordination compounds occurs in the solution, facilitating the monitoring of the complex formation and analysis of final products, including advanced fluorescence studies of some bidentate chemosensors based on the benzoxazol-5-yl-alanine (Figure 9C) [151,152]. For special purposes, the attachment of an already formed complex motif to the peptide assembled on solid support could be performed or the reaction with metal ions carried out with an immobilized peptide [153]. There are reports that the presence of metal complex may cause side reactions, including the non-standard cleavage of the Rink amide linker [154].

The development of water-compatible solid supports facilitates complexation reactions. However, the main drawback of these procedures concerns the analytical methods. The complexes formed on the resin could be cleaved and analyzed by standard procedures. However, there is no guarantee that the coordination moiety would stay intact. The study of resin-bound complexes could be performed using Raman spectroscopy and other surface-directed methods, although with a significant interference from the solid support [155].

The immobilized complexes are used as catalysts and analytical markers, taking advantage of the variability of amino acid derivatives, well-developed solid phase peptide synthesis procedures, and modular conjugate structures with multiple attachment points.

Solid-phase 2-oxazoline formation was applied in the synthesis of the on-resin phosphine-oxazoline palladium complexes (Figure 9D) for symmetric allylic substitution [10]. Palladacycle- and iridicycle-based amino acids were used in regular peptide synthesis, leading to hybrid catalysts [156]. Of note, cyclic peptide scaffolds bearing copper(II)-binding side-chains could also be used, as presented in a recent report on their activity in the Friedel-Crafts/conjugate addition [157]. The concept of asymmetric catalysis mediated by organometallic peptide derivatives was recently reviewed by Metrano et al. [158].

Peptide-based metal complexes are also used as markers in solid-phase screening assays. Organoplatinum(II) complexes attached to peptides synthesized on PEGA resin were reported as labeling agents (Figure 9E), with detection based on a colorimetric reaction with I_2, whereas the photolabile linker was used to facilitate MALDI-TOF product confirmation [159]. Carbonyl metalloimmunoassay (CMIA), an immunoassay method based on transition metal-carbonyl reporter groups (Figure 9F) and Fourier transform infrared (FTIR) spectroscopy as the detection method [155] is another example of the application of metal bioconjugation for analytical purposes.

In general, recent reports indicate a growing interest in nanostructural forms of metallopeptides. The self-assembly of peptides to form higher order structures is often facilitated by the coordination to metal ions [160]. Rather than bidentate ligands, which are often difficult to synthesize, monodentate aromatic ligands could be applied, arranged by location on the peptide chain and stabilized by hydrogen bond networks [158]. A pH-dependent formation of a core–shell nanoparticle–peptide@metal–organic framework was reported as a dual-recognition switch for monitoring the lysosomal cathepsin B activity [161]. A combination of IHIHIQI peptide with a cysteine–containing spacer and polar stabilizing fragment enabled immobilization of the resulting peptide on gold nanoparticles, and the resulting β-sheet dimers formed a three histidine zinc(II) recognition unit with carbonic anhydrase activity [162]. Polyferrocenylsilane block copolymers were conjugated with polypeptides to form self-assembling elliptical micelles [163].

Peptide-based catalytic units are deposited on electrode surfaces for catalytic reactions [150] or ultrasensitive detection [164]. A biohybrid catalyst formed as a biofilm on gold electrode by a modified metalloprotein with a Grubs-Hoveyda ruthenium unit was

successfully used in ring-closing metathesis in neat substrates [165], whereas a hybrid soft solar cell with bacterial channel protein and ruthenium(II) aminophenanthroline–viologen diads, was deposited on TiO_2 electrode [166]. The ultrasensitive detection of proteins was studied using a photoelectrochemical biosensor, with peptides immobilized on $CdTe/TiO_2$, and CuS nanocrystals providing amplification after selective cleavage [167], whereas a binding-induced emission resulted from the application of fluorophore-quencher pair attached to a specific peptide (Figure 9G) [168].

3. Discussion: Peptide Biosensors—Advantages and Applications

In this review, we concentrated on our recent research to develop materials functionalized with different functional groups, in order to effectively enrich the sample into the selected analytes [7,8,19,29,80]. Despite the significant development of mass spectrometry coupled with liquid chromatography (LC-MS) and bioinformatics methods, the analysis of biological samples, in particular, the search and identification of new biomarkers present in trace amounts, is still a challenge [11,28,42,43].

We focused on the selective enrichment and detection of biologically significant compounds that could be used as the markers of diseases, such as diabetes [7,43] or preeclampsia [19]. We applied commercially available solid supports (TentaGel R RAM, ChemMatrix Rink) as the starting materials, which were further functionalized with appropriate functional groups and linkers. The innovative approach involves the use of only one solid support for the synthesis of a linker containing the reactive group according to the Fmoc synthesis protocol, an efficient procedure for the concentration of a sample combined with the derivatization reaction to increase the sensitivity of the analysis, and ultimately the release of products from the resin and LC-MS and bioinformatics analysis.

We presented alternative methods of selective enrichment of compounds based on (i) reversible reactions, in which the compounds were released from the functionalized support in an unchanged form, such as deoxyfructosylated peptides [7] and (ii) irreversible reactions, in which the selectively bound analytes were released from the functionalized resin with an appropriate linker improving ionization properties during the LC-MS/MS analysis, such as carbonylated peptides [29], other compounds containing carbonyl group [8,29], peptides containing the SH group [19], and peptides containing the Tyr residue in sequence [80]. Moreover, the presented protocol may also be used for confirming the substrate specificity of executioner caspases, which suggests the applicability of the proposed methodology in the analysis of the on-bead substrate specificity and enzyme activity [169].

In this review, we present our research which is aimed towards the enrichment of the selected products in biological/clinical samples. However, the proposed procedure is more general and could be applied to various molecules present in complicated mixtures.

The solid phase peptide synthesis and solid supports offer various advantages in further applications of immobilized peptides and their conjugates, including selective enrichment, detection, catalysis, and light capture. The advantages include well-developed modular synthetic methods, wide range of support compatible with a large variety of solvents, including water, high chemical stability of support, and optimal swelling and mechanical properties. The combinatorial strategies and small scale synthesis are perfect for the creation and optimization of any type of peptide sequence to adjust the target requirements. However, the heterogeneity of solid support affects the access to the immobilized peptides and limits the available analytical methods for product monitoring.

Recent reports indicate that the answer to the disadvantages problem seems to involve replacing a typical synthetic support by various nanoparticles. The decoration of nanoparticles involves an additional step—immobilization of previously synthesized peptides, although this drawback is compensated by better dispersion and the added value of spectral or magnetic properties of nanoparticles, explored in the design of several sensors. A number of research results suggest the possibility of the practical application of peptide–nanoparticle conjugates in various areas of life, such as biology, chemistry, agriculture, and medicine [170–173]. The basic functions and properties of peptide functionalized nanopar-

ticles depend upon the physiochemical and biochemical properties of nanoparticles and peptides, which are integrated into peptide nanohybrids.

Analytical applications of nanoparticle-conjugated peptides include the detection of various molecules, from metal ions to proteins. The sample enrichment, very frequently observed in the case of solid-phase immobilization, is limited to the sensors based on surface modification [174], making the combination of capture and derivatization common for both approaches [175]. The magnetic nanoparticles, due to the additional separation feature, could be used in the preparation of electrodes for complex matrices, such as whole blood [176], since immunosensors with multilayer electrodes are gaining interest resulting in several papers with a title "*Recent Advances in Electrochemical Immunosensors*" [177–179].

Loading of different types of peptides onto nanomaterials have been used in the development of imaging nanoprobes in typical analytical chemistry applications, as well as medical techniques including near-infrared, fluorescence imaging, computed tomography, positron emission tomography, and magnetic resonance imaging [180]. In addition, the colorimetric assays based on peptide functionalized gold nanoparticles (AuNPs) were employed to determine the enzyme activity and act as screening inhibitors of enzymes (see Table 1). Moreover, several colorimetric assays based on peptide functionalized AuNPs have been developed for the detection of heavy metal ions, such as: Hg^{2+}, Pb^{2+} or Ni^{2+} [180]. In general, the detection occurs through fluorescence changes related to the peptide motif or colorimetry based on nanoparticle properties [164,181]. The recognition unit was initially based on antibodies [182], but less problematic protein aptamers [183], and, finally, regular peptides [184] are now used. In some cases, due to the competition between the nanoparticle and the analyte for the peptide probe, the spectroscopic or catalytic activities of nanoparticles are revealed to provide a detectable signal [106,183]. Representative examples of analytical sensors based on peptides deposited on nanostructures are presented in Table 2.

Table 2. Peptide-based sensors for non-enzymatic analytes. DOTA: (1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid); 4NP: 4-Nitrophenol; 4AP: 4-Aminophenol.

Analyte	Interaction Mode	Signal Output	Strategy	Signal Marker 1	Reference
H1N1 virus	Hemagglutinin affinity	Reflectance	Nanopillar optics	Refractive index	[174]
H1N1 virus	Peptide affinity	Colorimetry	Conformation shift	Polydiacetylene nanoparticle	[185]
Cyclin A	Cationic probe affinity	Colorimetry	nanoparticle aggregation	Au nanoparticle	[106]
Cyclin A	grove recognition	Fluorescence	intermolecular sensitization	Tb^{3+} chelating macrocycle DOTA/Trp	[186]
Anti-HIV antibodies	Epitope recognition	Fluorescence	Energy transfer	Fluorophore/quencher	[168]
Prostate specific antigen	Epitope recognition	Photoelectrochemistry	Peptide cleavage	Signal amplification	[167]
α-Fetoprotein	Epitope recognition	Electrochemistry	Immunoprecipitation	Amperometric response	[176]
Alzheimer's tau protein	Epitope recognition	Two-photon Rayleigh scattering, Colorimetry	Chromophore shape	λ_{max} shift	[182]
Gonadotropin	Aptamer affinity	Colorimetry	Redox catalysis	4NP/4AP	[183]
Nitropeptides	Chemoprecipitation	Mass spectrometry	Capture and labeling	Mass tag	[175]
Ni ions	Coordination	Resistive pulse sensing	His tag	Translocation velocity	[187]
Zn ions	Aggregation	Colorimetry	Aggregation	Au nanoparticle	[188]
Hg, Cu and Zn ions	Coordination	Fluorescence	FRET	Dansyl/Trp	[189]

However, it is the potential of innovation in the biomedical field that attracts attention to the advanced nanostructural peptide assembly. Nanoscale delivery vehicles may improve the half-life and the stability of antimicrobial peptides [190]. The bioconjugation of coordination modules with peptides, proteins, antibodies, and aptamers is studied in drug delivery systems [132,172].

Nanoparticles have shown their potential to serve as conjugate scaffolds that not only improve the functionality of peptides, but also implement abiotic characteristics, often resulting in synergistic effects. Peptide–nanoparticle conjugates often show good biocompatibility and present a low degree of cytotoxicity. In the case of peptide-conjugated nano-delivery systems (NDS), the augmented flexibility due to the linker allows for a wide selection of drugs, including compounds that are insoluble in water. A relatively low cost of production and high product reproducibility make peptide-conjugated NDS a rational choice and many of these conjugates have been developed for various applications, including therapeutic drug delivery, inhibition of pathogenic biomolecular interactions, molecular imaging, and liquid biopsy [191–193]. Depending on the construction of conjugates, the release of the peptide from the solid surfaces can take place due to physiological changes in the environment, such as temperature or pH or by an enzymatic action, and they can be used in diagnostic systems and mainstream therapeutic as carriers of therapeutic molecules or as therapeutics [171].

The conjugation of peptides to the NDS has been found to provide an added benefit towards the targeted delivery of chemodrugs from the injection site to their intracellular targets in tumorous cells for cancer therapy. The simultaneous delivery of peptide-conjugated NDS and nano probes has shown potential for the diagnosis of the malignant progression of cancer. The peptide functionalized nanoparticles can also be used as a positive tumor-targeting radiation dose enhancer in cancer radiotherapy. A recent paper by Gaurav et al. reviewed the promising nanoparticle-peptide conjugates based on cancer type, their specific receptor, and the conjugated peptide [171].

Antimicrobial peptides (AMPs) are good candidates to overcome antibiotic resistance. They often exhibit low cytotoxicity and broad-spectrum antimicrobial activity against a variety of pathogens. However, a common flaw in the druggability of AMPs is their poor pharmacokinetics. The combination of AMPs with carrier nanoparticles to improve delivery may overcome this problem and enhance their half-life, decreasing the dosage and, reducing production costs and toxicity. Attachment of AMPs to polymers improves AMP solubility, shields peptide constituents from protease degradation, and yields larger conjugates that avoid rapid renal filtration to prolong circulation in the bloodstream. AMP conjugation to neutral hydrophilic polymers can also reduce interactions with eukaryotic cellular membranes, thereby decreasing potential cytotoxic effects against mammalian cells [194–197].

Glutathione (GSH) decoration has been used extensively to deliver nanoparticles, particularly drug-loaded liposomes, across the blood-brain barrier (BBB), e.g., for Alzheimer's disease therapy [193,198]. Another approach to the design of effective amyloid inhibitors was based on hybrids of multiple peptide inhibitors coupled onto gold nanoparticles [199].

Recently, we also reported that peptide-nanoparticle conjugates could contribute to the development of environmentally friendly pest control methods [173]. In our work, we demonstrated that nanodiamonds coupled with *Neb*-colloostatin (SIVPLGLPVPIGPIVVGPR), an insect hemocytotoxic and gonadoinhibitory peptide isolated from the ovaries of the gray flesh fly *Neobellieria bullata*, can penetrate the insect cuticle. In various in vivo bioassays, we showed that this conjugate introduced into the hemocoel of the insect through the cuticle retained specific hemocytotoxic activity and disturbed the cellular and humoral immune responses of all the developmental stages of the *Tenebrio molitor* beetle [200].

The advantages of peptide-conjugated nanoparticles in the in vivo applications combine the benefits from peptide properties, i.e., modular structure, established geometry, facile derivatization, and tailoring to specific targets, with the nanoparticle features, bringing membrane permeability, increased half-life, and bioavailability from a broad range of materials used as carriers, from gold nanoparticles to graphene [201]. This relatively new area of research leaves some important questions still open, mostly concerning long-term toxicity, biodistribution, and environmental impact. Addressing these problems, as well as developing novel nanostructural solutions, is the only path available from well-studied analytical applications to biological effects and diagnostic tools [174,202]. The challenges

waiting on this pathway involve scale and costs of nanoparticle production, the control over the load stability, long-term physiological impact studies, and the validation of analytical nanoparticle systems.

The review of current trends in the application of immobilized peptides in qualitative and quantitative studies of biomarkers offers a novel tool for researchers and clinicians working on the improvement of diagnostic accuracy, and the early diagnosis of disease. The insights into the possible role of peptide-nanoparticle conjugates in analytical studies, engineering, and medical research bring together peptide chemistry and material science that lead to microdevices and nanomaterials.

4. Conclusions

In the design of advanced sensors, peptides offer several advantages: Structural versatility, optical activity, well developed synthetic methods for peptide assembly, and chemical modification. Through their sequences, they act as recognition units for other biomolecules, as well as carriers for specialized functional features. Once the affinity is explored, the reporting properties of peptide conjugates are revealed. The arrangement of functional groups in a peptide scaffold, as well as its decorations create a unique environment, which could serve as a synthetic center for the biohybrid catalyst. The classical synthetic solid support limits the possible applications of peptide sensors due to steric hindrance and solvent incompatibility. However, the evolution towards the nanostructural arrangement of functional peptides, combining selectivity and sensitivity, is visible in recent research trends [164].

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