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Optimized sample treatment protocol by solid-phase peptide libraries to enrich for protein traces

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Abstract Combinatorial peptide ligand libraries have been extensively used for the enrichment of very low-abundance proteins, while concomitantly reducing the concentration of major species. A number of biological extracts have been reported with great success. Nevertheless, there are examples where the enrichment was not as good as expected. It has been demonstrated that the protocol itself may be responsible for the final results and is not necessarily applied as it should. In this paper, technical details along with important suggestions are given for an optimized enrichment of gene products present at trace levels among very many other proteins.

Keywords Protein enrichment · Peptide libraries · Dynamic range · Proteomics · Protein markers

Introduction

It is already almost 8 years since the pioneering paper on the use of solid-state combinatorial peptide ligand libraries (CPLL) for the reduction of the dynamic concentration range of proteins from various biological extracts (Thulasiraman et al. 2005) has been first reported. This was, and interestingly still is, a real need, called enrichment of protein traces. Finding polypeptides that are currently undetectable because of their extremely low concentration compared to high-abundance proteins (in biological fluids whose dynamic range can span over 12 orders of magnitude) is actually a critical step in elucidating biological pathways (Freour et al. 2013), protein–protein interactions, detection of early stage protein markers from biological disorders (Meng et al. 2011; Glaser 2011) and also finding traces of species representing impurities within biopharmaceutical products and/or food/beverages (Agrawal et al. 2013).

This technical process is based on the capability of solid-phase ligand libraries, established as a mixed bed, to address and adsorb all proteins present in a sample. This straightforward approach becomes extremely powerful when the initial protein load exceeds by far the binding capacity of the individual sorbents (see principle below). When a large sample is put in contact with a limited volume of a mixed bed of a peptide ligand library the harvested proteins after desorption have a similar composition as that of the initial sample but the concentration difference among species is largely diminished. By that way many protein traces are significantly enriched. The success of the process is, however, highly dependent on adsorption and elution conditions, both critical for the final result.

The thermodynamic, kinetic and physicochemical mechanisms at the basis of the molecular process of protein binding involve a quite large number of parameters extensively described in a recent book (Boschetti and Righetti 2013) to the point that it is necessary not only to explain the theoretical fundamentals, but also to give practical recipes to the end users.

The exploitation of this very peculiar but powerful principle is in fact not very obvious for non-initiated scientists with limited experience in chromatographic interactions and affinity separations. This is why the success of CPLL was obtained at a price of multiple descriptions of

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the process attempting to demonstrate the mechanisms of action, and to show real applications to a large number of biological materials (Righetti et al. 2010a, b; Righetti and Boschetti 2012, 2013). In spite of the published documents and the detailed description of the technology, there are still reports where the technology is applied far from optimized (and often wrong) conditions.

Since several approaches could be taken for the capture and elution phases, various methods have been devised; over time the methodology was optimized, which means that the recommended protocols that have been published in 2008 (Guerrier et al. 2008) are now somewhat outdated.

With under-optimized protocols disappointments have been eported (Bandow 2010; Ernoult et al. 2010; Chen et al. 2013), which did not correspond to a technological problem or limitation but rather due to an irrational application of the method as elegantly demonstrated by comparative protocols (Di Girolamo et al. 2011a, b).

The present document deals with a detailed protocol for the optimized efficiency of the CPLL technology applied to proteomics investigations. The recommended protocol is not single, but can easily be adapted by the user in conformity with the recommendations and explanations given in the technical sections.

This document contemplates two main parts: (1) detailed protocols for protein capture and (2) detailed protocols for protein elution from the CPLL beads. Variants and alternatives are also described.

Technology principle

A solid-phase ligand library is a mixed bed of a large number of affinity media each of them grafted with a different ligand. In the present case the ligands are hexapeptides obtained according to the combinatorial approach defined as split-graft-recombine (Lam et al. 1991). The number of different hexapeptides depends on the number of diverse amino acids. For example with 15 amino acids the number of possible combinations is between 11 and 12 millions. Each bead carries a unique hexapeptide ligand at a relatively large density (around 80 μmol/mL of settled wet beads).

When such a mixed bed is put in contact with a large excess of a protein extract (e.g. blood serum) proteins are captured by their corresponding affinity ligand. However, the most concentrated proteins saturate rapidly the beads while the least concentrated proteins will not. The latter eventually saturate their corresponding beads as the protein loading increases. Once the system reaches equilibrium, the excess of proteins is eliminated and the beads with their corresponding captured proteins are separated. Desorption of adsorbed species allows harvesting a sample composed of theoretically the same protein mixture but with a dynamic concentration much reduced. Low-abundance proteins are hence largely enriched compared to the initial high-abundance ones which, in turn, are diluted.

A number of parameters govern this technology. For instance, the extent of the reduction of dynamic concentration range depends on the amount of proteins offered to a given volume of beads. Environmental conditions (pH, ionic strength, temperature, chemical agents) markedly influence the affinity constants of proteins for their peptide partner. The hexapeptide ligands are complex structures acting via various interaction possibilities (electrostatic interactions, hydrophobic associations, hydrogen bonding, structural docking, Van der Walls interactions, etc.) rendering the specificity for a given protein much more complex compared to a single-feature ligand. These peptide ligands are in effect mixed-mode interacting structures promoting an intense competition among proteins displaying a degree of affinity. This property is governed not only by the dissociation constant but also by the individual protein concentration by virtue of the mass action law. All of these critical points that contribute to the enrichment of low-abundance species are extensively described by Boschetti and Righetti (2013) and Righetti and Boschetti (2013).

Materials

Reagents

- ProteoMiner, a collection of peptide ligand libraries as a mixed bed is available from Bio-Rad Laboratories, Hercules, CA, USA. (www.bio-rad.com/prd/en/FR/LSR/PDP/1dd94f06-7658-4ab4-b844-e29a1342a214/)
- Sodium phosphate dibasic dihydrate (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb 71643). www.sigmaaldrich.com/chemistry/
- Potassium phosphate monobasic (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb P9791)
- Ammonium sulfate (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb A4418)
- Protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb S8820 or Roche Diagnostics, Basel, CH, USA; cat Nb 11836145001)
- Sodium chloride (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb S3272)
- Tris(hydroxymethyl) aminomethane (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb 154563)
- Dithiothreitol (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb D0632)
- Methanol (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb 34860)

- Chloroform (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb 650498)
- Thiourea (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb T8656)
- Guanidine (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb G3272)
- Iodoacetamide (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb I6125)
- Formic acid (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb 251364)
- Urea (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb U5378)
- Sodium dodecyl sulfate (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb L3771)
- Ethylene glycol (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb 85978)
- Glycine (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb G8898)
- Ammonium bicarbonate (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb 09830)
- Rapigest SF (Waters Corp., Milford, MA, USA; cat Nb 186001861). www.waters.com/waters/partDetail.htm?partNumber=186001861
- CHAPS (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb C9426)
- Citric acid (Sigma-Aldrich, Saint Louis, MO, USA; cat Nb C0759)
- Spin columns (Pierce Biotechnology, Rockford, IL, USA; cat Nb 69705). www.thermoscientificbio.com/nap

Reagent setup

The sample

This protocol applies to a large variety of biological fluids such as serum and protein extracts obtained in normal physiological conditions of pH and of ionic strength. The sample should be clear and not contain lipids in suspension such as in milk or in bile fluid. In this case, a delipidation step is recommended. Large amounts of nucleic acids or viscous polysaccharides when present should also be removed using current methods. Samples should not contain large amount of detergents or denaturing agents. For example non-ionic surfactants are tolerated at concentrations not exceeding 0.2–0.5 %; urea is tolerated at a concentration not exceeding 3 M.

- Equilibrate the protein sample with the selected buffer to be used for the incubation with CPLL beads. This operation can be achieved by dialysis or diafiltration or by desalting chromatography.

□ **NOTE**: in the event that the sample contains proteolytic enzymes it is recommended to add a protease inhibitor cocktail at a rate that is suggested by the supplier (e.g. in the case of the complete protease inhibitor cocktail from Roche Diagnostics one large tablet for 50 mL of sample solution).

CPLL beads

This solid-state material can be supplied dry or pre-wet in a buffer.

The mixed bed comprises very hydrophilic beads on where ionizable amino acids dominate. It comprises also very hydrophobic beads based on combinations of non-ionic amino acids. From these two extremes all possible combinations co-exist. Therefore, the rehydration of these beads may not proceed similarly. Before use they need to be rehydrated according to the following protocol:

- To 2 mL of methanol add 100 mg of dry CPLL beads and shake for about 30 min at room temperature.
- Add 2 mL of 25 mM phosphate buffer, pH 7. Gently shake overnight (do not use magnetic stirrers).
- Wash extensively and repeatedly the rehydrated beads with the buffer selected for the capture of proteins.
- Store at 4 °C and before use sample out the right volume of settled beads (no supernatant) that is most generally 100 μL.

If the CPLLs are supplied as aqueous slurry, the only operation to perform is to equilibrate the beads with the same buffer selected for the capture of proteins. To do so the beads have to be washed extensively and repeatedly with the buffer selected for the capture of proteins.

Buffers for protein capturing

Below the recommended buffers to be used according to the objective of the experiment are described. Different other buffers could also be used providing they do not contain chemicals that enter in competition with the interaction of proteins with the CPLL beads.

- PBS (phosphate buffered saline): dissolve 2.5 mmol (or 445 mg) of sodium phosphate dibasic dehydrate and 15 mmol of sodium chloride in 100 mL of distilled water. Dissolve 2.5 mmol (or 340 mg) of potassium phosphate monobasic and 15 mmol of sodium chloride (or 870 mg) in 100 mL of distilled water. Take 20 mL of the first solution and add the second solution up to pH 7.2 ± 0.1.
- Neutral phosphate buffer: dissolve 2.5 mmol (or 445 mg) of sodium phosphate dibasic dehydrate in

100 mL of distilled water. Dissolve 2.5 mmol (or 340 mg) of potassium phosphate monobasic in 100 mL of distilled water. Take 20 mL of the first solution and add the second solution up to pH 7.2 ± 0.1.

- 50 mM acetate buffer pH 4: dissolve 5 mmol of sodium acetate (or 410 mg) in 70 mL of distilled water. While measuring the pH add slowly dropwise a dilute solution of acetic acid (e.g. 200 mM). Once pH 4 is reached, complete to 100 mL with distilled water.
- 50 mM Tris–HCl pH 9.3: dissolve 5 mmol of Tris base (or 605 mg) in 70 mL of distilled water. While measuring the pH slowly add dropwise a dilute solution of hydrochloric acid (e.g. 200 mM). Once the pH 9.3 is reached, complete to 100 mL with distilled water.
- 1 M ammonium sulfate: dissolve 10.4 grams of pure ammonium sulfate in 100 mL of distilled water.

Elution solutions

- SDS-DTT solution: dissolve 4 g of sodium dodecyl sulfate in 50 mL of distilled water. Add 5 mol of dithiothreitol (or 770 mg) while stirring. Complete to 100 mL with distilled water.
- 1 M NaCl solution: dissolve 0.1 mol (or 5.8 g) of sodium chloride in 50 mL of distilled water. Complete to 100 mL with distilled water.
- 50 % ethylene glycol: mix 50 mL of pure ethylene glycol with 50 mL of distilled water and mix up to complete homogeneity.
- 8 M urea: To 0.8 mol of urea (this is 49.6 g) add distilled water while stirring. Complete to 100 mL total volume. The solubilization of urea may take some time since it is close to saturation. Perform this operation at room temperature.
- 200 mM glycine–HCl buffer: dissolve 20 mmol of glycine (or 1.5 g) in 50 mL of distilled water. While stirring, dropwise add a solution of 1 M hydrochloric acid to reach pH 2.5. Complete to 100 mL with distilled water.
- TUC solution (thiourea-urea-CHAPS): mix under stirring at room temperature 0.2 mol thiourea (or 15.2 g), 0.7 mol urea (or 42 g) and 4.4 g CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) in 60 mL of distilled water. Add distilled water up to a final volume of 100 mL and continue stirring at room temperature up to the complete solubilization of all products.
- Acidic urea elution solution (UCA): dissolve under stirring 0.9 mol of urea (or 54 g), 5 mmol of citric acid (or 960 mg) and 2 g CHAPS in 50 mL of distilled water. Add distilled water up to a final volume of 100 mL and continue stirring at room temperature up to the complete solubilization of urea.

Procedures

The use of solid-phase combinatorial peptide ligands covers different aspects depending on the objective and on the following analytical methods adopted to decipher the initial proteome. This is why here below the process is divided into two main blocks: the protein capture procedures and the protein harvesting procedures. Figure 1 schematically depicts the two main block-processes and their possible combinations.

Protein capture procedure

Protein capture can be operated under different physicochemical conditions and thus adapted to comply with the final goal. As extensively described (Boschetti and Righetti 2013), the extent of reduction of dynamic concentration range depends on the initial volume of the sample to treat for a fixed amount of solid-phase peptide ligand library. For commodity we describe here the example of blood serum using 1 mL of biological material and 100 µL of wet settled beads. The user has just to consider here that the volume of serum can be much larger (several hundreds of mL) with an expected much larger number of very low-abundance species that become detectable. The volume of beads, fixed to 100 µL, is here given for commodity; however, the user can adopt procedures involving larger volumes of beads with the consequence of enlarging the ligand diversity and hence the possibility to fish out from the initial sample more proteins. Nevertheless, the minimum amount of proteins to expose to beads should not be lower than 5 mg per 100 µL of beads. Using 100 µL beads the amount of proteins recovered is large enough to easily perform several electrophoresis analyses and mass spectrometry determinations.

□ **NOTE**: selection of the incubation buffer. The buffer where the protein capture is operated drives the affinity constants of all proteins to be captured by the beads. While for some proteins the affinity for a given peptide may increase, for others the affinity constant may decrease; moreover the peptide partner may be different from one environmental condition to another. The specificity is influenced by the pH, the ionic strength and the presence of lyotropic salts. Buffers of acidic pHs enhance the capture of acidic proteins, while alkaline pHs influence the adsorption of proteins with dominant basic isoelectric point (Fasoli et al. 2010). Low ionic strength, whatever the pH, promotes the electrostatic interaction of proteins with the ionizable peptide ligands (Guerrier et al. 2006; Rivers et al. 2011; Di Girolamo et al. 2011a, b). Physiological ionic strength counter-balances the ionic effect and essentially mimics the interactions that are naturally present within the living cells. The presence of lyotropic salts such as ammonium sulfate promotes hydrophobic associations and

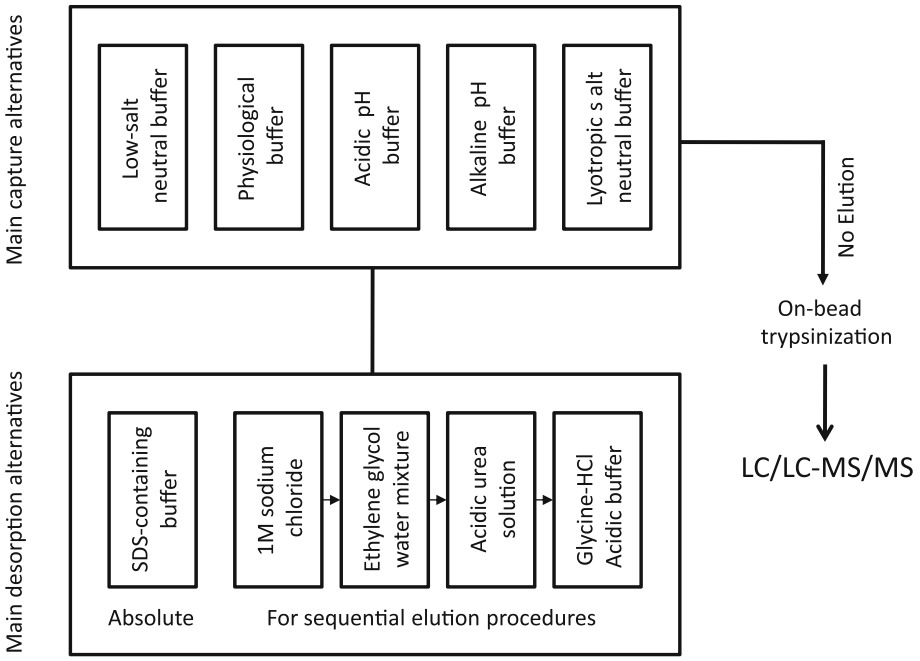

Fig. 1 Block diagram of the two main steps of the CPLL enrichment process. The described capture alternatives address different objectives as for instance to favor the enhancement of acidic proteins or hydrophobic proteins. The use of these alternatives contributes to capture all species. Both desorption alternatives focus on the full collection of captured proteins. SDS-based elution is a stand-alone approach while the sequence is a way to both collect different fractions complementary to each other and also allowing a complete desorption at the end of the sequence. A third way can also be considered: this is the determination of all captured proteins by a direct complete trypsinization on beads. In this case a tandem liquid chromatography followed by a tandem mass spectrometry identifies the adsorbed species

enhances the capture of hydrophobic proteins (Santucci et al. 2013). Nevertheless, ammonium sulfate should be used at a concentration that does not induce protein precipitation. In practice 1 M concentration is suggested.

Conditions of protein capture can thus be modulated according to the objective of the study. Therefore, this first phase is here described for each of the above mentioned possibilities (Fig. 2): under physiological conditions, at low ionic strength, at pH distant from neutrality (acidic or alkaline conditions) or under lyotropic conditions.

Protein capturing in physiological conditions

- Wash CPLL beads with PBS (phosphate buffered saline) to remove contaminants and stabilizers or preservatives. To this end suspend 100 µL of CPLL with 2–3 mL of PBS; mix thoroughly and then remove the supernatant by centrifugation. Repeat this operation three times.
- In parallel equilibrate the sample (e.g. 1 mL blood serum) with PBS by dialysis or other appropriate equivalent methodologies.

▲ **CRITICAL**: if traces of proteases are supposed to be present it is recommended to add protease inhibitors as cocktails (see "Materials").

- Mix the buffer-equilibrated CPLL beads with the blood serum and gently shake for 2–3 h at room temperature.

□ **NOTE**: temperature of incubation is critical when one wants to compare samples especially when the operations are performed over several days. It is recommended to operate under controlled temperature since it influences the affinity constant among the prey proteins and their respective hexapeptide ligands.

- Separate the CPLL beads from the supernatant by centrifugation at 2,000 g for 5–10 min. The CPLL beads are rapidly washed twice or three times using 300 µL of PBS to remove the excess of unbound proteins.
- If the CPLL beads with the adsorbed proteins are not used immediately they could be stored in the cold at 4 °C.

Protein capturing in a low ionic strength environment

- Wash the CPLL beads with a phosphate buffer (e.g. 25 mM phosphate buffer pH 7.2) to remove contaminants and stabilizers or preservatives. To this end suspend 100 µL of CPLLs with 2–3 mL of the same phosphate buffer mix thoroughly and then remove the

supernatant by centrifugation. Repeat this operation three times.
- In parallel equilibrate the sample (e.g. 1 mL blood serum) with phosphate buffer by dialysis or other appropriate equivalent methodologies.

▲ **CRITICAL**: if traces of proteases are supposed to be present it is recommended to add protease inhibitors as cocktails (see "Materials").

- Mix the buffer-equilibrated CPLL beads with the blood serum and gentle shake for 2–3 h at room temperature.

□ **NOTE**: the temperature of incubation is critical when one wants to compare samples especially when the operations are not performed simultaneously. It is recommended to operate under controlled temperature since it influences the affinity constant between the prey proteins and their respective hexapeptide ligand.

- Separate the CPLL beads from the supernatant by centrifugation at 2,000 g for 5–10 min. The CPLL beads are rapidly washed twice or three times using 300 µL of 25 mM phosphate buffer, pH 7.2, to remove the excess of proteins.
- If the CPLL beads with the adsorbed proteins are not used immediately they could be stored in the cold at 4 °C.

Protein capturing at pHs distant from neutrality

- Wash CPLL beads with either 25 mM acetate buffer pH 4.0 or 25 mM Tris–HCl, pH 9.3 to remove contaminants and stabilizers or preservatives. To this end suspend 100 µL of CPLLs with 2–3 mL of one of the selected buffers above, mix thoroughly and then remove the supernatant by centrifugation. Repeat this operation three times (Fig. 2c, d).
- In parallel equilibrate the sample (e.g. 1 mL blood serum) with one of the buffers described above used for the CPLL washing, by dialysis or other appropriate equivalent methodologies.

▲ **CRITICAL**: if traces of proteases are supposed to be present it is recommended to add protease inhibitors as cocktails (see "Materials").

- Mix the buffer-equilibrated CPLL beads with the blood serum and gently shake for 2–3 h at room temperature.

▲ **CRITICAL**: the pH of the CPLL-sample mixture must be the same as the one of the selected buffer. In case of differences it is advised to correct by adding small amounts of dilute acetic acid or dilute Tris base solutions.

□ **NOTE**: the temperature of incubation is critical when one wants to compare samples especially when the operations are performed over several days. It is recommended to operate under controlled temperature since it influences the affinity constant between the prey proteins and their respective hexapeptide ligand.

- Separate the CPLL beads from the supernatant by centrifugation at 2,000 g for 5–10 min. The CPLL beads are rapidly washed twice or three times using 300 µL of the selected buffer to remove the excess of proteins.
- If the CPLL beads with the adsorbed proteins are not used immediately they could be stored in the cold at 4 °C.

Protein capturing under lyotropic conditions

- Wash the CPLL beads with 1 M ammonium sulfate to remove contaminants and stabilizers or preservatives. To this end suspend 100 µL of CPLLs with 2–3 mL of this solution, mix thoroughly and then remove the supernatant by centrifugation. Repeat this operation three times (Fig. 2e).
- In parallel equilibrate the sample (e.g. 1 mL blood serum) with the same ammonium sulfate solution used for CPLL washing by dialysis or other appropriate equivalent methodologies.

▲ **CRITICAL**: if traces of proteases are supposed to be present it is recommended to add protease inhibitors as cocktails (see "Materials").
! CAUTION: in the presence of such a high concentration of lyotropic salt it is possible to observe some precipitation of proteins by salting-out. In this case it is advised to use a bit more diluted ammonium sulfate solutions such as for instance 0.8 M instead of 1 M. Moreover in case one or more very high-abundance proteins are present they could be removed first to reduce the precipitation risk.

- Mix the CPLL beads with the equilibrated sample and gently shake for 2–3 h at room temperature.

□ **NOTE**: the temperature of incubation is critical when one wants to compare samples especially when the operations are performed at a different time. It is recommended to operate under controlled temperature since it influences the affinity constant between the prey proteins and their respective hexapeptide ligands.

- Separate the CPLL beads from the supernatant by centrifugation at 2,000 g for 5–10 min. The CPLL beads are rapidly washed twice or three times using 300 µL of ammonium sulfate solution described above to remove the excess of proteins.

- If the CPLL beads with the adsorbed proteins are not used immediately they could be stored in the cold at 4 °C.

? TROUBLE SHOOTING

Problems	Reasons	Possible solution
Protein precipitation	Environmental conditions promote protein precipitation over time (sample unstable)	The sample must be better equilibrated with the initial solution prior to contact with the CPLL beads
High viscosity	Presence of mucins or nucleic acids that can prevent the proper capture of proteins because either they compete with the proteins or clog the bead pores	Proteins should be cleaned up by for instance precipitation with various means such as ammonium sulfate or other means as indicated in Boschetti and Righetti (2013)
Difficult to capture proteins	Possible presence of competition agents. This is frequently the case with proteins extracted from plants	See above for protein cleaning up. The protein extract should also be concentrated when possible
Formation of aggregates	Protein coagulation (e.g. blood plasma)	Addition of anticoagulation agents should be made before incubation with CPLL beads except heparin which may compete with the protein capture. The use of EDTA is recommended (Leger et al. 2011)

Harvesting proteins captured by CPLL beads

A critical key success factor of the above-described procedure is to collect all proteins that are captured by the beads.

Several approaches are possible as depicted in Figs. 1 and 3. A single exhaustive elution is based on the use of a buffer containing sodium dodecyl sulfate. When a fractionated elution is operated at least three desorbing agents are used sequentially. Alternatives for sequential elution agent's are possible as described by Boschetti and Righetti (2013).

Single stringent protein elution

- Prepare an aqueous solution of 3–4 % of sodium (or lithium) dodecyl sulfate (SDS) containing 25 mM reducing agents such as dithiothreitol.
- Mix the 100 µL CPLL beads carrying the captured proteins with 300 µL of SDS aqueous solution and mix to homogeneous suspension.
- Boil the beads suspension (boiling bath) for 5–10 min. All captured proteins are thus desorbed and solubilized in the supernatant.
- Collect the supernatant by centrifugation at 2,000 g for 5–10 min.

□ **NOTE**: the obtained protein solution is directly analyzable by SDS–polyacrylamide gel electrophoresis. In case the presence of SDS is not compatible with subsequent analyses (e.g. two-dimensional electrophoresis, MALDI) the elimination of SDS can be performed as described below (see also Fic et al. 2010).

- To one volume of clear protein solution containing SDS add four volumes of cold pure methanol while stirring vigorously for few minutes. Then add three volumes of pure cold chloroform while continue stirring for another few minutes.
- Add three volumes of deionized water. The precipitation process is the left to completion for 10–15 min at room temperature.
- Centrifuge at 15,000 g for about 5 min at 4 °C. The protein precipitate will be located at the liquid interface.
- Pipette out the top aqueous layer and discard it.
- Add four volumes of methanol and stir the blend again.
- Centrifuge at 15,000 g for about 5 min at 4 °C and remove the maximum volume of liquid without disturbing the protein precipitate.
- Dry the protein pellet by speed-vac or comparable means.
- Re-dissolve the proteins using an appropriate buffer compatible with the analytical determination.

□ **NOTE**: The SDS elution method has been proven as being extremely effective with urinary proteomes where a large panel of low-abundance proteins is present (Candiano et al. 2009). Single elution with SDS was demonstrated as being superior or equivalent to the sequential elutions using the TUC elution mixture followed by the UCA elution mixture and by 6 M guanidine-HCl, pH 6. Actually the latter allows collecting traces of proteins still left on the beads.

Other stringent protein elution methods

A second choice for complete or almost complete elution from CPLL is the use of 6 M guanidine-HCl (generally at neutrality, pH close to six). This solution competes with electrostatic interaction by its high ionic strength. Guanidine also destroys hydrogen bonding and by its action on

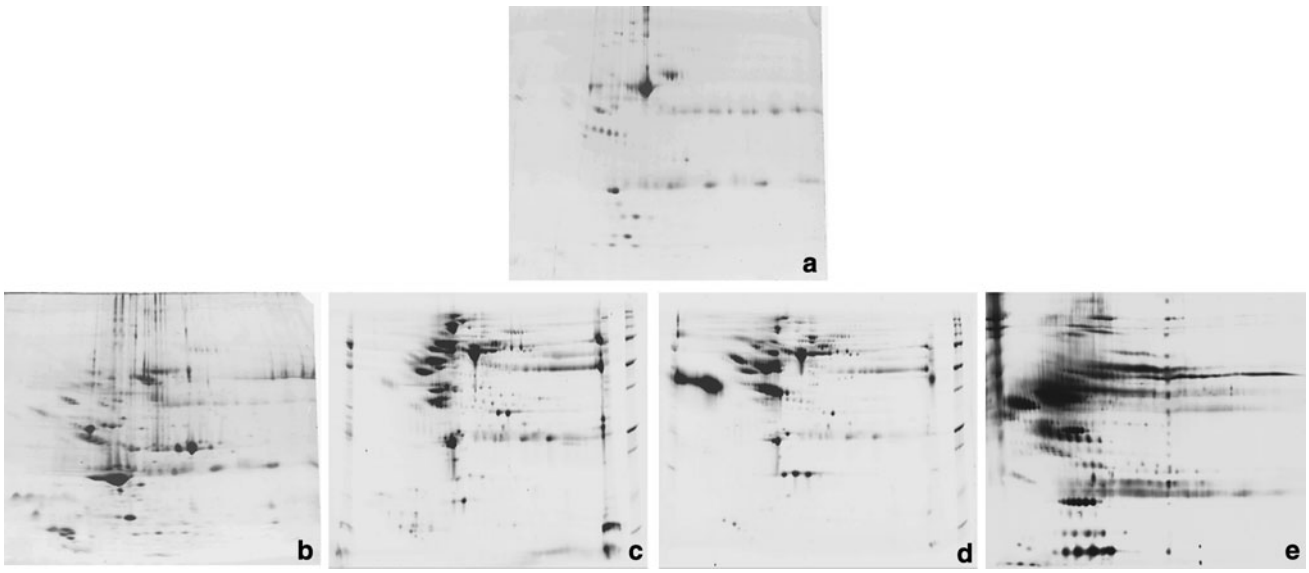

Fig. 2 Illustration of the influence of capturing conditions on the pattern of captured proteins. **a** Two-dimensional electrophoresis (2-DE) of non-treated human serum. **b** 2-DE of CPLL-captured proteins under physiological conditions of pH and of ionic strength (adapted from Thulasiraman et al. 2005). **c** 2-DE of captured proteins at pH 4.0 (adapted from Fasoli et al. 2010). **d** 2-DE of captured proteins at pH 9.3 (adapted from Fasoli et al. 2010). **e** 2-DE of captured proteins in the presence of ammonium sulfate (adapted from Santucci et al. 2013). In this latter case the initial serum sample was preliminarily treated for the elimination of albumin that was considered detrimental for the subsequent steps. Two-dimensional electrophoresis made within a pI range of 3 and 10; staining: coomassie blue

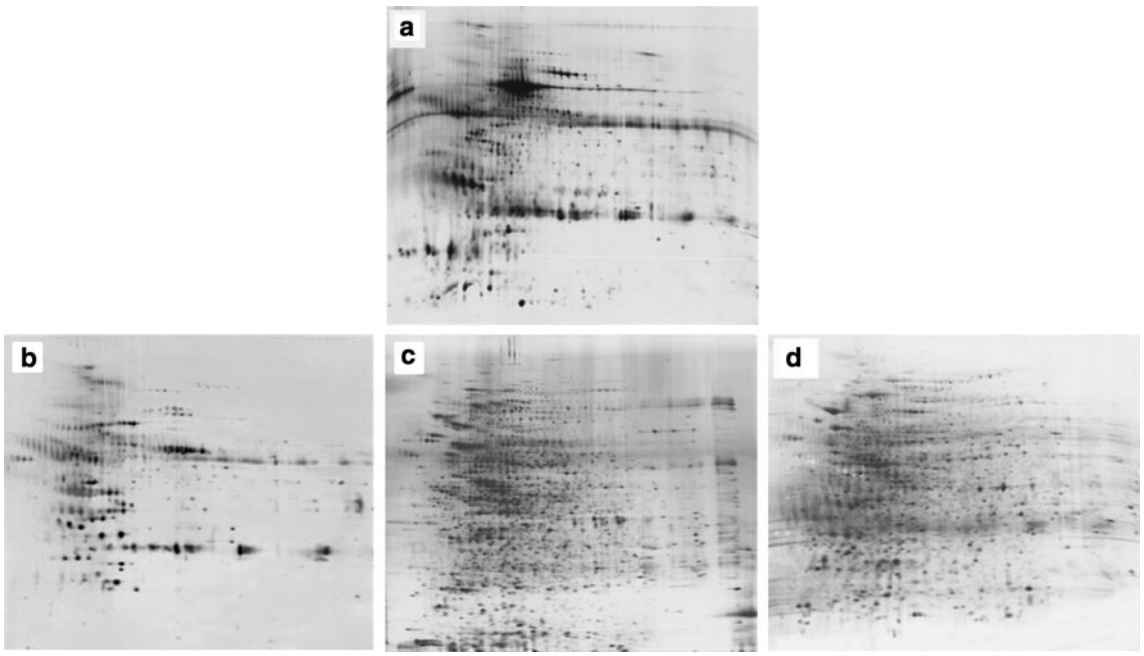

Fig. 3 Examples of the elution methods efficacy for urinary proteins. After collection, urines have been dialyzed against 25 mM phosphate buffer pH 7.4 and then treated with CPLL beads. 2D gel **a** non-treated urinary proteins. 2D gel **b** elution of captured proteins was performed using a solution composed of 2 M thiourea, 7 M urea, 4 % CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). Adapted from Candiano et al. (2009). 2D gel **c** elution of captured proteins was performed using a mixture of following amino acids lysine, arginine, glutamic acid and Aspartic acid at 50 mM, 50 mM, 25 mM and 25 mM concentrations, respectively. Adapted from Candiano et al. (2012). 2D gel **d** elution of captured proteins was performed using a solution composed of 4 % sodium dodecyl sulfate containing 50 mM dithiothreitol. Adapted from Candiano et al. (2009)

the structure of water it weakens hydrophobic associations with consequent desorption. In spite of a good elution efficacy, the proteins collected in presence of guanidine are difficult to analyze by current methods such as SDS-PAGE, 2-DE, mass spectrometry.

A TUC solution added with cysteic acid can also be used instead (Farinazzo et al. 2009). In this case 2-DE can directly be applied since cysteic acid does not disturb the formation of the pH gradient of the first migration dimension, a critical first step for this analytical method.

Alternative sequential methods for total protein desorption

The main reason why one would want to desorb proteins sequentially is when the number of proteins after CPLL treatment is very large preventing an exhaustive analysis of each component. A fractionated elution from the beads actually concurs to the simplification of the single fractions in term of number of proteins present and hence the following steps are more reliable and of an easier interpretation.

Sequential elution by interaction challengers

Several sequences have been described in the literature, each of them having their merits. For proteins extracted with current aqueous buffers, the most logical sequence should comprise different steps, each one addressing the elementary protein-hexapeptide interactions: one step for electrostatically adsorbed proteins, one step annihilating hydrophobic associations and one step to dissociate hydrogen bonding. A last eluting solution should also be used when all the interactions possibilities are present with no dominance. Actually due to the mixed-mode character of each peptide ligand, several types of interactions with comparable intensities may occur on the same ligand.

The recommended sequenced protocol is as follows:

- Mix 100 µL of CPLL carrying the captured proteins with 200 µL of neutral 1 M NaCl (or KCl) and shake gently to maintain beads in suspension for about 10 min at room temperature (0.5 M salt solution could also be used instead of 1 M).
- Separate the beads by centrifugation and collect the supernatant. Treat again the bead pellet with the same volume of the same salt solution and repeat the protein desorption process.
- Mix the second supernatant with the first and store in the cold or frozen for further analysis.

□ **NOTE**: this first elution allows collecting proteins that are dominantly adsorbed by an electrostatic interaction effect and can be modulated by the pH.

- Mix the CPLL pellet with 200 µL of 50 % ethylene glycol in water to desorb the dominantly captured proteins by hydrophobic associations. 20–50 % ethylene glycol in water can also be used instead, with lower efficiency, but also lower risks of protein denaturation.
- Shake gently the CPLL bead suspension for about 10 min at room temperature and collect the supernatant by centrifugation. Operate a second protein extraction using the same procedure. Mix the second eluate with the first and store in the cold.
- Mix the CPLL pellet with 200 µL of 8 M urea to desorb the dominantly captured proteins by hydrogen bonding.
- Shake gently the CPLL bead suspension for about 10 min at room temperature and collect the supernatant by centrifugation. Operate a second protein extraction using the same procedure. Mix the second eluate with the first and store in the cold.
- Mix the CPLL pellet with 200 µL of 0.2 M glycine–HCl buffer, pH 2.5, for the elution of other proteins that are tightly adsorbed by a variety of concomitant molecular interactions.
- Shake gently the CPLL bead suspension for about 10 min at room temperature and collect the supernatant by centrifugation. Operate a second protein extraction using the same procedure. Mix the second eluate with the first and store in the cold.

□ **NOTE**: 200 mM glycine–HCl buffer pH 2.2–2.5 is a deforming buffer used extensively in affinity chromatography to dissociate antigen–antibody complexes.

At this stage, the CPLL beads are empty of proteins and can be disposed. In case it is wished to check if all proteins are desorbed from the beads, the latter can be added with 100 µL of SDS Laemmli buffer and boiled for about 10 min. The supernatant is then used for a SDS-PAGE analysis.

The first three steps can be modified in their order of use. For instance, hydrophobic elution can start first instead of electrostatic challenging.

Sequential elution by increased stringency

A number of sequenced elutions alternative can be performed as extensively described in Boschetti and Righetti (2013) where the selection of desorbing agents is made by their increased stringency up to elution completion.

- In a case a mixture of 2 M thiourea, 7 M urea, 2 % CHAPS was used as first step. Then 9 M urea pH 3 by acetic acid allowed desorbing additional different proteins. Finally, a third step using a mixture composed of 6 % acetonitrile, 12 % isopropanol, 10 % ammonia at 20 %, and 72 % water was performed achieving thus

the collection of all adsorbed proteins (Mouton-Barbosa et al. 2010; Roux-Dalvai et al. 2008).
- In a second case Ernoult et al. (2010) used 4 M urea, 1 % CHAPS, 5 % acetic acid as a first step followed by 6 M guanidine-HCl, pH 6.0.
- In a third case (Boschetti et al. 2007) proteins captured from mouse serum under physiological conditions were desorbed sequentially using 1 M sodium chloride, 3 M guanidine-HCl pH 6.0 and then 9 M urea titrated with citric acid up to pH 3–3.5.

In all cases although the composition of protein fraction was complementary with the detection of many new species, they comprised a quite large overlapping of common proteins.

? TROUBLE SHOOTING

Problems	Reasons	Possible solution
Desorption of proteins is not complete	Certain proteins are very tightly adsorbed	Use SDS-DTE desorption solution at boiling temperature as final step
Proteins cannot be properly analyzed	Eluting agents present with proteins generate troubles during the analysis	Remove the elution agents by protein precipitation or any other equivalent mean as described by Boschetti and Righetti (2013)
Repeatability of experiments is difficult	Small treatment changes from sample to sample can generate large effects	Check first that temperature is strictly the same. This influences in an opposite manner both hydrophobic associations and electrostatic interactions

On-bead digestion of captured proteins instead of protein elution

The identification of proteins captured by CPLL beads can be obtained without elution. Peptides can be produced by full trypsin digestion followed by LC/LC–MS/MS analysis (Fonslow et al. 2011; Meng et al. 2011). This approach naturally precludes any possibility to visualize protein spots by SDS-PAGE or 2D-PAGE or by MALDI.

The detailed procedure is as follows:

- Mix 100 µL of CPLL beads carrying the captured proteins with 200 µL of 100 mM ammonium bicarbonate containing 0.1 % Rapigest SF (this is not mandatory, but it facilitates the proteolysis process). Vortex for a dozen of seconds.
- Add 300 µL of 10 mM DTT and then mix. Heat at 65 °C in a water bath or incubator for 1 h under gentle stirring or occasional shaking.
- Add 300 µL of 55 mM iodoacetamide; mix thoroughly and store in the dark for 60 min.
- Add 60 µL of 0.2 µg/µL of sequencing grade trypsin. Vortex the suspension for a dozen of seconds and incubate overnight at 37 °C under gentle shaking.
- The day after add 200 µL of 500 mM formic acid; vortex for a dozen of seconds.
- Incubate for about 40 min at room temperature and then recover the supernatant by filtration (30,000 MWCO) under centrifugation (12,000 rpm for 20 min) to separate peptides from insoluble material and beads.

□ **NOTE**: To fully extract the remaining peptides within the bead pores, a wash is recommended under centrifugation using 100 µL of 500 mM formic acid. This second filtrate is mixed with the first one.

- Dry the peptide solution by speed-vac and re-dissolved in 20 µL HPLC solvent for LC-mass spectrometry analysis (a centrifugation to get a clear solution may be necessary).

Anticipated results

The efficiency of the process to evidence low-abundance proteins first depends on the conditions of the capture step. It is in fact common that some proteins that are detectable before CPLL treatment are absent after the treatment of the sample. Under physiological conditions the amount of "lost" proteins could reach up to 20 % of high-abundance proteins with an average of <10 %. This lack of capture phenomenon could be extended to low-abundance proteins that are by definition not detectable; therefore its extent for rare species is by definition unknown. The exploration of a quite large range of pH values makes the protein capture more effective covering most of the species present. In practice the protein capture is performed at three different pH values: pH 4.0, 7.2 and 9.3 (Fasoli et al. 2010). This approach is played essentially on electrostatic-based capture which is only marginally adapted for hydrophobic-driven capture. To cover this last aspect a very powerful process involves the capture in the presence of lyotropic salts, namely 1 M ammonium sulfate (Santucci et al. 2013). By that way no high-abundance protein losses have been recorded and presumably this would be true also for low-abundance undetectable species.

A rational explanation can be found on the fact that ionic interactions, classified as long-range interactions, are

among the first to occur in an aqueous medium. They then need to be further strengthened by hydrogen bonds and hydrophobic associations otherwise they might be discouraged, due to the screening effect of the water solvent. Conversely, when hydrophobic interactions occur first (e.g. under a lyotropic environment) they are not broken in an aqueous milieu unless the dielectric constant of the solvent is drastically changed.

Whatever the process of protein capture the number of identified species is always much higher compared to the untreated sample. The reduction of the dynamic concentration of the proteins can reach up to 4–5 orders of magnitude. Naturally this reduction depends very much on the volume of the initial sample to treat to get available enough proteins present in trace amounts as recently underlined (Righetti and Boschetti 2013).

When low ionic strength capturing conditions are used instead of physiological buffers more proteins are captured by virtue of ionic interaction enhancement and increase of binding capacity of the beads (Guerrier et al. 2006; Di Girolamo et al. 2011a, b).

When low-pH environment capturing conditions are used more acidic proteins are captured as a result of net charge change for many proteins that can be determined from their titration curve. On the contrary, when high-pH environment capturing conditions are used, more alkaline proteins are captured because of the same behavior of ionizable amino acids residues phenomenon.

The efficiency of the process is also dependent on the elution method. All adsorbed proteins must be desorbed for a complete analytical determination. Molecular interactions at the basis of the protein adsorption do not all of them react similarly to given desorbing agents; interestingly while certain interactions are weakened by one eluting agent, other molecular interactions are strengthened. This is the reason why several options are given above, one being a full protein denaturation another a sequence of eluents and a third approach based on the complete trypsinization of all proteins captured to perform the analysis of resulting peptides.

An incomplete protein capture followed by an incomplete protein desorption, as occasionally reported, will not allow for very effective low-abundance protein enrichment even if a certain reduction of dynamic concentration range is effectively reached. Although the capture phase can easily be managed, a complete desorption is more difficult to assess. This phenomenon is the main reason of the poor performance of the CPLL-sample treatment as recently reported by Chen et al. (2013). Clearly here the authors desorbed only very partially proteins from the beads when they use a solution composed of 9 M urea and 2 % CHAPS. This elution solution weakens hydrogen bondings; hydrophobic associations are somewhat weakened but probably not enough to allow desorbing all proteins; electrostatic interactions are probably mostly unchanged. Thus, their conclusions that immuno-depletion with MARS14 performs better than a CPLL treatment is based on wrong data and very poor knowledge of the CPLL technique. To check that all proteins are collected it is advised to treat the final supposed "empty" beads with SDS-PAGE Laemmli buffer and analyze the supernatant by one-dimensional or two-dimensional electrophoresis.

Incomplete protein capture followed by an incomplete elution has dramatically negative effects on the detection of very low-abundance biomarkers that normally sign the early stage of an upcoming disease (see the review of Boschetti et al. 2012).

To enhance the low-abundance proteins enrichment, a final word is here due on the volume of beads and the volume of the biological extract. Although the bead volume increases the diversity of ligand collection, its impact in the final result is relatively limited. On the contrary, an increase of the biological sample is highly recommended if one wants to concentrate species that are present only in few copies per unit of volume.

Conflict of interest The authors declare that they have no competing financial interests.

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