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Review

Affinity chromatography techniques based on the immobilisation of peptides exhibiting specific binding activity

Cinzia Tozzi*, Laura Anfossi, Gianfranco Giraudi

Department of Analytical Chemistry, University of Turin, Via P. Giuria 5, 10125 Turin, Italy

Abstract

Affinity chromatography is one of the powerful techniques in selective purification and isolation of a great number of compounds. New challenges in scientific research, such as high-throughput systems, isolation procedures that allow to obtain a single substance from a complex matrix in high degree of purity, low costs and wide availability, have led to the discovery of new tailor-made synthetic recognition systems. In this review the design, synthesis, purification and characterisation of peptides with recognition properties are discussed. Applications of peptide ligands are described and analytical tools mentioned.

Keywords: Reviews; Immobilisation; Specific binding capacity; Peptides

Contents

1. Introduction

The development of new therapeutic drugs, the growth of the biotechnology industry and the initial results surrounding the impact of genomics and proteomics are a few examples of fields that will require the development of new analytical and preparative tools. These new approaches should allow the resolution and the characterisation of complex sets of molecule mixtures in a high-throughput mode and the subsequent purification of the target molecule. The second key challenge relates to these new scientific goals in terms of price, scale and quantity by struggling to contain the rising costs of manufacturing. Financial pressures engender a more

* Corresponding author. Fax: +39-011-670-7615.
E-mail address: cinzia.tozzi@unito.it (C. Tozzi).

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cautious approach to adopting high-cost techniques and a general agreement among the scientific community that lowering the number of purification steps and increasing yields at each step by re-thinking the purification processes could be a good way to obtain well-characterised compounds. The choice of a purification strategy is determined by economics, its speed of introduction, effectiveness and robustness. Conventional purification protocols based on precipitation with salts, temperature, pH, high relative molecular mass polymers are now being substituted with highly selective and sophisticated strategies based on affinity chromatography. This technique simulates and exploits natural biological processes such as molecular recognition for the selective purification of a target molecule. In nature, molecular recognition properties were showed by cavities that perfectly matched a part of the substance, which is, in turn, recognised. In affinity chromatography, molecular recognition properties are exploited to prepare an immobilised molecular derivative, which retains the ability to bind specifically to a mobile substance, and which mimics natural behaviour. When such specificity is achieved, powerful separation methods are available for preparative isolation of the desired species, for pre-concentrations of trace compounds, for the purification of analytes from complex mixtures and also as a method of analysis [1–4]. Affinity chromatography is probably one of the best techniques currently able to address key issues in high-throughput procedures. However, despite the fact that affinity methods managed to eliminate steps, increase yields and ease the experimental protocols, they suffer from other problems. The most widely used affinity columns are prepared by exploiting the antibody–antigen interaction [5]; the antibodies are immobilised on the solid-phase and their great affinity for a specific molecule permits them to retain the analyte. Furthermore, heparin, proteins and receptors are, together with antibodies, also commonly employed adsorbents that offer defined selectivity and specificity. These kinds of affinity columns are rather expensive, because these ligands require purification in their own right, can be contaminated, show lot-to-lot variations and can be fragile and costly to produce. These ligand characteristics may cause degradation of the affinity column, shortening column life and in some cases contamination of the end product. Moreover, difficulties are often experienced in translating the efficacy of the ligands identified by laboratory screening assays into that dictated by large scale production, different matrix samples and environmental conditions of application. All these factors have contributed to the widespread opinion that new synthetic systems, which could mimic recognition properties of natural ligands, had to be studied and developed [6,7]. Thus, over the last few decades, scientific research has tried to substitute the classical affinity devices with synthetic ligands [8–11] that combined the selectivity of the natural ones with high capacity, durability and cost-effectiveness of the synthetic systems. The best known examples of synthetic ligands are biomimetic textile dyes [12], developed about 30 years ago. These compounds, including the Cibacron blue F3G-A, contain a triazine scaffold substituted with polyaromatic ring systems with sulphonate or carboxylate functions together with electron withdrawing or donating groups. The triazine dyes are inexpensive chemicals that can be easily synthesised and immobilised onto solid-phases to generate high capacity columns. These immobilised ligands mimic the binding of natural anionic heterocyclic substrates such as nucleic acids, nucleotides, coenzymes and vitamins [13–15]. However, concerns over selectivity, purity, leakage and toxicity limited their use and led to the search for new and improved biomimetic systems.

In attempts to mimic biological ligands, the scientific community has developed tailor-made supramolecular organic structures. Extremely high selectivity is often obtained if, as in nature, a cavity exists that has been shaped to match that of the substance to be embedded in it and with the binding sites in a definite spatial arrangement. In molecular imprinted techniques [16] similar cavities can be created in highly cross-linked organic polymers: in fact complementary shaped polymers can be formed around a molecule that acts as a template, and then this template is removed. An imprint containing reactive groups with binding capacity remains behind in the polymer. After the removal of the template, the cross-linked three-dimensional structure forms remain unchanged by allowing reversible binding with the template molecule. The imprinting of organic polymers is today a promising area that includes many research fields like chromatography [17,18], catalysis [19], and biosensor technology [20]. The disadvantage of the molecular imprinted polymers (MIPs) is the necessity for a great quantity of imprinting molecule with a high degree of purity. This aspect, above all, limits the use of this technique for preparing MIPs imprinted with very toxic molecules, or with compounds that are low in purity or very expensive. Anyway, all artificial systems based on this kind of technique are rigid organic polymers that work successfully in organic solvents, but are far from the natural molecular recognition systems based on protein structures.

Since the introduction of the first random peptide library in 1986 [21], peptide chemistry has witnessed a great upswing in technological progress. The numerous possibilities for research by using synthetic peptides has become better recognised and many variants of the first peptide library have been developed and used successfully for the detection of ligand binding peptides [22–25]. When using the peptide libraries the ligand is given the opportunity to select any compound, which is able to fulfil the three-dimensional requirements for recognition. Random libraries are well suited for the generation and screening of large numbers of random peptides, in some cases the number of synthetic peptides could be smaller, however, they allow optimal flexibility due to the variation of the amino acid level and may therefore be used to focus on a binding motif when the initial binding sequence is available. Moreover, synthetic peptide libraries of limited complexity could be used for the rapid elucidation

of the rule of peptide binding and for the discovery of a high affinity ligand binding sequence. In this sense synthetic peptides have played a key role in our understanding of the regions of protein involved in antibody binding [26–28]. The elaboration of a peptide library prepared by either chemical or biological methods has become a format which is useful for the discovery of peptides with specific recognition properties because of powerful synthesising [29] and screening [30] and is the first step for preparing affinity media by using peptides as small synthetic recognition systems.

Well-designed peptides have great potential as capturing agents because of their varied chemical properties and functional groups, their different physical properties, their well-known and sufficient number of synthetic approaches. Moreover, many amino acids could be easily purchased with a low cost, even if some specific sequences could be obtained only by difficult syntheses that need expensive reagents, above all to prepare a sufficient amount of peptide.

Even if we can read about peptide synthetic works almost daily, the use of the peptides as mimetic reagents or antibody-like systems in affinity chromatography is not so common. This review would like to focus the attention on the strategy to provide new affinity media by exploiting peptide recognition properties. We apologise to some others who have made important contributions that the space available does not allow us to discuss or mention.

2. Design of tailor-made peptides

The choice of the amino acid pattern from which the peptide library is synthesised is very important to reach a ligand with good affinity and selectivity. Selection of an appropriate target site and the design, synthesis and evaluation of a complementary affinity ligand is a semi-rational process, because many unknown factors could be met during the research work. The chemical and physical properties of the target molecule have to be taken into account when we are thinking what the best amino acid sequence to bind it could be. Modern design techniques can greatly improve the rational design of the peptide library by exploiting different knowledge about the structure and the chemical properties of the target molecule and by using advanced computational tools. In this way the design of tailor-made reagents becomes more feasible, powerful, logical and faster. The overall research also involves identifying potential binding sites on the target molecule, such as an active site, solvent-exposed region or a motif on a protein surface or also a site involved in binding a natural complementary ligand. Modern software [31] provides the wherewithal to calculate, visualise, formulate and hypothesise about the interactions between the molecule and the peptide ligand. Knowing interactions between the analyte and proteins could help us to find an amino acid pattern from which we could start to build our peptide library. Sophisticated modelling and three-dimensional visualisation packages (i.e. SWISS-PROT [32], SCOP [33] and UCL [34]) and software for investigating protein–ligand complexes (LIGPLOTS [35]) can help to identify the possible exploiting interactions of the new affinity device. Moreover, automated docking programs (DOCK [36] and LUDI [37]) allow prediction of the structure, mode and free energy of the binding of ligand–protein complexes, while there is also the possibility (HOOK [38]) of generating possible ligands based on chemical and steric characteristics of the protein binding site. Calculating molecular parameters via classical functions or quantum mechanics is often used by exploiting programs such us CHARMM [39], MM2 [40], MM3 [41] and AMBER [42]. In an easier way, modelling software packages can help to determine the most useful amino acid sequence with evaluating energy minimisation, autodocking and homology programs (QUANTA [43], SYBYL [44], MACROMODEL [45], INSIGHT [46], RASMOL [47]).

Less explored but crucially important is the application of library data for the discovery of new substrates and binding partners for the molecule in question. Existing database searching programmes (i.e. BLAST [48]) can scan databases for short peptide sequences within proteins, but a high score requires either an exact match or mismatched positions with closely related residues. Recognition motifs tend to have only some positions that are stringently selective, and some residues with more flexible positions will not always share recognition properties. Thus, searches with single peptide sequences, even if selected from synthetic library or phage studies, can be unsuccessful in identifying downstream ligands. Novel approaches to the use of these databases have been developed by taking advantage of the greater depth of information obtained from the library. An internet-based program [49] enables protein sequence databases to be searched by using matrices derived from peptide library data. These matrices consist of a selectivity value for each amino acid, each of up to 14 residues surrounding a single fixed position. This procedure allows us to take advantage of the large numbers obtained from peptide and phage display libraries. Furthermore, inputting matrices based on our peptide library data, restricting the search by relative molecular mass or by isoelectric point or by using other site motifs are other facilities at our disposal.

Although design studies allow us to define and reduce the numbers in the library with meaningful changes in strategic points to reduce experimental work, in many cases there is inadequate or insufficient information on the interaction between the target molecule and a substrate, inhibitor or binding site. Thus, designing a library from which ligands could be selected involves ensuring that we can find complementary functionality to target residues from the amino acid sequences. The best approach is, of course, to explore as great a number of amino acid combinations as possible. Hence the combinatorial work becomes very large and an intelligent approach may be the investigation of the properties of similar compounds on which computational design

can be exploited. Furthermore, recognition properties of natural sequences towards the target molecule or compounds of its class may be used, such as a reference starting mark to prepare a first exploratory library. From this first level we can identify an amino acid motif which shows greater binding properties than the others; from the selected motif a new level of peptide library may be prepared by exploiting other combinations. The work can be repeated for as long as necessary to identify a ligand with sufficient affinity.

In the end, the design procedures involve some further steps after the selection of the molecule towards which the peptide ligand must be constructed. The first one is modelling the interactions between the target molecule and the probable peptide ligand by using computational tools, taking into account natural and known interactions, considering binding properties of similar compounds. From this study we obtain a focused library from which to screen a first generation of ligand. The second step is to prepare a second level library that allows better recognition of the affinity properties. These steps should be repeated and in some cases refined by new experimental information.

3. Preparation of peptides with the designed amino acid sequence

3.1. Syntheses

The synthesis of peptides has been a challenge to organic chemists since the turn of the century. The early endeavours were stimulated by the emerging theories of protein structure [50]. By the middle of the century, however, the realisation that other biologically important molecules had simpler amino acid sequences increased the stimulus; in the early 1950s the isolation, structure and synthesis of the lactogenic nonapeptide amide hormone oxytocin [51] initiated a new era in both biology and chemistry. Since then the peptide synthesis has become more and more complex, efficient and widespread.

Peptide synthesis can be described as a multistep process consisting of the synthesis of partially protected amino acids, activation of the reactive group, along with coupling and final detachment of any protection group (Fig. 1). Synthetic protocols have been developed in solid-, solution- and liquid-phases and also biological combinatorial synthesis methodologies can be used [52]. All these three approaches

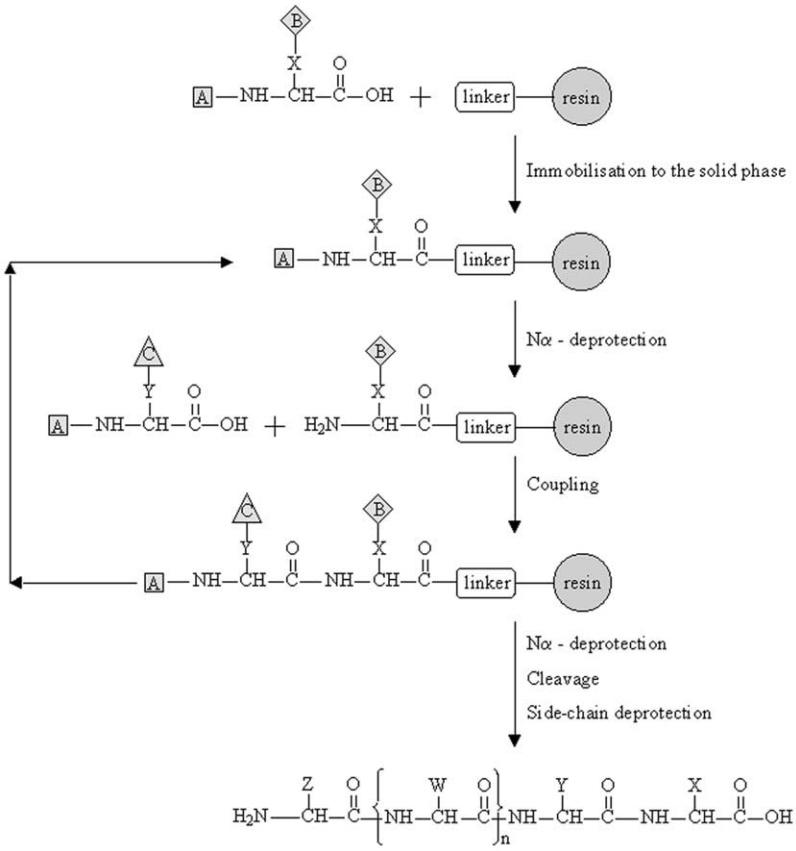

Fig. 1. Solid-phase peptide synthesis. The first Nα-protected amino acid is immobilised to the solid support (resin) via a spacer arm moiety (linker). The Nα-protecting group is removed (deprotection step) and so the subsequent amino acid can be coupled to the first amino acid. These two steps (deprotection/coupling) can be repeated until synthesising the desired amino acid sequence. Finally, the peptide is cleaved to obtain the free peptide and ideally the cleavage step should remove all the amino acid side-chain-protecting groups.

have advantages and disadvantages and precise evaluation of positive and negative aspects should be done before starting with the synthesis of the peptide—above all in our case where also large libraries have to be prepared. Solution- [53] and liquid-phase [54] synthesis can be a good choice for small libraries because the number of purification and separation steps are reduced and the technology is normally less expensive. Even if these approaches are feasible, the binding properties showed by the synthesised compounds might not be conserved when they are immobilised on a solid support. Soluble ligands could lose their binding properties when a complex three-dimensional matrix environment modifies their chemistry, geometry and steric hindrance. Hence, it is preferable to prepare the peptide library by following the solid-phase protocols.

Solid-phase peptide synthesis (SPPS) was developed for the first time in 1963 by Merrifield [55] who published his first work on solid-phase synthesis to overcome the restriction of peptide synthesis in solution. Essentially, an Nα-derived amino acid is attached to a solid support via a linker moiety. The Nα-protecting group is removed (deprotection step) and after washing steps the next amino acid is coupled in the presence of an activator by an amide bond to the immobilised amino acid. The second amino acid can also be pre-activated before introducing it into the reactor. Washing steps are again performed to allow the removing of unreacted materials. The deprotection–coupling cycle can be repeated until the desired amino acid sequence is reached. There are many protocols to combine suitable temporary and permanent protecting groups, but the two most extensively used are the Merrifield strategy (tertiary-butyloxycarbonyl-chemistry, Boc) and the Sheppard strategy (9-fluorenylmetholoxy carbonyl-chemistry, Fmoc) [56]. The value and usefulness of these two approaches can be easily found in many works, but a comparison was made [57] by involving 40 laboratories where a peptide containing 16 amino acids was prepared by following both Boc and Fmoc techniques. This test peptide has potential sites for post-synthesis modification and multiple sites for problematic or slow couplings. The results showed that over 33% of the crude cleavage products made by Boc chemistry did not contain any of the desired peptide and over 44% of the Boc-derived peptides were unable to achieve greater than 25% purity. In contrast, 31% of the samples made using the Fmoc procedure had over 75% of the desired compound. Although the best peptides made by Boc chemistry were comparable with that of the best made by Fmoc chemistry, those results suggested that, in skilled and experienced hands, both methods can give good results, but for the "average user" the Fmoc procedures seemed to be more accessible and more likely to succeed in routine syntheses. On the other hand, the milder conditions of the Fmoc protocols have led to its being preferred by peptide laboratories [58] but certain deleterious side reactions are more prevalent in this approach [59]. In our opinion, an easy and well checked synthesis should be the best choice for the preparation of peptide ligand because of the amino acid sequence having to be definite. Thus, the evaluation of the synthetic protocol should be done each time by taking into account all the experimental and laboratory variables. Moreover, the quantity of peptides necessary to create the library and to check binding properties is on a laboratory scale, so also difficult synthetic procedures can be followed with care. Nevertheless, the syntheses in solid-phases are more advantageous as the washing and filtration steps are easy and allow the application of excess reagents, thus quantitative reactions may be achieved. Furthermore, multistep syntheses can be completed in a single vessel avoiding losses by repeated reagent transfer, and in the end each synthetic passage can be automated. There are different approaches to the creation of a peptide library by exploiting semi-automated or completely automated syntheses. Important development in parallel synthesis technologies were the multipin [23,60,61], the "tea-bag" method [62] and the "diversomer" approach [63]. Moreover the introduction of the "split and mix" [64,65] and the "one-bead–one-peptide" strategies [8] for the combinatorial peptide synthesis, showed themselves to be well-used methods for preparing compounds on a large scale. Recently, new planar solid support for the creation of combinatorial libraries have been introduced, such as polystyrene grafted fluoropolymer microtubes [66], mobile polymer or copolymer grafted to rigid plastics [67], polyacrylic acid grafted polypropylene pins [68], and above all the SPOT synthesis concept [69,70]. In the SPOT synthesis method different peptides are synthesised at different locations, i.e. the spots, in a single sheet of cellulose paper. The dispensed volumes create a specific spot size by determining both the scale of reaction and the number of compounds that can be arranged on the area of a membrane. Surface techniques are advisable for rapid and inexpensive parallel syntheses of a huge number of peptides or peptide mixtures, that can be screened in parallel with different assays and allow us to study numerous aspects of molecular recognition. The necessity to decrease quantities of substances and increase the number of synthesised compounds has brought the development of miniaturised devices [71]. Although this technology looks promising, it still does not show its real potential; however microdevices will be a challenge to reach a greater synthesis speed, better performances, high throughput, low cost and easy automation. For a discussion of the technological proposals in the automation of peptide syntheses see the interesting review of Cargil and Lebl [72] or the web-site http://lab-robotics.org. Automation efforts are in some cases still very expensive, with high maintenance costs and laboratory modification. Thus, semi-automated devices, such as reaction block systems, are more suitable because of their reduced cost and steady high synthesis degree.

Regarding the biological approach to the peptide libraries there is a set of methods in which DNA is manipulated and transferred from the parent organisms into the host microorganisms. Phage display technology has been a powerful

approach to isolate new ligands for many protein targets and to study protein–protein interaction (i.e. epitope mapping). Highly diverse peptide libraries can be prepared with this technique that may offer multiple binding solutions, above all when the target molecule is a protein. Examples of the application of the phage display peptide library to the development of biosensors by using peptides as counterparts of the antibodies are still present in the literature [73–75]. Anyway, biological methods also need a check of the binding properties of the selected peptide when it is immobilised on a solid support.

The peptide library can be created with one of the described synthetic strategies by choosing the more suitable for our target molecule and for our laboratory experience. By following even easier but well-known procedures, the probability of obtaining the desired peptide pattern, from which the best peptide ligand could be selected, should increase.

3.2. Identification

Carefully selected assays should be employed to optimally screen libraries. It is essential to have an accurate screening method that permits us to check small and large libraries rapidly to successfully isolate a few ligands from all the other peptides of the library. Peptide libraries should be tested by several assays that when used in series can help to narrow down the potential ligands and then retest them for biological effect and cross-reactions with other unwanted compounds. The choice of the procedure largely depends on the synthetic procedures used to construct the peptides (Fig. 2); there is availability of different reagents such as enzymes, antibodies, radiolabelled molecules and biological targets.

In solid-phase libraries, binding assays are the method of choice for identifying the binding amino acid sequence. The molecular target can go from a small molecule [76] to

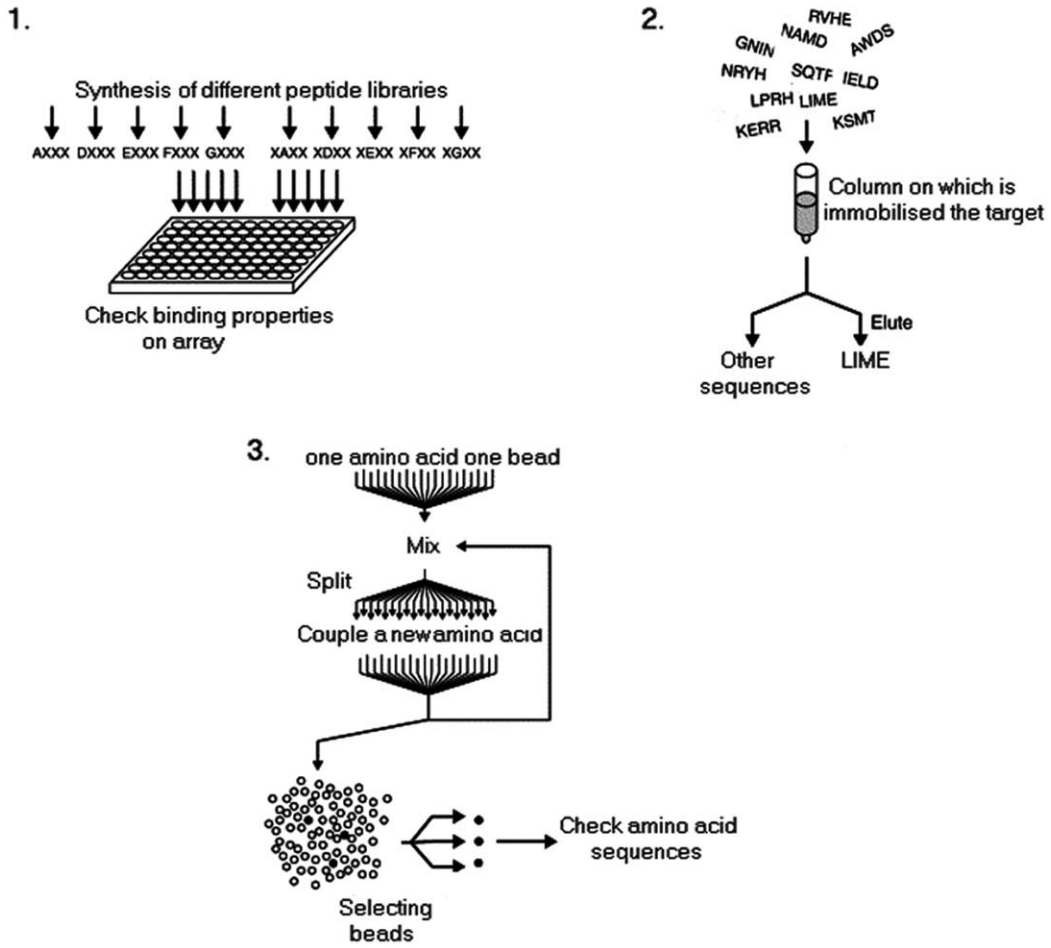

Fig. 2. Different methods to identify the peptide with the best binding activity in three combinatorial approaches. In the first example different peptides were obtained by changing one amino acid in a specific position of the sequence. Then, they are screened on a suitable array by using, for example, a binding assay. In the second example a peptide library prepared in solution is applied to a column on which was immobilised the target molecule. The peptides that have binding activity towards the target molecule are retained within the column. On the contrary, the other sequences are eluted in a few millilitres. The retained peptides can be recovered by changing mobile phase. The third scheme shows the combinatorial approach called "one-bead–one-peptide", so each bead carries a single amino acid sequence. Beads are screened towards the target molecule by exploiting, for example, a fluorescent-labelled target molecule. If a peptide shows binding capacity towards the labelled target molecule, the bead becomes fluorescent and can be recovered. Then, the amino acid sequence immobilised on it is determined.

an intact cell [77] or a whole organism, such as bacteria or virus [78]. In this kind of assay small molecules or proteins are first incubated with a library of immobilised ligands; target molecule and peptide interactions can be visualised with an appropriate group such as biotin, an enzyme [79], a fluorescent probe [80], a coloured dye [81], or a radiolabelled molecule [82]. Microorganisms and intact cells are also incubated with the peptide library. A labelled antibody or a specific dye that stains such a microorganism may be useful to identify binding amino acid sequences. On the contrary, probes are not needed for intact cells as they can be visualised under a dissecting microscope [83]. Moreover, chemical properties of the target molecule can be exploited, such as natural UV adsorption or fluorescence [84]. Another approach could be the labelling of peptides with a fluorescent probe [85] or biotin [86], in particular for small libraries where the greater synthetic work does not complicate or increase the synthesis step too much. Furthermore, fluorescence or coloured solid support can be used above all as a detection system for the "one-bead–one-peptide" procedure. In addition to the binding assays, functional assays have been developed to identify specific substrates [87].

In solution libraries, numerous assays are available: radioimmunoassay, radiolabelled ligand binding assays [88], competitive ELISA assay, scintillation proximity assay [89], fluorescence polarisation assay [90], time-resolved fluorescence assay [91], affinity chromatography [92] and cell-based bioassay [93]. Many of these techniques are adapted to high-throughput devices and they can be applied also to solid-phase libraries after the cleavage step.

Biological libraries, such as phage display libraries, are generally screened by examining a target protein-coated Petri dish [94] or by looking for cell surface binding with intact cells in cell culture [95]. In some cases, functional assay can also be used [96].

In the end, the screening procedure is directly linked to the synthesis strategy and to the chemical and physical characteristics of the target molecule. We have always to take into account that we are searching for a peptide ligand that will provide a new affinity media for chromatography. Thus, if the screening is performed when the peptide is in solution, the binding properties have to be checked again when the peptide is immobilised onto a solid support by using binding assays.

4. Purification and characterisation

When the peptide ligand is identified, the synthesis has to be repeated on a great enough scale to prepare an affinity column. The synthesis procedures are the same as those described in the previous paragraph for preparing the libraries. At the end of the synthesis the peptide should be in solution in order to characterise it and a purification step is necessary to separate the peptide with given amino acid sequence from residual synthesis products that can interfere with recognition properties of the peptide. Also, solid-phase strategies need a purification step because chain assembly is followed by at least one further step to remove the peptide from the resin and to cleave side-chain protecting groups. Purification becomes more difficult as the crude product is more impure. Early experience shows that highly heterogeneous or over-ambitious synthesis may be particularly intractable and not too amenable to any separation technique. Optimisation of the chain assembly and the final cleavage procedure, above all when the chain is complete, will pay dividends when the purification stage is reached. Conventional separation procedures based on molecular size or charge differences continue to be of great value in peptide purification. They are now complemented most powerfully by the more recent developments in reversed-phase liquid chromatography, which depend on hydrophobic differences. Other techniques, such as affinity chromatography and counter current distribution, are also applicable, and every laboratory has its own favourite procedures. However, nowadays, the most widespread purification procedure is the high-performance reversed-phase liquid chromatography, which allows both purification and characterisation of the peptide. Moreover, for great sample quantities, it is possible to use preparative medium/low pressure chromatography. Solid phases are C_{18} [97], C_8 [98] and C_4 [99] reversed-phase silica columns with analytical (e.g. 150×4.6 mm I.D.) or semipreparative (e.g. 250×10 mm I.D.) dimensions. The mobile phase can be different grades between distilled water and acetonitrile, often with 0.05–0.1% of trifluoroacetic acid. Flow rates can range from 0.5 to 5 cm^3 min^{-1} and the UV detector is normally set at 220 or 280 nm. Moreover, the sensitivity of the method can be greatly enhanced by using fluorescence or chemiluminescence detection in place of UV and visible absorption of the peptide, which has to be labelled [100]. Significantly more efficient use of hydrophobic phase extraction can also be achieved by applying the displacement mode of chromatography (displacement chromatography, SDC) to reversed-phase separations of peptides [101]. Recently, SDC has also been adapted to modular solid-phase extraction (SPE) technology to develop stationary phases for a rapid, simple and cost-effective procedure for the efficient and parallel purification of multiple phase preparative peptide mixtures [102]. The characterisation of the peptide can be done with different procedures such as amino acid sequencing, biophysical techniques (e.g. gel permeation chromatography [103], circular dichroism [104], NMR spectroscopy [105]), electrophoretic methodologies (e.g. capillary electrophoresis [106,107]) or more often by mass spectroscopy [108]. Capillary electrophoresis (CE) provides invaluable tools for characterisation of peptides because of its superior resolving power, selectivity and speed. The use of different techniques such as applying different separation modes and the selection of the buffer pH allows for adjustment of the selectivity for every individual separation problem [109,110]. While CE provides a number of significant advantages, the small-diameter capillaries limit the amount

of material that can be analysed, thus rendering the method difficult to use in a preparative mode. Several methods for micro-preparative CE have been described and demonstrated to be useful [111,112]. Coupling CE to mass spectroscopy permits a further characterisation of separated protein [113–115].

Due to its great sensitivity (<100 fmol) and mass accuracy (up to 0.01%), mass spectroscopy (MS) is ideally suited to the analysis of trace quantities. Recent developments of new generation mass spectrometers designed for use in biological laboratories and continuously advancing desktop computing power provide powerful analytical tools. In the late 1980s two new ionisation techniques were developed for MS that revolutionised the analysis of proteins. Both were termed "soft" ionisation processes in that large, charged molecules could be introduced into the gas phase for subsequent mass analysis with minimal fragmentation. These techniques were matrix assisted laser desorption/ionisation MALDI [116–118] coupled to a time-of-flight mass analyser and electrospray ionisation (ESI) [119,120] coupled to a quadrupole mass analyser. In MALDI-TOF ions are formed in a vacuum from a solid state after co-crystallisation of the sample with a matrix compound by irradiation with either a UV- or IR-laser source. MALDI-TOF is relatively tolerant of small relative molecular mass contaminants such as buffers and salts which are excluded during the co-crystallisation process. In ESI-MS ions are formed from a sample solution sprayed into the orifice at low flow-rates at atmospheric pressure. Although less tolerant of contaminants, ESI-MS has the advantage that it may be directly coupled to either reversed-phase HPLC (LC–ESI-MS) or capillary electrophoresis, as these methods are compatible with the above method for sample introduction. These two MS techniques provide rapid, sensitive, and accurate determination of molecular masses and chemical modifications of proteins and peptides [121–123]. A review of the relative merits of the two techniques in the identification of proteins can be found in Ref. [124]. There are many interesting reviews in the literature about the use of mass spectroscopy in the peptide characterisation [125] and also about the screening of large libraries of compounds [126].

5. Chromatographic techniques

5.1. Solid supports

The solid supports should be chemically and physically stable, possess good mechanical strength to allow high flow-rates and should have low non-specific adsorption and interaction [127]. Polymer flexibility and immobilisation of peptide ligands should be such that high peptide accessibility is achieved and the properties of the peptide are not destroyed [128]. The ideal support should maximise the surface area available for target adsorption while minimising that available to contaminants. For application on an analytical scale, pore and particle-size distribution have to be taken into account. Moreover, low cost, high binding capacity, maximum throughput and ability to regenerate and effect cleaning procedures are other important parameters, above all for a preparative scale. The solid-phase includes two aspects: the solid support and the stationary phase that is chemically or physically immobilised onto the core and carries the necessary functional groups. The base support plays a decisive role in the mechanical, chemical and thermal stability of packing materials. Classical affinity chromatography supports are both organic and inorganic and can also be used for immobilising peptide ligands. Some of the materials most used are polysaccharides, such as agarose, cellulose and cross-linked dextran [129,130]. These materials are stable for a wide pH range (pH 3–13) and possess a high content of hydroxyl groups available for activation and derivation. This hydrophilic surface generally does not interact with proteins and shows low non-specific adsorption. A disadvantage is the poor mechanical strength related to swelling ability. Examples of commercially available solid support are reported in Table 1.

Organic polymers, such as polyacrylamide, polyacrylate, polystyrene with different percentages of cross-linker and polyvinyl polymer are more resistant to pressure than the polysaccharides, even if, in comparison to inorganic materials, they show a lower pressure tolerance. These materials exhibit swelling differences in the presence of organic solvent, a broader pore-size distribution, a decreased efficiency and non-specific interactions due to the hydrophobic character of these polymers. These last two problems can be partially solved by coating the polymer with a suitable cover [76] or by a final blocking of the residual functional groups. Polystyrene properties have been changed by grafting polyethylene glycol (PEG) chains onto low-cross-linked polystyrene; these new solid-phases were Tentagel resins [131]. Tentagel resins have good swelling characteristics in both organic and aqueous media; the ability of the resin to swell in aqueous media is an essential feature for many binding tests. On the other hand, new organic resins have been prepared by incorporating flexible polytetrahydrofuran (PTHF)-based cross-linkers [132–135]. JandaJel™ are commercial resins (available through Aldrich) and the insertion of PTHF into the polymers allows us to increase the overall polarity and thus render the resins more suitable for their use with organic solvents.

The inorganic polymer silica is the most widely used chromatographic material. Silica is very stable under pressure and can easily be derived to introduce functional groups [136]. On the other hand, silica is unstable at mild alkaline pH values, dissolves above pH 8 and non-specific interactions occur above pH 4. Also in this case a variety of protocols have been developed throughout the years to modify the surface of silica support [137,138]. A recent article described a new solid-phase support for affinity separation by using alumina as a base material [1]. Alumina shows high mechanical and physical stability and, in comparison to

Table 1
Examples of surface functional groups available on commercial affinity supports

Functional group	Solid support	Commercial product	Supplier
Carboxyl	Agarose	Affi-Gel 10, Affi-Gel 15, AC-Ultrogel ACA, AC-Magnogel ACA, Activated CH Sepharose 4B	BioRad, Amersham Biosciences, IBF Biotechnics
	Cellulose	CMR cellulose, CM cellulose, Cellex CM	Sigma, BioRad
	Dextran	CM-Sephadex	Amersham Biosciences
	Polyacrylamide	CM BioGel	BioRad
	Silica	Carboxy-CPG	CPG
Amine	Agarose	Affi-Gel 102, HMD-Ultrogel ACA, HME-Magnogel, Novarose Act High, Novarose Act Low, Sepharose AH, EAH Sepharose	BioRad, Amersham Biosciences, IBF Biotechnics, GROM Analytik+HPLC
	Cellulose	Cellex-PAB	Biorad
	Polyacrylamide	A-E-BioGel P, Enzacryl-AA, Enzafix P-AB	BioRad, NBS Biologicals
	Silica	CPG-aminopropyl, CPG-aminoaryl	CPG
Hydroxyl	Agarose	BioGel A, Ultrogel A, Ultrogel ACA, Magnogel, Sea Kem, Sepharose 2B, Sepharose 4B, Sepharose 6B	BioRad, IBF Biotechnics, FMC BioProducts, Amersham Biosciences
	Cellulose	Avicel, Cellex	Bioprobe, Biorad
	Dextran	Sephadex	Amersham Biosciences
	Polyacrylamide	BioGel P	BioRad
	Polyvinyl	Fractogel	EM Separations
	Silica	CPG, CPG-dextran, Unisil	CPG
Aldehyde	Agarose	Act-Ultrogel ACA, Act-Magnogel ACA	IBF Biotechnics
Thiol	Agarose	Affi-Gel 401, Thiopropyl Sepharose 6B, Activated Thio Sepharose 4B, Novarose Act High, Novarose Act Low	BioRad, Amersham Biosciences, GROM Analytik+HPLC
	Polyacrylamide	Enzacryl-PT	NBS Biologicals
	Silica	CPG-thiol	CPG

silica, higher chemical stability. The stationary solid-phase consisted of a cross-linked water-soluble polymer to which a 13-atom hydrophilic spacer, which showed a suitable functional group at the end of its chain, was coupled. Also monolithic material was used as a support for peptide synthesis, screening [139] and affinity chromatography [140].

5.2. Immobilisation

The functional groups for immobilising a peptide ligand are widespread and exploited a different chemical approach. The peptide is generally linked by using the carboxylic group from the C-terminal or amino group from the N-terminal; other less frequently used possibilities are carboxylic groups of aspartic and glutamic acids or ε-amino groups of lysines. When an immobilisation reaction is performed, great attention has to be paid to all the amino acid side chain groups; they have to be protected or must not react in the same experimental conditions in which the peptide is immobilised. Usually the groups present on the solid support are activated separately, then, after a washing step to remove the residual activating agents, the peptide is added in a higher concentration than that of the activated groups present on the solid-phase. The concentration of the peptide can even be 10 times higher than the surface groups for ensuring a reaction as quantitative as possible. This approach should prevent cross-reaction between the peptides in solution and thus it is not necessary to protect all the peptide functional groups but it is sufficient to protect the ones that could also bind to the solid-phase. Instead if a peptide carboxylic group or amino group is activated, great care must be given to permit only the reaction between the desired functional group on the peptide and the solid-phase by protecting all the other reactive groups. Protecting groups and procedures are well described in the literature [141] and it is also possible to synthesise the peptide with automated instruments, as described in Section 3.1, and avoid long synthesis work by purchasing protected amino acids. In some cases there are no competitive or problematic reacting groups on the amino acid sequence, hence the synthesis is simpler and without protecting/deprotecting steps [84].

In Table 1 examples of functional groups on different commercial solid matrices are shown. Many manipulations of the solid surface chemistry are possible, in fact the functional groups present on the solid support can be treated as organic chemicals and may be modified by changing their reactive group into another or by introducing a spacer arm. Many resins already have a spacer arm immobilised on their solid support and a good rule is to provide it, when it is lacking. A spacer arm moves the amino acid sequences away from the bead surface, thus the mobility of the peptides during their interaction with an affinity partner residing in the mobile phase should improve by showing better binding capabilities. A careful choice of spacer arm may even help to prevent non-specific adsorption. The concentration of the reactive groups on the solid support are on average some micromoles per gram, but it is also possible to work with solid-phases that have a higher surface concentration of functional groups (mmol/g). A partial coating of the surface groups could be necessary for working with smaller quantities of coupling reagents and peptide, nevertheless a final blocking step could be useful to avoid different interactions from the affinity binding of the peptide. Blocking procedures are well described in the literature [130,142].

Carboxylic groups can be activated by carbodiimide to intermediate *O*-acylisourea which can react further in subsequent steps; an extensive application of the carbodiimide can be found in the literature [143]. An important aspect of the carbodiimide coupling is its use together with *N*-hydrosuccinimide [144] that is able to stabilise the intermediate *O*-acylisourea, hence the reaction of the activated carboxylic group is longer giving higher yields of the peptide–solid-phase reaction. Amino groups are often activated by using glutaraldehyde [145] that can couple two amino groups. Moreover, other activating agents are cyanogen bromide (hydroxyl group), *N*,*N*-carbonyldiimidazole (hydroxyl and carboxylic group), tresyl or tosyl chloride (aldehyde group), hydrazine (amino group). Another common surface group is epoxy that will be hydrolysed in aqueous suspension with time because of its high reactivity and therefore has a limited shelf life after synthesis. Pre-activated resins are also available on the market.

The same activating and condensing agents can be used for the activation of the peptide in solution. Another approach can also be the building of the amino acid sequence on the solid support itself by exploiting the synthesis procedures described for the synthesis of the peptide. Fmoc and Boc strategies can also be easily used to prepare affinity media by using the described solid supports. After the immobilisation of the peptide the resin must be washed many times to remove unreacted compounds and residual peptides, then the resin is ready for packing into an affinity column.

5.3. Applications

Peptide columns in chromatographic techniques were used as affinity media for antibodies [25,146–150], proteins [97,139,151–155], low-molecular-mass analytes [76,84], peptides [156–161] and they could be used for widespread applications because of their practicability and different available approaches. We would like to quote some different examples.

Protein A has long been the ligand of choice in the affinity purification of IgG monoclonal antibodies. Despite this, recent work [162] showed the possibility of using a phage display library approach to identify peptides to use them as an alternative to protein A in the affinity purification of monoclonal antibodies. In this study the constant region of an IgG_1 monoclonal antibody, denoted humanised anti-Tac (HAT), was used as the target for the phage display library. After subsequent biopannings against pFc′ fragments of HAT, a bioinformatic technique was used to determine the homologies among and between phage-derived amino acid sequences and Protein A. These studies selected four peptides that were immobilised by using aldehydo-NuGel derived from the commercially available amino-NuGel. Affinity chromatography of HAT was performed on the four-peptide columns and binding capacities were checked. The results showed that the amino acid sequences with the highest homologies did not show HAT binding, while the best sequence shared 42% homology with protein A. Interestingly, this sequence fitted the linear region and partly extends into both helical structures of the Fc binding domains of protein A, moreover all molecular modelling yielded similar structures that were linear in the middle and helix-like on both ends. These results demonstrated the feasibility of small peptides that share similar binding properties with protein A but are less expensive, simpler to prepare and more stable for ease of regeneration. Antisense peptides have shown recognition properties towards sense peptide and proteins with significant selectivity and affinity. This kind of recognition was applied in many peptide and protein separation techniques by affinity chromatography [157,163]. Although there were several successes in the use of antisense peptide-based affinity separations, an antisense peptide with a high binding constant towards its corresponding sense peptide is difficult to obtain. Some efforts are being made to increase interactions between sense and antisense peptides [158–160]. It has recently been noticed that degeneracy in antisense peptide can exist, as a degeneracy in genetic code exists. The existence of the degeneracy and the possibility to increase the affinity between the sense peptide and the antisense peptides, by modifying the sequence of the antisense peptides according to the degeneracy of the genetic codes, were demonstrated [164]. This effort was focused to develop a simple and efficient method for selecting affinity ligands at low cost and with long life and high specificity. A sense peptide and four antisense peptides were taken into account as models for analysis of the affinity interactions by evaluating them through high-performance affinity chromatography. Then an application of this model was checked with a fusion peptide from the influenza virus A. Syntheses of the peptide were performed by following Fmoc strategy and they were purified by reversed-phase liquid chromatography and mass spectroscopy (MALDI-TOF). The peptide affinity columns were prepared on poly(glycidyl methacrylate) beads (PGMA) by following suitable immobilisation procedures [164]. Ethylenediamine was chosen as a spacer arm. Binding assays showed different affinity between the antisense peptides for the corresponding sense peptide and the blank column showed no retention. One of the selected sequences showed higher affinity and it was characterised by the presence of arginine. On the other hand, sequences in the presence of glycine and serine did not show recognition properties. The same results were obtained with the fusion protein from the influenza virus A. Hence, the affinity recognition properties of the peptide ligand towards native peptides or proteins was strictly correlated to the amino acid sequence and can be improved by varying the amino acid sequence of the starting antisense peptide.

Furthermore, the development of the ligands with affinity towards soluble macromolecular targets, such as proteins, is an important goal and, about 10 years ago, a process for finding them was reported [8]. In this approach a peptide

combinatorial library was created and the protein in question was incubated with each peptide. All peptides were immobilised on microscopic beads, so, in these cases, the kind of linkage and distancing arm can play a decisive role and also make, as previously said, an important contribution to the binding capacity. Thus binding properties can be influenced by different linkers, by the manner of attachment or by poor accessibility of the ligand. In this sense studies about the linkers and their chemistry must to be taken into account when an affinity column for macromolecules is projected. A recent work [165] described the study of the properties of the linkers towards seven-protein targets: three proteases, one metabolic receptor, one kinase, one extracellular matrix (ECM) receptor and one protein subcellular localisation domain (SH2). Peptide linkers and a ligand library were made by using Fmoc strategy on amino-Tentagel beads. Different amino acid sequences were used as linkers and their binding properties were studied as was the frequency of the residues at each position of the linker for all the seven proteins. In the first two positions the cationic residues (diaminobutyric acid, diaminopropionic acid) dominated. Moreover, the diaminobutyric acid was less common than diaminopropionic acid and it is reasonable to suppose that the longer side chain hindered the interaction between the protein epitopes and the ligand. Aromatic or hydrophobic groups were also slightly common. In the end of the work the authors concluded that in their cases the best linker for the display of peptide ligands is one that has positive charge and minimal side chain size or aromatic and hydrophobic bulk.

Still on the theme of proteins, in recent years there has been a growth in studying proteomics and in the production of recombinant proteins. The efficient recovery and purification of these proteins are difficult tasks because the target molecule is often present at low concentrations in complex streams that contain many proteins with similar characteristics. Varied peptide ligands can be created by exploiting different functional groups and so it is more probable to obtain a peptide that shows good selectivity towards similar proteins [85]. Also, it is possible to develop a peptide ligand by using a small quantity of recombinant protein, which is expensive in general and difficult to find. In the case of proteomics, affinity chromatography with peptide binding columns could be helpful, but an accurate screening methodology must be followed [97]. In this work a procedure consisting of three screening steps was described. The first screen probes a solid-phase combinatorial peptide library synthesised on modified chromatography resin for binding to the target protein, the fibrinogen, in a column format. Then, in the second screen the relationship between the density of peptides on the resin and the selectivity of peptide binding were evaluated. In the end the influence of the peptide binding properties on the purification of the fibrinogen was evaluated. These procedures allow us to optimise not only the peptide sequence, but also its density on the resin, the binding and elution conditions, the column dimensions for the highest yield, purity, throughput and functional activity in the following production process.

In our laboratory, peptide ligands for small-molecular-mass analytes such as estrogens [76] and aflatoxins [84] were developed. In our work we exploited both combinatorial and molecular imprinting techniques by performing a combinatorial solid-phase synthesis. For the selection of a peptide that was able to bind the estrogens, we used the amino acids present in the estradiol-binding site of the human steroid binding protein as monomers for the library. The number of amino acids present in a protein binding site is not so high and the amino acids involved in the binding with this target molecule are usually four or five. Thus we prepared an amino acid library with a pattern of eight monomers and we used commercial cross-linked polystyrene as solid support. We used the *N*-hydroxy-succinimide ester method [166] to build amino acid sequences by activating superficial carboxylic groups. We used 4-aminobutyric acid as a spacer arm. We created a first peptide library (Fig. 3) and from it we selected an amino acid sequence by checking the binding properties and the selectivities towards structural homologues of all compounds. These steps were performed

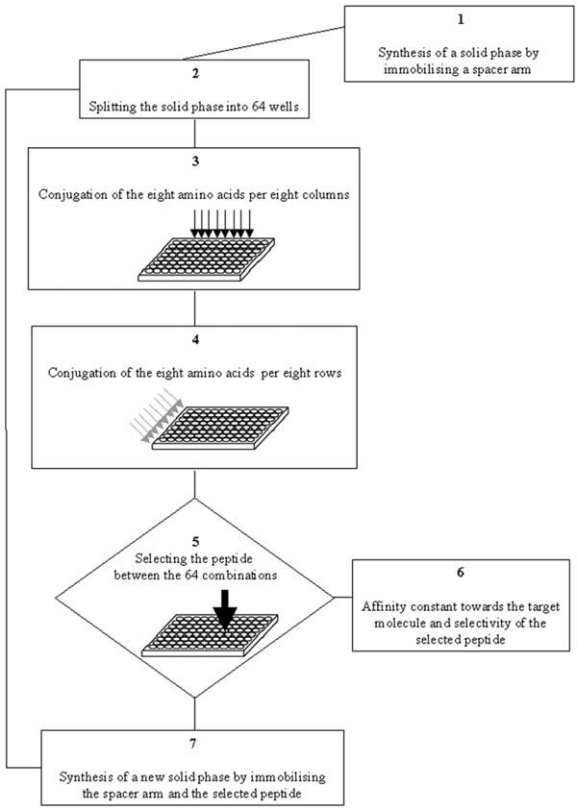

Fig. 3. Flow chart of the experimental work. The working sequence, which was followed to select a peptide with binding properties, is made by first synthesis steps to prepare the amino acid library, the binding and selectivity properties of the 64 combinations are then put through a checking phase. The selected peptide is used as a starting solid-phase for a new working sequence. This working scheme can be repeated until synthesising the desired amino acid sequence.

by binding assays by labelling the target with a marker (a radiolabelled probe). The selected peptide immobilised on the solid support was used as the starting solid-phase for the creation of the following library. Again a new peptide was selected and this procedure (creation of the library, selection, new library) was repeated. We obtained peptide ligands that showed high binding constants and which can be successfully used in affinity chromatography. The affinity media was prepared by following the synthetic procedures used for the creation of the library. The solid support is the same and was packed into small columns (11×17 mm) on a low-pressure device. The buffer alone is not able to elute the analytes from the column but it was necessary to introduce a percentage of solvent into the mobile phase to recover them. The recoveries of the analyte were quantitative and comparable with ones reported in the literature for the immunoaffinity column. We prepared blank columns and they did not show any retention, moreover we checked binding properties of selected peptides towards molecules with similar structures and we observed no binding towards them. In the end we analysed some real samples (for example tap and river water) and the efficacy of the peptide ligand was the same shown in buffer solution. Moreover, even if we selected the amino acid sequence by using only one estrogen (i.e. the estradiol), the peptide affinity column was able to bind all the natural estrogens and also some synthetic ones. In the end we would like to underline that the affinity chromatography results were obtained with a solid-phase on which a sequence of four amino acids was immobilised and that the affinity constants and the selectivities of the peptides towards the estradiol started to decrease after the hexapeptide and after the tetrapeptide, respectively. This behaviour seemed to agree with the observation about the number of amino acids involved in the protein binding site. Also, our peptide column, as in the first example, was easier to prepare, more robust towards elution conditions and less expensive than classical immunoaffinity devices.

6. Electromigration methods: affinity capillary electrophoresis

Affinity electrophoresis [167] denotes all techniques in which some kind of biospecific interaction between an electrophoresed component and another component (ligand) present in the medium occurs. The interaction results in the mobility of the electrophoresed substance compared to its mobility in the absence of the specific ligand in the medium. The observed effect should be most pronounced when the two components, the ligand and the target molecule are of similar size or when the ligand is highly charged. Affinity capillary electrophoresis (ACE) [168] has the advantage of being faster still and requiring less sample than the conventional types of electrophoresis. Moreover, ACE serves as a mild and sensitive tool for the investigation of molecular interaction and biomolecular recognition [169]. ACE is sensitive to both size and charge as capillary zone electrophoresis, but can also exploit binding interaction between a ligand and a specific molecule. The binding of charged ligands can be quantified directly, but for a neutral ligand it is necessary to perform a competition with well-characterised charged ligand. For a summary of its application for the characterisation of protein and peptides see Ref. [170]. Samples with high purity or quantified ones are not requested in this technique, because the analysis is based on the changes in the migration time rather than the peak areas [171]. ACE, as classical capillary electrophoresis, can be used in the characterisation of amino acid and peptide mixtures and it is a powerful tool for binding studies and binding constant determinations. The determination of the kinetic and equilibrium constants relies on the shift in migration times and the peak shape. Monomer and dimer forms can be easily distinguished by differences in respective migration times [172,173]. The analysis of the affinity between a peptide and its ligand can be another application where ACE can give a good contribution by evaluating the influence of the amino acid sequence on the binding capacity [174] with structural homologues of the natural peptide. These studies can be very useful to approach the studies of unknown peptide ligand, which should be exploited in the development of new affinity electrophoresis. In a recent work [175] ACE using mobility shift analysis was utilised to characterise the binding of peptide ligands to cyclophilins. Then the authors faced the study of a peptide library obtained by scanning the sequence of *Drosophila melanogaster* protein called CAPPUCCINO. The protein CAPPUCCINO was suspected as being a potential ligand because of its high content of proline residues in the amino acid sequence of the protein [176]. The recognition of proline-containing binding motif is a fundamental property for the peptidyl-prolyl *cis*/*trans* isomerase [177]. The library was prepared by following the spot strategy and the peptides that interact with the target protein were detected by labelling them with a fluorescent probe. Three of the seven peptides that showed recognition properties were selected and two of these were tested by ACE. One peptide managed to change the electrophoretic behaviour of the cyclophilins, even if recognition of the fluorescent probe was also present. ACE could also be used to select new ligands by screening libraries [178] although this application is not yet widely used because of the necessity of sophisticated instrumentation.

7. Evaluation of the analytical results

Affinity chromatography techniques can take great advantage of the development of peptides with specific binding properties. As said in the first part of the review, the chemical synthesis and purification of an amino acid sequence can exploit a large literature base and scientific work that make the preparation of the ligand a standardised and well-checked process. Moreover, technological advances in all instrument

areas allow us to design, synthesise and characterise peptides with high analytical purity. Checking the amino acid sequence and the peptide structure can be performed by a wide number of analytical and biological methods that can cross-reference between themselves.

The versatility of different approaches to prepare a peptide is a very important starting point to obtain good results in the development of a ligand that can be applied in widespread fields. A significant point is the possibility to shape design, synthesis and selection procedures to the target molecule, even by using toxic compounds or very expensive or rare substances. All these characteristics contribute to decrease the time necessary to develop the ligand. Anyway, this time is generally shorter than that which is useful to obtain a classical biological counterpart. Furthermore, as the properties of peptide ligands can be easily defined, the reproducibility of the synthetic procedure and of the ligand features increases.

The applications of the peptide as a mimic of biological systems showed longer life, reusability and low cost for the column. In comparison with classical affinity chromatography the peptide affinity systems show similar recovery data, specificity and precision, but the binding constants are lower than those shown by antibodies. This property can be exploited in favour of an easy recovery without using strong elution conditions, hence a higher speed of elution. Also peptide ligands are compatible with the use of solvents and they can resist a wider temperature, pH and ionic strength range. In this way, as was said before, the life of the columns can be improved by avoiding bleeding effects and reusability becomes a real advantage. Peptide affinity columns give the possibility of being interfaced with other techniques (i.e. HPLC, MS, CE) by finding applications with fewer problems.

All these considerations seem to conclude that peptide affinity systems will be a success in the future, but we are more inclined to think that they will be a great opportunity and certainly a challenge. In fact from an analytical point of view, there is little chemometric data and the results about recoveries, sensibility and so on are comparable with classical affinity columns, but there are no detailed studies. Moreover, peptide devices were often applied to samples prepared in buffer or serum solution but other real samples with complex matrices have to be faced in a significant way. The potentialities of these systems can be larger than the applications shown until now and, in our opinion, there is a lack of analytical approach, as peptides are not seen as artificial binding systems yet.

8. Future perspectives and conclusions

This review has identified procedures that allow us to rationalise the discovery and the project of selective peptide affinity ligands that can be used in affinity chromatography techniques. The design strategy could comprise different approaches, above all when there is little or no information about known ligands, target sites, and biological interactions, thus a modelling step is hard or not possible. Also, the synthesis and purification strategies are widespread, and, in many cases, well known. Hence, they can be adapted to the necessities of each laboratory. Moreover, technological advances allow us to use MS or CE for the characterisation and the study of large peptide libraries as well. These bases give a strong starting point for the development of peptide affinity media that can also exploit a large literature base about classical affinity media, supports and columns. Furthermore, peptide ligands offer a number of advantages for their application in affinity techniques, as they are less expensive, used in different scales, durable and reusable over multiple cycles. Peptide ligands, in our opinion, should to be thought of as a mid-way point between antibodies and molecular imprinted polymers because of their intermediate properties, hence, they should be handled as classical synthetic ligands. The future direction(s) in affinity techniques will undoubtedly depend on new technology development, such as miniaturisation and nanotechnologies, possibility of multi-peptide media, higher throughput systems and that which will facilitate the meeting of different objectives in challenges of quality, quantity and diversity. In this sense peptide ligands will also be successfully used in affinity sensors and novel detection microarrays. In the near future, the quality of these new affinity analysis systems will be improved further, as these approaches will become more popular to solve many different chemical and biological problems.

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