Combinatorial Peptide Ligand Libraries as a "Trojan Horse" in Deep Discovery Proteomics

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UPDATED HIGHLIGHTS

It is of common and widespread knowledge that proteomes are populated by not only a very large number of proteins but more importantly by a few highly concentrated proteins and a majority of species of extreme low abundance. This situation derives from the difference in gene transcription and represents a technical obstacle to the detection of diluted species that are below the sensitivity of current methods. Thus, the detection and the identification of very dilute proteins one-by-one from the ca. twenty three thousands of protein coding genes represents a challenge and remains the global goal of proteomics specialists. Many efforts have been performed with sophisticated prefractionation approaches or sample treatment methods.

Separation of a protein extract or biological fluid by simple fractionation schemes (precipitation, chromatography, electrokinetic technologies) has extensively been described.[1,2] Elimination of high-abundance proteins as a way to evidence the hidden proteome (also called subtraction or depletion with its specific immunodepletion approach) has also focused the attention of scientists and contributed to important findings about interesting biomarkers. Within the various technological developments, the enrichment of low-abundance species while concomitantly decreasing the high-abundance ones allowed one to deepen the investigations toward the rarest and most dilute proteins. To alternatively circumvent the difficulties related to the very large dynamic concentration range of expressed proteins, attempts have been deployed toward the transcriptome investigations considering that the latter could represent the blue print of the proteome. Nevertheless this approach contributed to largely underestimate the low-abundance protein situation, as recently demonstrated.[3,4] Contrary to what was described by Schwanhäusser et al.[5] and largely taken as a basis of knowledge, demonstration was made of the poor correlation between transcriptomics and proteomics. While investigating in parallel the transcriptome, translatome, and proteome of the same cultured cells, Li et al.[3] demonstrated the large contribution of mRNA in protein abundance accounting for up to several dozen percent of the differences from previous data. This important finding incidentally suggests continuing using performant technologies to directly investigate the proteome composition and concentration. Then correlations between the transcriptome, the translatome, and the proteome could allow elucidating the complex mechanisms leading to both the difference and significance in individual protein expression.

The use of combinatorial peptide ligand libraries (CPLL) for the reduction of the protein dynamic concentration range in biological samples in view of detecting very low-abundance species has been proposed a few years ago and successfully applied to numerous situations. Variants to the standard mode of use have been described with the benefit of improving the global efficiency of the technology or to make specific focuses on given groups of proteins. However, all the reported technical advancements have never been associated within a single review to analyze each singular contribution to results improvements. For a current use of the technology and on the basis of what is known to date, unconventional utilizations and pertinent associations are here proposed in view of enlarging the proteome coverage and of simplifying the experimental manipulations. To date it remains one of the methods of choice for the detection of very low-abundance protein species. In addition, a remarkable interest of the technology was found also in glycoproteomics for general purposes as for instance focusing essentially on the

glycoproteins as a whole group[6−8] or targeting specific groups of glycoproteins such as those comprising fucose molecules.[9]

The combination of sample treatment with CPLL with performant analytical methods is also more and more the focus of the low-abundance protein analyses, especially for the detection of early signs of given diseases.[10−14] It is within this context that the following sections assemble the most recent approaches on how to use the CPLL technology to reach the best results by just playing on initial conditions or specific elutions.

LIMITS OF THE CURRENT USE OF SOLID-PHASE COMBINATORIAL PEPTIDE LIGAND LIBRARY

Several hundreds of published papers report the use of combinatorial peptide ligand libraries (CPLL) for the reduction of dynamic concentration range allowing access to very low-abundance proteins otherwise undetectable. An extensive list of published papers is available from Boschetti and Righetti.[15] The principle is today quite well-known and is based on the concomitant concentration of low-abundance proteins and the reduction of high-abundance ones thanks to the mechanisms of mixed-bed affinity chromatography in a large overloading situation. Many examples of applications have been reported, such as the extension of the list of proteins present in various proteomes of animals,[16] plants,[17] microbial origin,[18,19] and cell culture supernatants.[20] Similarly the discovery of expression modification of certain proteins of low abundance upon biological disorders (see following sections), the evidence of foreign unexpected proteins in food and beverage preparations,[21] the discovery of novel allergens,[22] and the alteration of protein expression during the embryogenesis development[23] have been described.

In spite of the great success of the technology, several issues are to be underlined. Sometimes they are related to the incorrect use of this methodology; however, there are examples where the limitation is due to the current peptide composition of CPLL. The key success factors are described repeatedly but quite randomly, and they here deserve to be classified and discussed all together, which is the aim of this paper. They can be classified into five distinct categories.

(1) On the technology point of view it is necessary to make a fundamental difference between a true library and a mixture of the same ligands on the same substrate. Moreover, one has to make a distinction between a mixed-mode ligand and a ligand library.

(2) With a given peptide library there are two main operational conditions that need to be met: the sample overloading of the binding capacity to unbridle the molecular competition to take place and the exhaustive elution of captured proteins.

(3) When using a given library under optimized parameters, the initial physicochemical conditions can be modified according to certain criteria to make a focus on different categories of proteins.

(4) The current library can be chemically modified and be used for the same difference concentration compression purpose.

(5) Finally the current peptide library can be complemented with other solid phase sorbents with an extended capability of protein capture.

In spite of the emergence of a very large number of undetectable low-abundance proteins, one observed limit of the technology is that certain proteins easily observable without the sample treatment are "lost".[24] This limitation is considered quite critical because it is unclear if this phenomenon affects also low-abundance proteins that are intended to be "amplified".

It is within this context that the use of CPLL has been considered with the goal of enhancing the power of the technology and maximizing thus the capability of finding additional pertinent discoveries. This is primarily important for novel biomarker discovery that are of very low abundance because they allow determining the early stages of a metabolic disease: pregnancy-related protein markers,[25−27] veterinary identification of diseases,[28−31] obesity and related diseases,[32−34] and many others. A growing number of papers are dominantly published with focus on different types of cancers with the aim of finding relevant markers for early stage detection such as in pancreas,[35,36] liver[37,38] lung cancer,[39−41] breast,[42] ovarian[43] and prostate cancers.[44]

Without the possibility to find modifications of protein expression at their initial stage, no early detection of disease is possible, but this is not enough without a rigorous reproducibility of the technology as demonstrated repeatedly,[45,46] which is an essential guarantee for the process validation.

The results reported in this paper have been partially already described singularly, while others have not been published to date. The latter should serve as experimental support to demonstrate the flexibility of the technology and to trace the path for further developments in view of a larger coverage and a simplification of the experimental manipulations.

LIGAND LIBRARY BEHAVIOR IS DIFFERENT FROM THE SAME LIGAND COLLECTION COLLECTIVELY GRAFTED ON THE SAME SUBSTRATE

The concept of reducing the dynamic protein concentration range is based on the use of a ligand library made by using amino acids. These molecules are interesting for the fabrication of the diversity because they are easy to assemble with very different side chain structures capable to produce any type of molecular interaction such as electrostatic, hydrophobic, hydrogen bonding, and van der Waals. Each single bead carries a unique type of hexapeptide. The number of diverse hexapeptides is the result of full possible combinations of amino acids used as building blocks. All different beads are assembled thus as a sort of mixed bed composed of a very large number of affinity-like sorbents. Each bead can comprise a single or multiple types of interaction sites that are located differently along the peptide chain. The type and the topology of interaction points are at the origin of the specificity of interaction for the targeted proteins. The interaction is operated at the level of a quite restricted zone of a protein surface which means that several proteins could have similar sites for docking to the same hexapeptide. Probabilistically these similar interactions differ, however, in terms of affinity constants suggesting a quite intense competition among species for interacting with the same grafted ligand as governed by their respective concentration according to the well-known mass-action law. Nonetheless a legitimate question would be whether the peptide ligands need to be grafted separately (one-bead-one-peptide) or grafted all together on all beads (one-bead-all-peptides) to contribute to the expected reduction of concentration of high-abundance proteins and concomitant increase of the low-abundance ones. Intuitively only a mixed-bed library as defined would produce the expected job as a series of affinity chromatography sorbents used separately.

To demonstrate the different behavior of these two grafted peptide ligand configurations specific experiments have been designed. Starting from the same mixture of peptides two types of sorbents were made: the standard one called one-bead-one-peptide and the second one where all peptide mixtures were

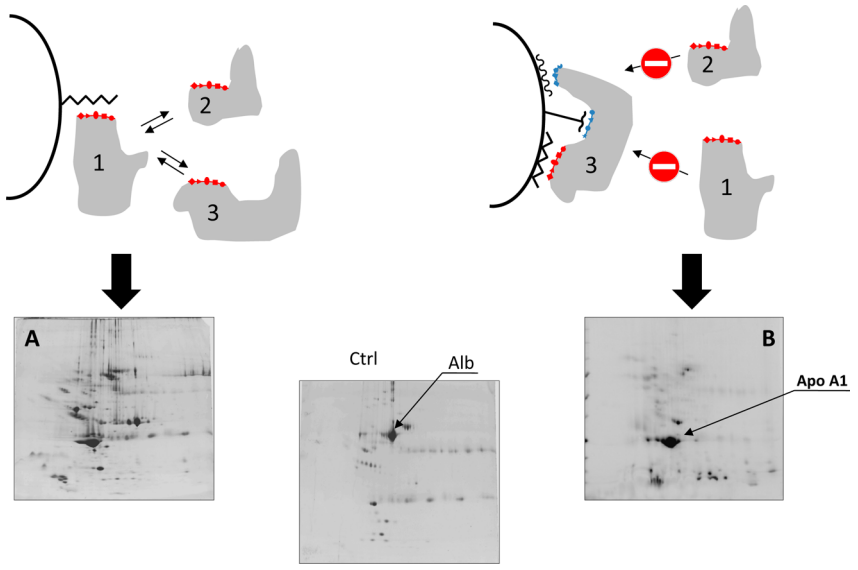

Figure 1. Behavior difference between a combinatorial peptide ligand library versus the same collection of peptides grafted all together on all beads illustrated as 2D electrophoresis patterns of treated human serum samples. Picture "A" represents the collected proteins from a CPLL column and picture B is the collected proteins from all hexapeptides randomly grafted. The upper part of the figure is an illustration of the protein docking mechanism interpretation of one situation versus the other. 1, 2, and 3 are three model proteins sharing the same exposed sequence of amino acids interacting with the hexapeptide sequence of a ligand (they compete for the same hexapeptide position; left side). On the contrary, protein model 3 comprises two other epitopes that interact with two other hexapeptide sequences grafted on the same bead (no full competition is possible; right side). The two-dimensional electrophoresis images representing the serum protein pattern (p*I* range between 3 and 10; M_r range between 10 and 250 kDa) are from the experimental work of the authors of this review and are adapted from Righetti et al.[47] The desorption of protein was performed with 9 M urea, 50 mM citric acid titrated to pH 3.3; staining with colloidal Coomassie blue. Ctrl, Control panel obtained with untreated human serum; Alb, serum albumin spot; ApoA1, apolipoprotein A1 spot.

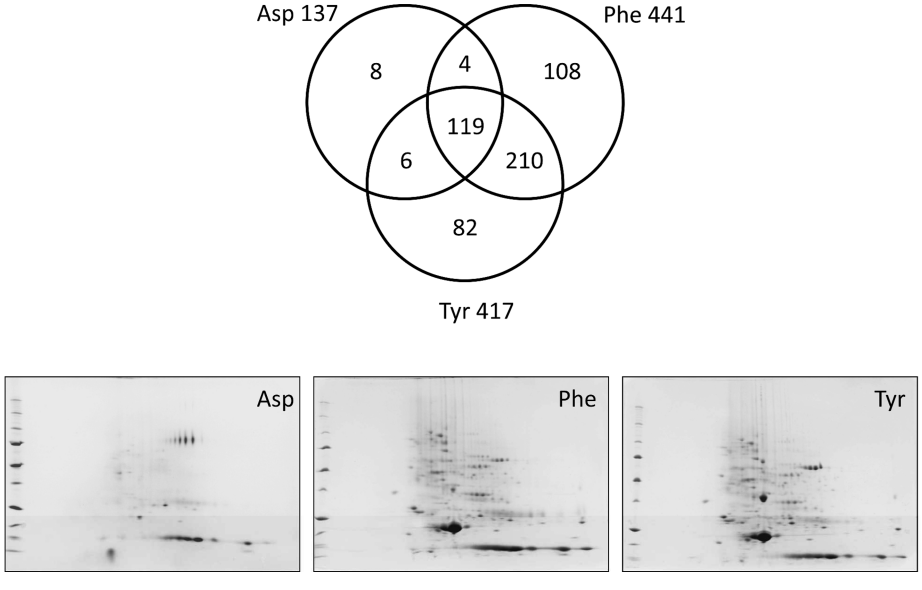

Figure 2. Effect of amino acid differences on the pattern (lower part of the figure) of protein capture for the same crude initial extract. 2D electrophoreses were made within a p*I* range between 3 and 10 and a M_r range between 10 and 250 kDa. The protein desorption was performed by using a solution of 9 M urea with 50 mM citric acid titrated to pH 3.3; staining with colloidal Coomassie blue. The upper Venn diagram illustrates the proteins in common as well as the exclusive proteins from each amino acid tested with a red blood cell protein lysate. The numbers indicate the gene products found and identified by LC–MS/MS. The experiments have all been performed under physiological conditions of ionic strength and of pH in order to minimize the electrostatic effect of the interaction. Asp, aspartic acid; Phe, phenylalanine; Tyr, tyrosine. Unpublished data.

grafted on the same bead. The standard protocol produced a mixed bed and the second protocol generated a homogeneous multiligand affinity bed. Both beds have been similarly overloaded with human serum and, after an extensive washing, the captured species were globally desorbed and analyzed in parallel by two-dimensional electrophoresis as illustrated in Figure 1. Interestingly, the obtained protein patterns are very different. While the level of albumin (high-abundance protein) is reduced in both systems, the mixed bed (Figure 1A) clearly captures a much larger number of proteins of low abundance (not detectable from the nontreated serum sample, Ctrl) than the homogeneous beads.

The protein capture process is dependent on an intense competition among protein species; however, with the use of these two versions of the same set of hexapeptide the competition mechanism is very different (Figure 1, upper cartoon panel). Actually in a bead comprising single peptide structures, the competition is resolved by both the difference of affinity for the same ligand and the concentration difference between competitors. In the second case where a bead comprises all peptide structures close to each other, a protein interacts with several different peptides at the same time in accordance with compatible atomic distances. The competition for a single peptide ligand is here practically absent, thus the saturation phenomenon due to overloading conditions appears only as a secondary (and probably quite marginal) effect on the enhancement of low-abundance proteins. In fact a protein that docks by means of different interacting ligands is difficult to displace by another protein that may not have the capability to interact with the same set of peptides with the same degree of affinity for each site. The resulting practical effect is that the eluted protein pattern is largely different from what results from the one-peptide-one-bead library[48] (Figure 1B). One main consequence of such a situation is that the sorbent version with all peptides on a single bead does not facilitate the capture of low-abundance species because of the poor possibility of displacement. Moreover, proteins that have a natural good propensity to interact with a large number of structures (e.g., apolipoprotein A1) have the advantage over other proteins and become very abundant after the treatment of serum.

Another distinction that deserves to be recalled is a difference between a mixed-mode and a mixed-bed adsorption. The former uses a single ligand carrying various docking points, while the latter comprises different ligands that are singularly grafted on different beads. The basis of this approach has been described a few years ago[49,50] with the comparison of combinatorial peptides library of different length where the most simple was the single amino acid. The reduction of the length of peptide ligands (from hexapeptides to dipeptides) diminishes the number of captured species due to the reduced ligand structure diversity; however, even with very short peptides or single amino acids the capture was quite significant. This is the result of the gregarious effect of each ligand, all of them acting together for the final dynamic compression effect on the protein concentration difference. Rationally amino acids of similar structure will capture proteins giving a similar pattern, as evidenced by two-dimensional electrophoresis analysis, but very different structures will produce complementary patterns. Figure 2 (lower panel) attests for this effect. Grafted aspartic acid (Asp) with its simple structure, its anionic character (p*K* 4.07) and its hydrophilicity, adsorbs fewer proteins in comparison to tyrosine (Tyr) (an aromatic non ionic, p*K* 10.46, and more hydrophobic amino acid with propensity to generate H-bonds by the presence of an OH group on the phenyl ring). Hence the quality of captured proteins is very different. This is not the case when comparing two quite similar aromatic amino acids such as tyrosine and phenylalanine (Phe). For these ligands a large number of redundant protein spots are found (Figure 2, upper panel). As an extension of this study, all other amino acids have been compared and it was possible to observe that the sum of all separated elutions was exceptionally similar to the mixed bed of all of them in terms of protein pattern.[50]

IMPORTANCE OF THE PROPER UTILIZATION OF A PEPTIDE LIBRARY

The performance of CPLLs depends on two major parameters: (i) a certain degree of overloading ensuring the enhancement of low-abundance proteins by progressive proportional concentration (the larger the overloading the better reduction of dynamic range) and (ii) the total elution in an exhaustive manner of all captured proteins.

The binding capacity for bead saturation is around 3 ng of protein per bead under physiological conditions. This translates into a binding capacity of about 10 mg of proteins per milliliter of settled bed of beads. A number of biological samples comprise many different proteins individually covering a very large concentration range extending up to more than 10 orders of magnitude. Obviously if one wants to bring the proteins present in trace amount to the detection level, the sample volume (and total protein concentration) involved should be large enough to allow for enough enrichment. The most concentrated species will be present in large excess but not affecting the capture process because they stay in solution and are eliminated by a washing step.[47,51,52] A spectacular effect of large overloading has been demonstrated by Roux-Dalvai et al.[53] when 1 288 gene products were identified (most of them present in very low traces) from a red blood cell lysate by loading about 5 700 mg of proteins onto 1 mL of CPLL beads.

In certain cases the enrichment process could take a different aspect such as the formation of novel high-abundance species when the amount of exposed proteins to the beads is very large (see Rivers et al., 2011)[54] as for instance in chicken pectoral muscles proteins. Nevertheless the compression of the dynamic protein concentration range was always produced with a shallower protein abundance distribution.

For a given overloading the initial conditions play also an important role. The nature of the equilibration buffer, the environmental pH and ionic strength, the temperature, and the presence of added chemicals influence the affinity constants of protein prays versus peptide ligand baits. All these variations will be discussed in the next paragraphs. What is important to keep in mind here is that a rigorous initial sample treatment is the price to pay for high result reproducibility, as described for the urinary proteome.[55]

From the beginning of the technology exploitation, the exhaustive recovery of proteins by elution has been a true dilemma. Various methods have been proposed with their merits and their limitations. Desorbing captured proteins implies the dissociation of various interactions, among them the most important being electrostatic, hydrophobic, and hydrogen bonding. They are present under various configurations: isolated and dominating the entire interaction or all together when allowed by the configuration of the interacting protein epitope. Since all possible combinations of interactions are induced by the peptide library, a general method with capabilities to weaken the association is hard to find. To understand the situation it is useful to make a brief recall of what happens at the molecular level. With CPLLs, covalent bonds are not considered since cysteine is never used for the preparation of hexapeptide ligands.

Physicochemical forces are essentially from the side chains of amino acids forming the peptide ligands and the exposed protein surfaces. The solid phase library comprises by definition all possible structures in a sort of structural continuum from homogeneous hexapeptides to highly heterogeneous ones. It ranges from very acidic to very alkaline structures and from

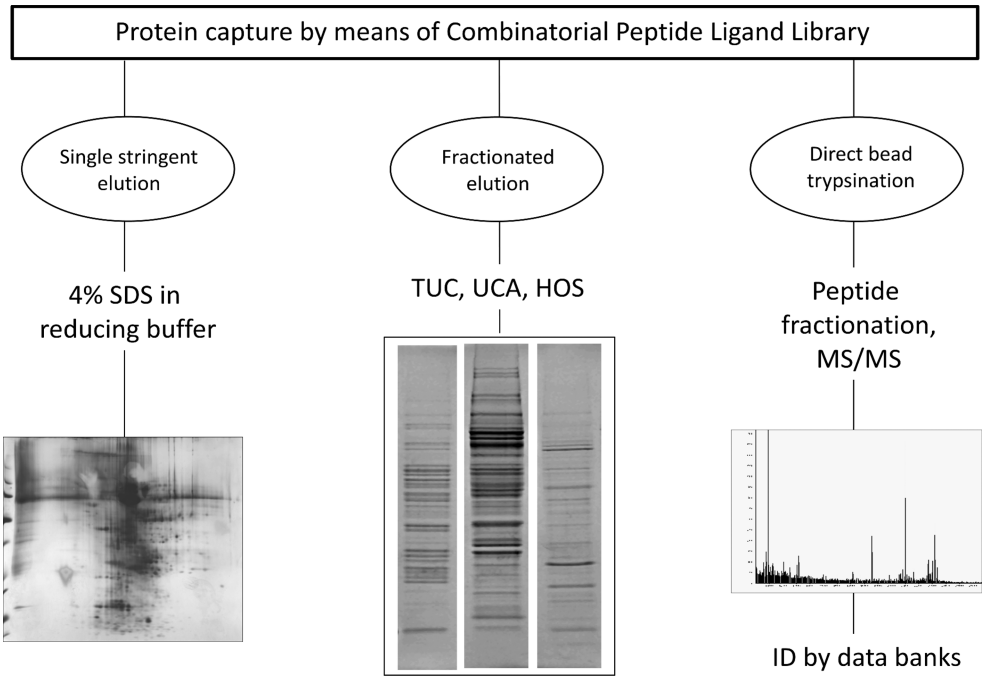

Figure 3. Protein elution options offered to the users of CPLLs independently from the conditions of capture. From the left to the right: (i) Global hemolymph protein desorption using a SDS-DTT solution at boiling temperature. The two-dimensional electrophoresis results are adapted from D'Amato et al.[58] (ii) Fractionated elution of a whole red blood cell lysate analyzed by SDS-PAGE. From left to right are proteins collected according to the sequence indicated above the picture (TUC, 2 M thiourea−7 M urea−4% CHAPS; UCA, 9 M urea−50 mM citric acid titrated to pH 3.3; HOS, 6% acetonitrile−12% isopropanol−10% ammonia−72% water). Extracted and adapted with permission from ref 53. Copyright 2008 American Society for Biochemistry and Molecular Biology. (iii) Direct trypsination of CPLL beads carrying proteins (no elution) in prior to fractionation by HPLC and protein identification by LC−MS/MS.

nonionic hydrophilic to nonionic very hydrophobic assemblies. Moreover the amino acids used as building blocks for the library comprise more than one specimen of each category enlarging the p*K* ranges as well as the hydrophobicity degrees. The most probable representative forces for the attraction between the ligand bait and the protein pray are electrostatic interactions. They are dependent on the net electrical charge of both the protein and the side chains of the peptide ligands at a given environmental pH value. The pH is a fundamental parameter for interaction because it drives the net charge values of the peptides and proteins due to their amphiphilic nature (possible concomitant presence of positive and negative charges randomly distributed). Depending on their isoelectric point, it is frequent to find polypeptides that have opposite complementary charges at a given pH to attract to each other or having the same charge (positive or negative) at another pH resulting in a sort of repulsion. Complementary electrical charges tend to associate and similar ones tend to repulse. Electrostatic interactions are at the basis of many mechanisms of biological communication. Beyond the pH value, electrostatic interactions are modulated by other physicochemical parameters such as salts (extensively used in ion-exchange chromatography)[56] and other small and large ions. Temperature also influences the intensity of electrostatic interactions. As an example when temperature increases the electrostatic interaction is weakened while it is intensified when temperature decreases. pH, salts, and temperature can thus play singularly or concomitantly with a quite strong synergistic effect.

Hydrogen bonding, another possible interaction between the peptide library and proteins, takes its origin from two electronegative charges that share the same hydrogen atom. For instance the protonation of aspartic acid produces a donating group contributing thus to the creation of a hydrogen bond. Many examples exist within the protein structures not only of intermolecular hydrogen bonding between two distinct chemical functions but also of intramolecular situations inside the peptide bonds of polypeptides.[57] Within the context of grafted hexapeptides and proteins in solution, H-bonds take place mainly at the level of exposed side chains of amino acids when the molecular distances are compatible (the shortest the distance, the strongest the hydrogen bond, with an optimal distance of about 0.3 nm). Chemical groups participating to the formation of H-bonds are carboxyls from aspartic or glutamic acid with −OH groups such as those from tyrosine, serine, and threonine. Other participating chemical groups are ═NH with carboxyls as well as ═NH with −OH groups.

As described for electrostatic interactions, the protonation of carboxylic acids depends on the environmental pH, thus the formation of certain hydrogen bonds is quite sensitive to pH changes. Nevertheless changes in pH do not necessarily annihilate all hydrogen bondings; on the contrary, the presence of strong competitors such as concentrated urea and/or concentrated guanidine are more effective.

Hydrophobic interactions among polypeptides are due to the presence of hydrophobic amino acids, generally grouped as clusters. Since side chains of hydrophobic amino acids tend to stay away from water they associate to each other forming thus highly ordered structures. This is not really a chemical link but rather an association of cooperative molecules. Water molecules around these associations form consequently a well-structured network contributing thus to the reduction of global entropy. CPLLs comprise more or less hydrophobic structures ranging from the presence of a single hydrophobic amino acid to the presence of six of them, identical or not, assembled randomly (leucine, isoleucine, valine, and phenylalanine). The strength of

hydrophobic associations depends on the configuration of the protein epitope, on the one hand, and on the hydropathy of the considered amino acid association, on the other hand. These interactions are modulated by external parameters such as the presence of structuring salts from the well-known Hofmeister's series that reinforce the links; they are also modulated by the modification of the temperature. An increase of temperature up to a certain level reinforces the interaction. Contrary to electrostatic interactions and hydrogen bonding, hydrophobic associations are not strongly influenced by the environmental pH. The disassembly of these peculiar molecular interactions is produced by agents capable to destructure the water network around the association and also by competing hydrophobic molecules such as heavy alcohols, glycols and detergents. A reduction of environmental salts may also contribute to weaken the strength of hydrophobic associations.

In practice, within the context of the present described technology, the grafted hexapeptides mostly comprise the same structural chemical groups capable to interact as a mixed mode. Thus, electrostatic interactions, hydrogen bonding, and hydrophobic associations are concomitantly present. This peculiar situation is very important because it drives the search for the elution conditions. In fact while trying to elute proteins by an increase of the ionic strength (addition of salts), only highly ionic species are harvested because salts may reinforce hydrophobic associations and have no or minimal effects on H-bonds. On the contrary when attempting to dissociate hydrophobic interactions, using for instance heavy alcohols or glycols, there is no effect on electrostatic interactions. This is also the case when decreasing the ionic strength which may in return even promote the reinforcement of electrostatic interactions. Taken globally the situation of the harvesting of CPLL-captured proteins depends on the capability to design elution compositions with complex effects capable to address all possible situations and working synergistically. Alternatively, a sequence of desorption solutions could be used, each of them addressing a given category of interactions the one after the other. Figure 3 illustrates examples of protein elutions from CPLLs. Desorption protocols have been recently updated and described in detail.[59,60]

Various published papers describe elution method options, including the complete protein trypsination directly on the beads, circumventing thus the elution dilemma.[42,61,62] Protein desorption must address at the same time (or sequentially) various molecular interactions that stabilize the protein–ligand complex. Salt solutions can easily dissociate ion exchange charge-based interactions; however, they intensify hydrophobic associations by the consequence of water structuring. The opposite effect is obtained when attempting to weaken hydrophobic interactions. Nonetheless solutions resulting from compromises have been obtained such as acidic and alkaline concentrated urea solutions,[63] urea–thiourea–detergent mixtures,[51,64] Tris–urea–thiourea–CHAPS cocktails,[65] and acidic solutions containing either sodium chloride or nonionic detergents. These approaches should be completed by a final wash with a deforming solution generally used in affinity chromatography for the dissociation of antigen–antibody complexes which is a 100–200 mM glycine–HCl buffer at pH 2.2–2.6.

Other possible chemical agents capable to desorb all proteins together is a concentrated solution of guanidine (6 M guanidine-HCl)[66] and urea–thiourea–cysteic acid solutions.[67] The efficiency of the former is based on the high ionic strength to dissociate electrostatic interactions and the strong chaotropic effect able to disrupt the water network around hydrophobic associations and to compete with H-bonds. The mechanism of the second proposal is also based on its strong chaotropic effect, which is associated with a quite low p*K* but compatible with two-dimensional electrophoresis.

With the combinatorial composition of peptide ligands present in huge number, a sort of a continuum of affinity interaction constants is present for all or almost all proteins from the biological sample probably throughout several orders of magnitude. This explains why it is not easy to find a general rule for desorbing all captured proteins at once.

Against the difficulty of collecting all captured proteins at once, a very effective alternative approach has been proposed.[55] Here the paradigm has been changed, and instead of trying to elute by means of competing agents, the use of sodium dodecyl sulfate (SDS) has been described. SDS is a detergent with a hydrophobic tail of 12 carbons and a strong anionic head (sulfonate group) with the property to strongly interact with proteins with resulting exposure of a whole negative charge in all cases. This is a single step protein desorption involving not only SDS but also some amount of reducing agents. Proteins are demonstrated as being quantitatively desorbed by consequence of the fact that sodium dodecyl sulfate micelles coat the polypeptide chains and deliver a full negative charge to all proteins, thus preventing their adsorption to surfaces.[55] Operationally this treatment is performed while heating; the presence of reducing agents, such as dithiothreitol, prevents the spontaneous formation of disulfur bonds among desorbed proteins. Although with such an approach all proteins are collected, they are embedded within SDS micelles that require to be eliminated to comply with the analytical methods that follow, such as two-dimensional electrophoresis and/or mass spectrometry and/or enzymatic treatment. This is the reason why simple protocols of the elimination of this detergent have been described.[68] Figure 4 illustrates quite clearly the cumulative desorption effect of SDS compared to a sequential elution where each solution partially contributes to protein desorption. The complete collection of proteins captured by the beads is actually a key factor to obtain all very-low abundance proteins as demonstrated by Di Girolamo et al.[69] Otherwise the interpretation of experimental results could be wrong or biased by apparent poor performance as described. All the above clearly teaches that is possible to maximize the performance of CPLLs by unambiguous overloading conditions followed by an exhaustive method for protein harvesting.

INITIAL CONDITIONS: A WAY TO DRIVE THE FOCUS TOWARD GROUPS OF LOW-ABUNDANCE PROTEINS

First of all it is recalled that biological samples comprise not only proteins (the target of the present technology) but also many other small and large molecules. This being said, the efficacy of CPLL technology can be hampered by the presence of species that prevent a proper capture of proteins and pollute the protein sample. Namely, they are sticky macromolecules such as polysaccharides, especially acidic ones (e.g., heparin), nucleic acids, polyphenols and lipids. It is advised to remove all these molecules prior to contacting the protein solution with peptide libraries. A number of simple removal methods are available; for protocol details see Boschetti and Righetti.[15] From a "cleaned" protein solution, the most common procedure for capture is to load the sample directly to the CPLL beads. This apparent easy operation must be under full control to ensure a good reproducibility. In fact the initial sample may not always be in

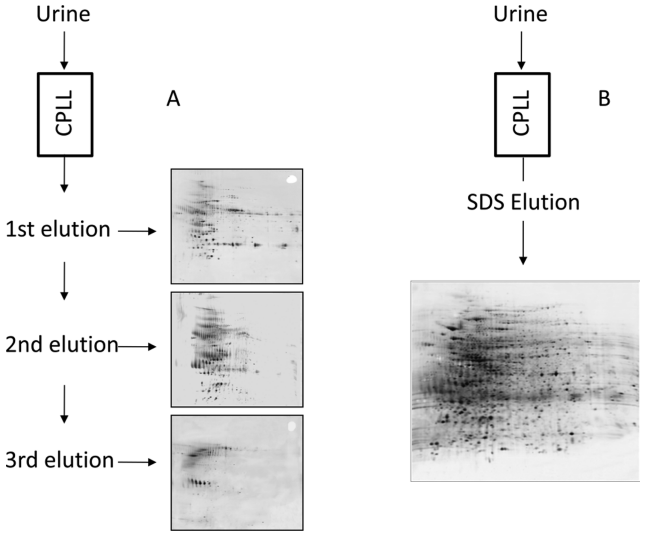

Figure 4. Efficiency of various protein elution methods applied to human urinary proteins. Panel A represents three sequential (and incomplete) protein desorption methods by the images of 2D electrophoresis patterns compared to a single exhaustive elution using a boiling solution of sodium dodecyl sulfate (panel B on the right). The sequential elution was obtained by three increasingly stringent agents: 2 M thiourea−7 M urea−2% CHAPS (first elution); 9 M urea−50 mM citric acid pH 3.5 (second elution); 6 M guanidine-HCl, pH 6 (third elution). Data and their representation are modified with permission ref 55. Copyright 2009 John Wiley & Sons, Inc. The pH gradient of 2D electrophoresis analysis was between 3 and 10 nonlinear; the protein spots detection was obtained by silver staining.

the presence of the same amount of salts and the same pH. Although there are samples that are probably very similar whatever the source (e.g., human serum), other samples, like urine, may vary during the day in protein composition, ionic strengths, pH, and contaminants. A quite significant difference among urinary proteomes treated with CPLL as they are or processed after dialysis against a phosphate buffered saline has actually been demonstrated. Therefore, the use of standardized conditions is in practice necessary. This fact suggests also that, by modifying purposely the initial conditions, different proteomes can be obtained with the "amplification" of a group of low-abundance proteins that may not be the same if initial conditions are altered. This is the case when the pH is modified, on the one hand, and when the salinity conditions are altered, on the other hand.

The first option can be used to promote the capture of dominantly alkaline proteins in alkaline conditions and dominantly acidic proteins in acidic conditions.[70] This approach has been adopted in a number of cases with the objective of enlarging the domain of protein capture. From a crude extract of cypress pollen, new allergens have been discovered upon use of CPLLs at three different pHs. The analysis of harvested proteins has been performed by SDS-polyacrylamide gel electrophoresis and two-dimensional electrophoresis followed by immunoblots against the serum of allergic patients.[71] The success of the extension of pH protein capture was confirmed in a number of other cases as for instance when analyzing the proteome of the hemolymph of *Limulus polyphemus*[58] where the capture of proteins at pH 4 and 9 together allowed an increase of gene products of about three times. Similarly a substantial increase of novel gene products has been obtained from various biological extracts by using different capturing pH values (including very acidic conditions, e.g., pH 2.2) such as in spinach (*Spinacia oleracea*) leafs,[72] in avocado[73] and banana pulp[74] with the identification of more than 1000 proteins, in globe artichoke,[75] and in a number of other cases.

The initial ionic strength may also modulate the capture of proteins. While the global binding capacity differs (binding capacity for serum proteins increases when the ionic strength decreases), proteins behave differently depending on their own physicochemical properties. It has been reported for instance[54] that for some proteins (titin and lactate dehydrogenase), the capture capacity varied considerably: it was increased when augmenting the ionic strength. In this particular example out of 222 proteins captured, 41 were exclusively bound at low high ionic strength, 107 proteins were bound at high ionic strength, and 74 were bound under both conditions.

Initial conditions could also comprise the presence of lyotropic salts that favor the hydrophobic associations between appropriate peptides and hydrophobic patches of the proteins from the sample. This procedure has been used to enlarge the capture coverage with standard hexapeptide ligand libraries. Interestingly it has been found that the method allowed to capture the proteins that were considered lost (detected in the initial sample but not in the CPLL-treated sample).

Most peptide ligands carry neutral, ionic, and hydrophobic amino acids. Even for those peptides comprising five or six amino acids with hydrophobic side chains, the overall hydrophobicity is modulated by the hydrophilic backbone and the terminal hydrophilic primary amine reducing thus the probability of hydrophobic binding events especially for mild hydrophobic proteins. However, by forcing the system to operate under a hydrophobic capture mechanism in the presence of lyotropic salts, the hydrophobic association is largely promoted like in hydrophobic chromatography using a hydrophilic support on which hydrophobic hydrocarbon chains are grafted. The salt environment is here constituted of 1 M ammonium sulfate, a strong structuring agent bringing the proteins close to "salting out".

Experimental data obtained while varying the initial conditions, especially pH and lyotropic conditions, unambiguously support the idea already suggested by Rivers et al.,[54] hypothesizing the possibility of using CPLLs as a genuine "two-dimensional" technique, operating either by dominant electrostatic (different pHs) or dominant hydrophobic binding. The latter possibility is true especially if induced by lyotropic salts from the Hofmeister's series (typically around 1 M). The validity of this approach has been recently reported[76] where the authors compared the populations of proteins captured in the presence of ammonium sulfate with those obtained using current physiological conditions. The two populations of proteins are reported as being completely different upon the comparative analysis of the ionically driven adsorption versus the hydrophobically engendered capture. The comparison of Gravy indexes have also been made; however, considering that all these proteins are in solution (human serum), the hydrophobicity degree difference is difficult to evidence. In spite of this situation it was clearly possible to distinguish at least five proteins Apo A2, Apo C2, Apo F, Apo L1, and Apo M whose main function is to bind hydrophobic ligands all of them only found upon capture under lyotropic conditions. Other lipoproteins such as Apo A1, Apo A4, Apo B, Apo C3, Apo D, Apo E, Apo A, and Apo H were also found predominantly in the same treated sample, but they are present as well, albeit in trace amounts, on the physiologically treated CPLL sample. This is not abnormal because they are

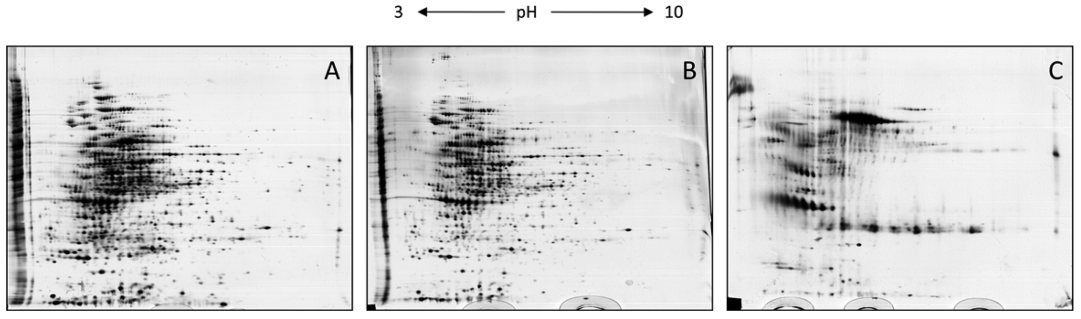

Figure 5. Two-dimensional electrophoresis analysis of a serum protein eluate using 10 mM EDTA in PBS from CPLLs in the presence of copper ions. (A) Control using native CPLLs and native serum sample; (B) CPLLs were first loaded with a solution of 20 mM copper acetate and the copper excess washed away using 50 mM sodium chloride. Then the peptide library was loaded with human serum and washed to eliminate the excess of proteins. (C) CPLLs were used as usual with no prior treatment; however, the serum sample was added with 5 mM of copper acetate and then loaded on CPLLs. The protein elution was performed in all cases using 10 mM EDTA in PBS. This eluting agent desorbs only partially the captured proteins; other still retained species were desorbed using a SDS-DTE solution as described.[55]The volume of CPLL beads used was 100 μL; the volume of serum sample loaded was 1 mL. Protein staining was with colloidal Coomassie blue.

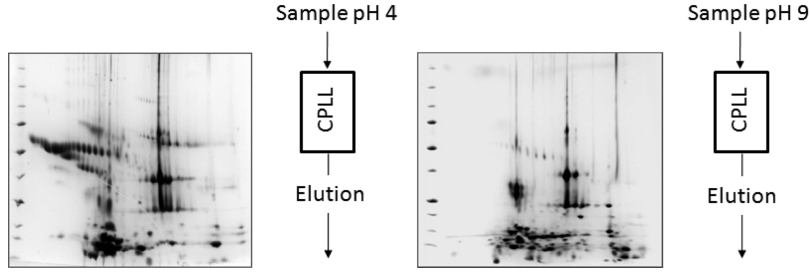

Figure 6. Influence of environmental pH on the adsorption pattern of proteins using commercial CPLLs. This example illustrates the behavior of snake *Bitis arietans* venom. When the protein capture is operated under acidic conditions (pH 4.0 in this case) more acidic proteins are captured. On the contrary under alkaline pH (9.3 in this example) basic proteins are favored. 2D electrophoresis analysis performed within a p*I* range between 3 and 10 and a mass range from 10 to 250 kDa. Spot staining was performed using colloidal Coomassie blue. Adapted with permission from ref 70. Copyright 2010 John Wiley & Sons, Inc.

freely circulating proteins in solution and hence have amphiphilic properties allowing also ionic interactions. Another interesting finding which confirms the hydrophobic character of the capture is that the identified novel species comprise mostly basic proteins. From physical chemistry it is in fact admitted that carboxylic groups from acidic species are more hydrated than amino groups present in alkaline proteins, the latter thus being slightly more hydrophobic.[77] This study allowed decreasing very largely what was qualified as "lost species" that were indicated as being unable to find hexapeptide ligands with affinity within the CPLL library.

As a general comment of this section it should be strongly underlined that the initial conditions for CPLL utilization are of outmost importance to maximize the capturing capability while enlarging the coverage to species that are very acidic or alkaline as well as to amphiphilic ones.

From all the above-described behavior it is clear that initial conditions can have a significant influence on the protein capture pattern. At this point, since imagination of scientists is now well stimulated, new opportunities are opened to extend the range of the reduction of protein dynamic range by for instance comparing the capture at different temperatures or building cases in the presence of various additives such as metal ions, dyes, sugars, amino acids, and other chemical agents.

As an illustration of this approach it has been demonstrated that by adding copper ions to a biological fluid containing proteins in solution it was possible to find different patterns (unpublished data). Interestingly when the copper ions were added to the CPLLs before sample loading, the capture of proteins from human serum that are desorbed by EDTA comprised fewer proteins than the control. Most, if not all, of them were also found in the control. However, when the sample was loaded with copper ions before contact with untreated CPLL, the protein pattern observed was largely modified: the number of proteins was lower but the number of isoforms was largely enhanced (see Figure 5). These variations on protein behavior suggest differences in properties and may deserve to be further studied to understand the common properties at the basis of this group.

In another example when the solid phase library was coated with bromophenol blue, the number of proteins and their positioning within the two-dimensional electrophoresis plate was significantly modified. Actually it appeared that many more acidic and low mass proteins were captured. The common property at the basis of these proteins remains also to be elucidated (work in progress).

CHEMICAL MODIFICATION OF PEPTIDE LIBRARIES AND EFFECT ON PROTEIN CAPTURE

Structurally hexapeptides of CPLLs are attached on the substrate by means of their terminal carboxylic group leaving free at the other end a primary amine.[49] The latter can easily be chemically modified with changes at the level of the affinity constants for their protein partners and also possible modulation of the specificity to the profit of other proteins. The chemical modification is intended not only at the level of all α-positioned terminal amines of the most distal building block but also to the primary amine of the lysine side chain residues (5–7% of

hexapeptides carry at least one lysine residue). For instance the acetylation of these amines is very easy by using acetic anhydride. As described in a recent book,[15] the resulting effects of such chemical modifications confer not only different physicochemical properties (modification of the titration curves) but also have functional effects. While acetylation has only a modest effect on the CPLL (neither significant modification of polarity nor changes in the hydrophobicity), a more in-depth change can be observed by a succinylation reaction[78] with more dominant anionic character, higher hydrophilicity, and large modification of the titration curve. This chemical modification is strong enough to change the protein pattern of captured proteins as assessed by one- and two-dimensional electrophoresis (see Figure 6). In several published papers, a side-by-side comparison was made between a regular amino-terminal CPLL and its succinylated version. Most generally a quite large number of proteins captured are common; however, the total number of gene product found is substantially increased by the fact that the succinylated version captures other proteins exclusively. Needless to say that by that way one of the drawbacks of the technology mentioned in the first paragraph of this paper, which is that around 10% of known proteins are lost, could be largely but not completely resolved.[79] Figure 7 assembles several

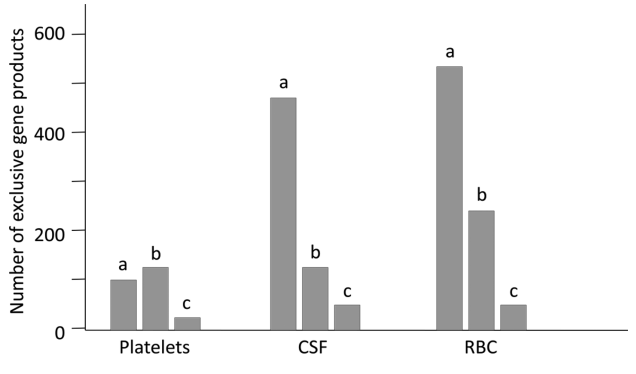

Figure 7. Number of proteins exclusively found upon utilization of either a primary amine terminal peptide library (a) or a carboxylic succinylated version of the same library (b) as well as proteins that were not found in the eluates but identifiable from the nontreated initial sample (c). The latter are qualified as "lost" proteins that should have been captured by the library. The same experiment performed in physiological conditions was made using a human platelet extract, a human cerebrospinal fluid (CSF), and a human red blood cell lysate (RBC). It is to be noticed that the % of lost proteins calculated on the basis of total proteins found before and after CPLL treatment represents, respectively, 5.8%, 5.1%, and 3.6%. Data was reconstructed from ref 15.

experimental data supporting this phenomenon. An in-depth analysis of novel proteins detected by the succinylated version of CPLL revealed that the additional captured proteins belong to a different population of species. This has been elegantly illustrated by placing proteins in a graph crossing over the isoelectric point and the hydrophobic Gravy index scales.[64] Basically, exclusive gene products captured by a primary amine terminal library were mostly located within the acidic space (p*I* 4–6 range); on the contrary species from the succinylated library were mostly located in the alkaline space. Only a few exceptions were found. Other proteomes (egg white,[80] bile fluid,[81] red blood cell lysate,[53] and cerebrospinal fluid[82]) have been analyzed in parallel with these two libraries with similar conclusions. Figure 8 illustrates the protein patterns of two proteomes. The interpretation of these data suggests that the differential effect of these libraries is not due to a dominant ion exchange effect because the experiments have been operated under physiological conditions of pH (neutral) and of ionic strength (150 mM sodium chloride). In fact the dominant negative charge of the carboxylic version is only marginal to the overall charge of hexapeptides that comprise also unmodified basic amino acids such as arginine and histidine. Nevertheless the modification of the structure induces a modification of molecular interactions and hence a profound change of affinity constants of the captured proteins.

COMPLEMENTING THE CURRENT CPLL TO ENLARGE THE PROTEIN CAPTURE

Since chemically modified CPLLs used under the same buffering conditions deliver quite complementary results or at least they allow discovering additional species that are exclusive of each modified library, the open question is whether such libraries could be used as a mixture with the same effect. As a corollary, a second question would be about the proportion of such a library mixture. A direct benefit of this approach would be time saving with just a single operation instead of two in parallel or in series (see ref 15, chapters 4.5.7 and 8.11).

To confirm the expectations of the use of library mixtures, some practical experiments have been performed. A carboxy-terminal library and a primary terminal library have been mixed in various proportions (100% each, 25–75%, 50–50%, and 75–25%) and used under comparative manner, namely, in physiological phosphate buffered saline. Desorbed proteins have then been analyzed classically by SDS-polyacrylamide gel electrophoresis. Interestingly each library used alone delivered a specific protein pattern while library mixtures delivered an overlapping of results depending on their proportions within the mixed bed. Whether this delivers similar results when compared to CPLL used under acidic and alkaline conditions has been extensively discussed with the assumption that from the ionic standpoint the two approaches would be substantially equivalent. Actually instead of ionizing the library by chemical modifications, the ionization would be induced on proteins by using different initial pHs. It is however argued that even quite mild ionization conditions (pH ranging from around 4 to around 9) could have some little influence on the protein folding with possible small modifications on hydrophilic–hydrophobic balance, hence minor modifications on the protein pattern are expected. The above-described experiment opens the way to many potential alternatives, the most powerful one being the use of various mixtures of two or more complementary libraries. Moreover these mixtures could be used at different initial conditions as described in one of previous sections with the possibility to enlarge the spectrum of low-abundance protein discovery.

TOWARD A MULTIDIMENSIONAL BIOLOGICAL SAMPLE TREATMENT?

The idea of performing a sort of orthogonal scanning of low-abundance proteomes came from a paper published by Santucci et al.[76] where a description of the enhancement of hydrophobic species was approached by the use of lyotropic initial treatment conditions (see paragraph above). This opposes the enhancement of hydrophilic traces of proteins obtained when using low ionic strength or physiological buffers. On a similar token, by using opposite pHs as initial conditions of treatment it is possible to focus on either acidic or alkaline species. Therefore, to cover

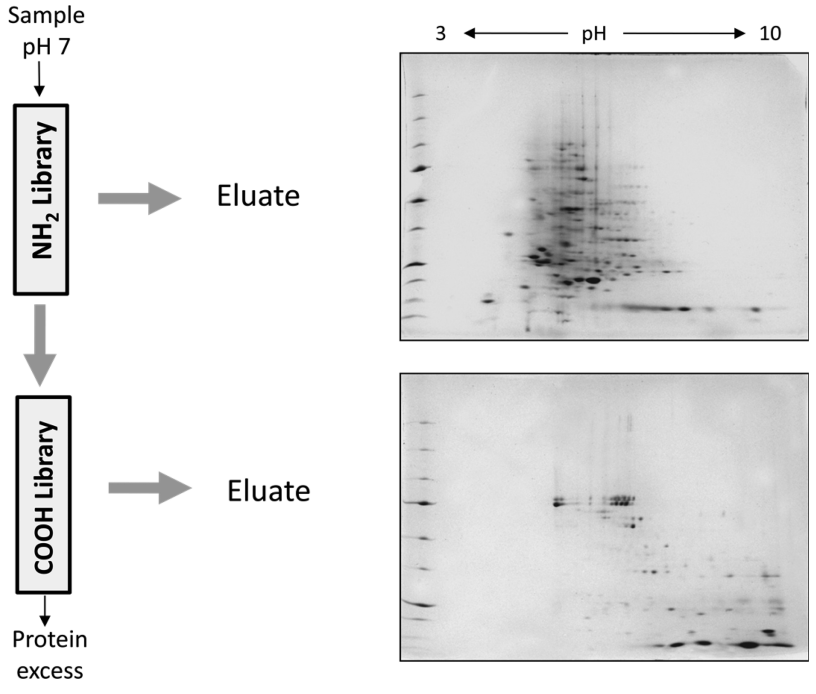

Figure 8. Two-dimensional electrophoresis analysis of a red blood cell lysate treated with two peptide libraries in series as illustrated on the scheme on the left. The first column was a primary amine terminal peptide library and the second a carboxylic succinylated version of the same. The amount of sample was very large exceeding by far the binding capacity of the beads. Notice the significant difference of isoelectric points of harvested proteins that were predominantly acidic with the primary amine library while proteins collected from the second library were predominantly alkaline. The isoelectric focusing gradient was between pH 3 and 10; the second dimension masses were from 250 to 10 kDa. Protein elution from beads was implemented by 9 M urea, 50 mM citric acid titrated to pH 3.3. Staining was with colloidal Coomassie blue. Adapted with permission from ref 15. Copyright 2013 Elsevier.

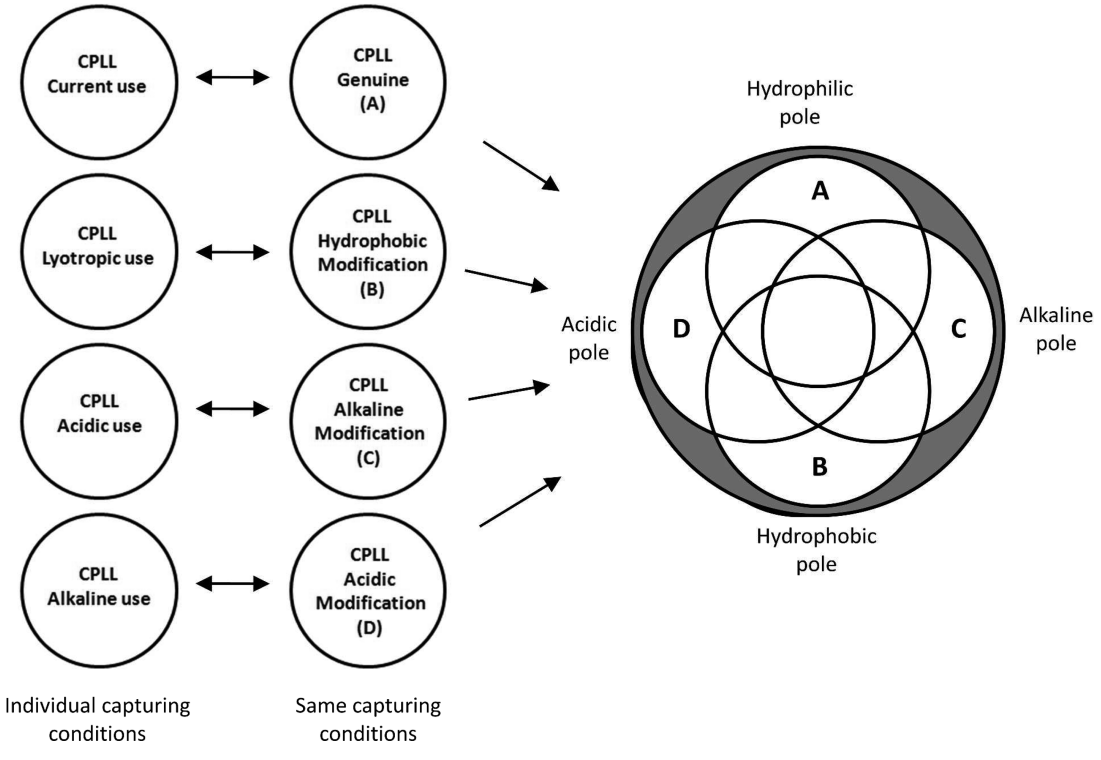

Figure 9. Schematic representation of a possible process toward a larger protein coverage from four distinct separated operations to a single sample treatment step (see details in the text).

the "map" four distinct operations are necessary, which is a work-intensive approach particularly when one wants to extend the study to differential proteomics. In addition, the large overlapping of proteins pollutes the interpretation of results and to obtain a whole picture of the situation a sort of virtual subtraction of redundant proteins must be made. Nevertheless this "two-dimensional" exploration of proteomes has a somewhat fascinating side that is its capability to attack all "extremities"

of a proteome even for proteins that are at the most distant positions of a hydropathy-pH map and are of low abundance.

The question is whether it is possible to obtain such a collection of proteins at once under close-to-physiological conditions. In other words, is it possible to cover all the extent of the protein diversity using a single treatment with a library? What has been demonstrated in a recent past is the capability of capturing alkaline species after having modified chemically the peptide library by the introduction of carboxylic acids at the level of primary amines (see previous section). The use of a mixture of such a modified library and the unmodified library allowed obtaining the sum of individual libraries. Although this is still to be quantitatively demonstrated, the trend is there. It is by consequence postulated that an addition of a second chemical modification with the introduction of cationic groups such as guanidine or even quaternary amines would further enlarge the low-abundance protein coverage. With this approach the question related to the extension of the library power to the largest pH coverage should be resolved especially if, instead of carboxylic groups, sulfonates functions are introduced.

The question of hydrophobic protein capturing is a second dilemma that remains to be resolved; however, hydrophobic chromatography teaches that by elongating the hydrocarbon chain it is possible to reduce the amount of lyotropic salts or even eliminate them. In such conditions it appears possible to introduce into a peptide library certain hydrocarbon chains (whose length needs to be determined) at the level of primary amine where the grafting reaction is relatively simple. Also in this case the resulting partially hydrophobic library should be mixed with the standard CPLLs to obtain a mixed bed capable to capture both hydrophilic and hydrophobic proteins. The dilemma of the second dimension would thus be resolved.

The last step would be to make a mix of four libraries: the current CPLL, the acidic version, the alkaline version, and the hydrophobic version as illustrated schematically in Figure 9. Such a mixture should capture all protein species using a single step followed by a single elution step. The protein desorption could also be fractionated in case one wants to elute sequentially acidic, alkaline, or hydrophobic species. Although this is not yet published, various available results suggest this trend which is with no ambiguity a way to empower the use of CPLL as a large concept. Experimental work is in progress in this direction.

CONCLUSIONS

Although there is considerable literature about the reduction of protein dynamic range compression using CPLLs, very little is written about the extension of the technology in view of increasing the protein coverage beyond what is the current expectation. A collection of data from published results associated with original experimental observations demonstrates the great potential of CPLLs toward certain categories of proteins generally ignored. The association of immunodepletion with CPLL has been described in various reports. However, nothing was indicated and demonstrated about what would be the ideal sequence of these operations. The book dedicated to CPLL technology[15] reports a special discussion on the question. In spite of the fact that this association appears as quite a nonsense, published papers investigated not only their respective merits,[66,83−85] but more importantly recently some authors compared the two possible sequences involving immunodepletion and enrichment with CPLL.[86] Both sequenced approaches have been compared to the singular technologies. The authors concluded that the best way to go is first to use immunodepletion followed by CPLL treatment. Reported data highlighted the importance of this sequence for a more powerful way to discover biomarkers in human plasma samples with good reproducibility.

All-in-all, however, a note of caution should be here stated on the proper use of CPLLs. It has been mentioned above that the matrix in which the various proteomes are embedded could have strong adverse effects on the CPLL capture (notably lipids, DNA, polysaccharides, polyphenols, and many other compounds present especially in plant extracts) and thus efforts should be made to eliminate them (or minimize their concentration) prior to CPLL treatment. To those one has to add in biological fluids the presence of pigments (e.g., bile pigments in urines) which are strongly adsorbed by the CPLL beads, thus hampering proper protein capture. These pigments are not easily eliminated by dialysis, since they are often adsorbed onto urinary proteins. One effective way to minimize their presence is to extract them with butanol. Another strongly interfering substance, typically found in nonalcoholic (e.g., Coca Cola, Pepsi and the like) as well as alcoholic (e.g., aperitifs and digestives) beverages is caramel. Caramel is a product of oligomerization of sugars, as induced by heat but also by chemicals. Such oligomers are disperse in size and their removal is not an easy proposition. Because of the fact that most caramels contain negative charges, they are strongly adsorbed to CPLLs, thus reducing their ability to capture proteins. Thus, one has to be aware that in plenty of samples there could be many "effectors" strongly interfering with CPLL treatment and that, for best results, their presence has to be assessed and every effort has to be made for their removal in order to maximize protein capture via this methodology.

The global analysis of the situation along with newly described concepts may constitute an unexplored or improved way to detect low-abundance protein expressions for a strong empowering of CPLL exploitation with additional discoveries of pertinent protein markers for a variety of metabolic alterations. In addition, the presented data also suggest rational modifications of the current peptide libraries with better performance and potential routes for further developments.

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Notes

The authors declare no competing financial interest.

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