

Fingerprint-Imprinted Polymer: Rational Selection of Peptide Epitope Templates for the Determination of Proteins by Molecularly Imprinted Polymers

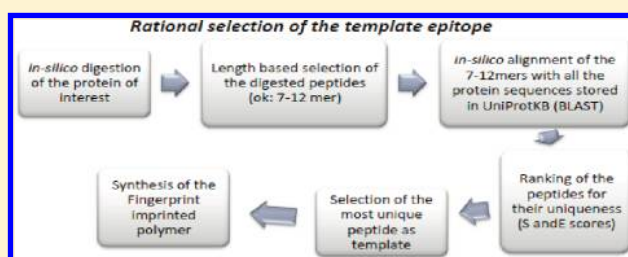
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S Supporting Information

ABSTRACT: The pool of peptides composing a protein allows for its distinctive identification in a process named fingerprint (FP) analysis. Here, the FP concept is used to develop a method for the rational preparation of molecularly imprinted polymers (MIPs) for protein recognition. The fingerprint imprinting (FIP) is based on the following: (1) the *in silico* cleavage of the protein sequence of interest with specific agents; (2) the screening of all the peptide sequences generated against the UniProtKB database in order to allow for the rational selection of distinctive and unique peptides (named as epitopes) of the target protein; (3) the selected epitopes are synthesized and used as templates for the molecular imprinting process. To prove the principle, NT-proBNP, a marker of the risk of cardiovascular events, was chosen as an example. The *in silico* analysis of the NT-proBNP sequence allowed us to individuate the peptide candidates, which were next used as templates for the preparation of NT-pro-BNP-specific FIPs and tested for their ability to bind the NT-proBNP peptides in complex samples. Results indicated an imprinting factor, IF, of ~ 10 , a binding capacity of 0.5–2 mg/g, and the ability to rebind 40% of the template in a complex sample, composed of the whole digests of NT-proBNP.



Molecularly imprinting consists of producing a polymer with specific recognition sites for a defined target molecule, called the template, by a template-assisted synthesis.^{1,2} Molecularly imprinted polymers (MIPs) for small molecules are well-established, whereas protein imprinting reported limited success.^{3–6} Despite some recent remarkable achievements in the field,^{7–9} no general rules have been set to successfully imprint a protein. This explains why the initial perception to revolutionize the diagnostic panorama with plastic antibodies (the colloquial locution for MIPs)¹⁰ remains more of a vision than a reality. The main drawback in the use of proteins as templates is their three-dimensional (3D) structure. The structure is complex and flexible, unfolds easily with changes, also minimal, of the physicochemical characteristics of the environment (transition native-unfolded for an average protein is estimated $\Delta G^\circ -5$ kcal/mol¹¹). These low energies are the careful product of the evolutionary process, selected by nature to guarantee an efficient turnover and to facilitate the switching on/off of protein functions in the cell. However, for imprinting purposes stable proteins with rigid 3D structure would be strongly preferred.

Decreasing the complexity of the template is perceived as the most promising strategy to overcome the aforementioned complications and to achieve successful protein imprinting. Thus, an elegant approach to the simplification of the protein structure was proposed under the name of “epitope

imprinting”.^{9,12,13} The “epitope imprinting” consists of the identification of key epitopes (short and exposed peptide sequences) on the surface of the protein and to prepare the MIP, using as template in the imprinting process the selected linear peptide epitope. The key to success is the identification of a proper epitope. Currently, the epitopes have been identified by crystallographic data and by analysis of antibody binding sites, both available only for a limited number of proteins (68 344 crystallographic structures in the Protein Databank, PDB), out of the vast number of known protein sequences available (D UniProt Knowledgebase: 519 348 entries from UniProtKB/SwissProt and 11 636 205 entries from UniProtKB/TrEMBL). UniProtKB is the reference database for proteome analysis. The outstanding achievement in proteomic is the ability, in theory, to identify each and every single protein expressed in the cell (or tissue, or organ), starting from a mixture of all the thousands protein candidates copresent in the sample, i.e., coexpressed in the cell in a particular phase of the cell life. Statistics software, like Basic Local Alignment Search Tool (BLAST),²¹ correlate each proteomic data (i.e., the mass of a peptide) with a probability score that indicate the probability of that peptide belongs to a

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protein, by comparing the peptide sequence with all the protein sequences stored in the database UniProtKB. The ability to identify every protein out of a pool of proteins (several hundreds to thousands) through a peptide strikes the attention and could be useful to improve protein imprinting.

It should be noted that conserved and unique peptide epitopes could be located at any position of the protein. Yet, in all current protein MIP approaches, internal epitopes were not accessible and, thus, have never been considered as templates, bringing to the loss of a huge number of potential epitope candidates. Furthermore, in many cases conserved epitopes of diagnostic interest are located at the core of the protein, where these are not exposed to the pressure of the immune system.

Here we present an approach to the selection of epitopes for protein imprinting that enables targeting all epitopes, either exposed or internal, with the aim of defining a rational method for identifying one (or more) unique peptide sequence(s) highly compatible with imprinting and by this improving the chance of obtaining a successfully working MIP. The MIPs would be referred to as fingerprint-imprinted polymers (FIP), in analogy with the fingerprinting analysis which refers to the use of the constituent peptides to identify a protein.¹⁴

The principle of FIP is here tested by taking as example the detection of the clinical marker NT-proBNP. The measurement of plasma B-type natriuretic peptide (BNP) and of NT-proBNP levels is accepted as clinical screening to assess risk of heart failure.^{15,16} The BNP hormone is synthesized by myocytes as a prepro-134-mer polypeptide chain. The mature form of the prohormone consists of 108 residues (proBNP); it is further processed into a 76 residue fragment, NT-proBNP, and the 32 amino acid biologically active form (BNP) that maintains the cardiovascular homeostasis. In commercial assays cross-reactivity between the mature forms and the precursor proBNP or of degradative forms is reported.¹⁷ The development of a diagnostic test for the NT-proBNP based on FIPs might offer an alternative to the current tests. In first place, this work is aimed at demonstrating the feasibility of the FIP concept and at offering an alternative to the use of MIPs for the detection of proteins.

■ EXPERIMENTAL SECTION

Materials. 2-(Diethyl amino) ethyl methacrylate (DEAEM), trimethylolpropane trimethacrylate (TEGMA), ethylene glycol dimethacrylate (EGDMA), methacrylic acid (MAA), phosphate buffer, and Tween-20 were from Sigma-Aldrich (Darmstadt, Germany); solvents were ACS grade from Carlo Erba (Milano, Italy).

Synthesis of FIP for the Peptide EVATEGIR. FIP and control (NIP) polymer for EVATEGIR (ER8) were prepared with the monomer (DEAEM) and with the recipe shown in Supporting Information Table S.2. The peptide ER8 was dissolved in dimethylformamide (DMF) with sonication for 2 min, added of monomer, cross-linker, and initiator (2,2-dimethoxy-2-phenylacetophenone), purged with argon for 20 min, and photopolymerized with UV light (180 W) for 1 h. After polymerization the polymer was ground and sieved (32–200 μm) with methanol. Particles were collected and washed with methanol and 80% acetonitrile (ACN)/water until complete removal of the residual template, verified with high-performance liquid chromatography (HPLC).

Synthesis of FIP for the Peptide LQESPRPTG. FIP and control (NIP) for LQESPRPTG (LG9) were prepared with the DEAEM or with MAA, with the ratio and the conditions

reported in Supporting Information Table S.2. After polymerization NIP and FIPs were ground and sieved as described above.

Polymers were washed in ACN (10 mL) for 1 h under mild shaking, then in ACN/water 1:1 (v/v) for 1 h. Finally, the polymers were recovered by filtration through Whatman paper, boiled in phosphate buffer 50 mM pH 7.0 for 1 h, and kept overnight in phosphate buffer. FIP3/NIP3 were crushed in ACN, washed in phosphate buffer in boiling condition, and kept overnight in phosphate buffer. HPLC analysis of the filtrates was used to assess the progresses of the washings.

HPLC Analysis. Peptides and digested NT-proBNP were analyzed with HPLC in reversed-phase on a Phenomenex Gemini C18 column (250 mm $\times$ 4.6 mm), using a gradient elution: solvent A, water trifluoroacetic acid (TFA) 0.1%; solvent B, acetonitrile TFA 0.1%; flow rate, 1 mL/min; injection loop, 20 μL ; detection wavelengths, 214 and 280 nm. The elution method was the following: 3 min, 5% B; 3–15, min gradient of 5–60% B, 18–23 min, gradient of 60–80%, 23–28 min, post run 5% B.

Rebinding of the Peptide Epitope ER8 on FIP A/FIP AA/NIP A. Amounts of 20 mg of polymers (FIPA, FIPAA, NIPA) were suspended in 300 μL of EVATEGIR 0.2 mg/mL in NaP 50 mM pH 7.0 and shaken at *T* amb. At the time points 0, 0.5, 1.5, 4.5, and 19 h, 10 μL of solution was withdrawn and microfuged for 2 min. A volume of 5 μL of the solution was diluted 1:1 with the buffer for capillary zone electrophoresis (CZE) analysis containing glycyl-aspartate as internal standard (ID) and run in CZE. Running conditions were the following: aspartic acid, 40 mM; applied voltage, 10 kV. Peak areas of ID and peptide were integrated. Each experiment was done in triplicate (more information is in the Supporting Information).

Rebinding of the Peptide Epitope LG9 with FIP1/FIP2/NIP1/NIP2. Amounts of 20 mg of polymers (FIP1, FIP2, NIP1, NIP2) were suspended in 600 μL of NaP 50 mM pH 7.0 containing 0.1 mg/mL LG9, and orbitally shaken at *T* amb. At the time points 0, 0.5, 1.5, 2.5, and 4.0 h, 40 μL of solution was withdrawn and microfuged with 0.2 μm Vivaspin (Sartorius, Italy) for 2 min. An volume of 15 μL from the solution was diluted 1:1 with the HPLC buffer containing ID (0.05 mg/mL), and 20 μL was injected to the HPLC for analysis.

Rebinding of the Peptide Epitope LG9 with FIP3/NIP3 and on FIP4/NIP4. Amounts of 20 mg of polymers (FIP3, NIP3, FIP4, NIP4) were suspended with 600 μL containing 0.066 mg/mL LG9 in phosphate buffer (pH 7.0, 50 mM) solution. The incubation and HPLC analysis was as described above for FIP1 and FIP2.

Digestion of the NT-pro-BNP. NT-ProBNP was digested with the enzyme thermolysin from *Bacillus thermoproteolyticus rokko* (T7902 Sigma, 50–100 U/mg protein). A 1 mg/mL stock solution of NT-proBNP was prepared by dissolving 250 μg of NT-proBNP in 250 μL Tris–HCl 50 mM, 0.15 M NaCl, 10 mM CaCl_2 , pH 7.4. The stock of thermolysin was prepared 1 mg/mL in the same buffer as above. An aliquot of 100 μg from the NT-proBNP stock was added to 10 μL from the thermolysin stock (10 μg), and the digestion was carried out at 68 $^\circ\text{C}$ overnight. Finally, the digested NT-proBNP was stored at $-20\text{ }^\circ\text{C}$.

Rebinding Experiment of Digested NT-pro-BNP Peptides with FIP1, NIP1, FIP2, NIP2. Twenty microliter aliquots of NTproBNP digest were used for the rebinding experiments. An aliquot was run in HPLC and used as a

Table 1. List of BNP Peptides Corresponding to the Criteria of Epitope Candidate (Selected Candidates in Bold)^a

sequence	no. of AA	proteases	score S	pI	hydrophilicity	2D structure
EVATEGIR	8	trypsin	30	4.3	0.6	coil
MVLYTLR	7	trypsin	29	9.8	−0.9	α -helix
ASDLETSG	8	thermolysin	28	3.0	0.5	α -helix
LQESPRPTG	9	thermolysin	34	7.0	0.5	coil
LGSPGSASD	9	pepsin	32	3.1	0.2	coil
SPGSASDL	8	chymotrypsin	29	3.1	0.2	coil
QESPRPTGVW	10	chymotrypsin	41	7.0	0.1	coil
PTGVWKSRL	8	Arg-protenase	33	11.5	0.1	coil
EVATEGRG	8	Arg-protenase	30	4.3	0.8	coil
KMVLTLR	8	Arg-protenase	32	10.4	−0.4	α -helix

^aHydrophilicity and structure were reported as from the ExPASy web site.

reference peptide map, from which the LG9 peak area was used to calculate the LG9 content in the digest NT-proBNP.

Spin columns were loaded with 12 mg of polymers (FIP1, NIP1, FIP2, NIP2) and incubated with 400 μ L of digested NT-proBNP (0.05 mg/mL) for 4 h at room temperature, under mild rocking shaking. At the end of the incubation the spin columns were microfuged. FIP1, NIP1 and FIP2, NIP2 were first washed with an equal volume of buffer with 0.02% Tween-20 in order to remove nonspecifically bound peptides. Next, FIP1, NIP1 and FIP2, NIP2 were incubated for 2 min with pure ACN (400 μ L) to strip off peptides, centrifuged, and the ACN was evaporated under N₂. The recovered peptides were redissolved in 100 μ L of sodium phosphate buffer 50 mM pH 7.0 and analyzed by HPLC.

Rebinding Experiment of Digested Serum with FIP1, NIP1, FIP2, NIP2. An aliquot of 100 μ L of serum sample of a healthy volunteer donor was diluted 1/40 in phosphate-buffered saline (PBS), added of 10 μ L of thermolysin (1 mg/mL), and digested as described above. Spin columns, loaded with 15 mg of polymers (FIP1, NIP1, FIP2, NIP2), were incubated with 400 μ L of digested serum supplemented with 2 μ g of LG9 for 4 h and treated according to the above protocol. Incubated solutions and bound peptides were analyzed in HPLC.

Theoretical Basis of the Method. The rational selection of the best epitope peptide candidate is based on the use of the free protein sequence databank UniProtKB and on probability scores attributed by the free software BLAST. At first the target protein is cleaved in silico into peptides at specific cleavage sites by a software such as PeptideCutter-ExPASy (http://web.expasy.org/peptide_cutter/). This would result in the generation of a specific library of peptide fragments *per* protein, termed the peptide fingerprint (FP) of the protein. Each peptide candidate would be evaluated for its suitability as a template for the imprinting by taking into consideration the following parameters: (I) The uniqueness of the epitope, determined by screening its sequence against all the sequences present in the database and by associating an individual probability score to each peptide, which would help in categorizing how unique and thus how suitable a peptide shall be as marker of the protein in question. (II) The length of the peptide will be evaluated to optimize the imprinting performance: not too short to avoid similar sequences in other proteins; not too long to allow for good imprinting.¹⁸ (III) The composition of the peptide will be analyzed according to the functional groups of its side chains to evaluate possible interactions with the functional monomers in the FIP. The in silico analysis would be repeated for a number of cleaving

agents, in order to maximize the probability to generate a unique peptide associated to the protein and thus to select the best epitope for imprinting. These MIPs produced through the in silico epitope selection process would be referred to as fingerprint-imprinted polymers.

RESULTS AND DISCUSSION

Rational Selection of the Epitopes. The sequence of NT-proBNP was cleaved in silico with different enzymes, generating a list of peptides per each enzyme (Supporting Information Table S1). The length of the peptide was considered a crucial parameter to attain a unique recognition.¹⁸ too long peptides have flexible structures that could impair the imprinting and/or later, the rebinding process; thus, a length of 7–12 amino acids was considered to be appropriate. Peptides were next selected for their hydrophilicity, as for the final goal of using the FIPs in clinical analysis (plasma, serum, biological fluids), recognition would in aqueous environment. Out of the hundreds peptides screened, the most promising ones were selected with the rational criteria of S and E scores related to the unicity of the peptide candidate (in bold, Table 1). The selection was (1) the epitope EVATEGIR (ER8) with pI 4.3, obtained by trypsin cleavage, since trypsin is the most diffused cleavage enzyme, and (2) the epitope LQESPRPTG (LG9), pI 7.0, thermolysin cleavage.

Synthesis of FIP/NIP Polymers and Selection of the Best FIP Polymer. The composition of the FIP/NIP polymers, reported in Supporting Information Table S.2, was based on the combination of a charged functional monomer with a bi- or trifunctional cross-linker,^{19,20} changing the molar ratio epitope/functional monomer with the intention of optimizing the polymeric material for the binding of the selected peptide epitope.

Binding Performance of FIP A, FIP AA, and NIP A to ER8. Polymers A and AA were prepared with the peptide epitope ER8 as template. The binding of ER8 to the polymers was evaluated on spin columns loaded with the polymer and with a porous septum (similar to SPE cartridges). After incubation the bound ER8 was stripped off the polymers and quantified. The results, in Figure 1, indicated binding reached equilibrium in 1.5–4 h; there was a distinctive imprinting effect in the FIPs (IFs 4.5–7). The binding capacity, calculated as quantity of peptide bound per quantity of polymer, was ca. 1 mg/g polymer, which is in line with commercial affinity resins. Overnight incubation seemed to favor aspecific binding on NIP; thus, all the following binding experiments were not monitored over 4 h.

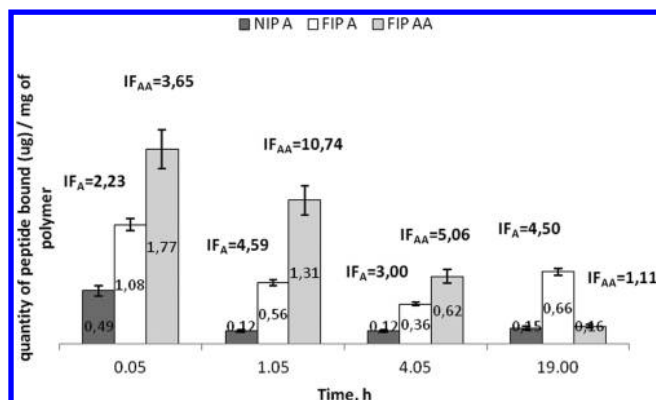


Figure 1. Capacity and IF values calculated for the rebinding of epitope ER8 to FIP A, FIP AA, and the control NIP A, monitored at different time points.

Binding Performance of FIP1/NIP1 FIP2/NIP2 to LG9.

The polymers prepared with the epitope LG9 were tested for their rebinding ability. FIP1 and FIP2 were prepared with DEAE or MAA, respectively, as functional monomers. Figures 2 and 3 show the binding capacity and IF for LG9

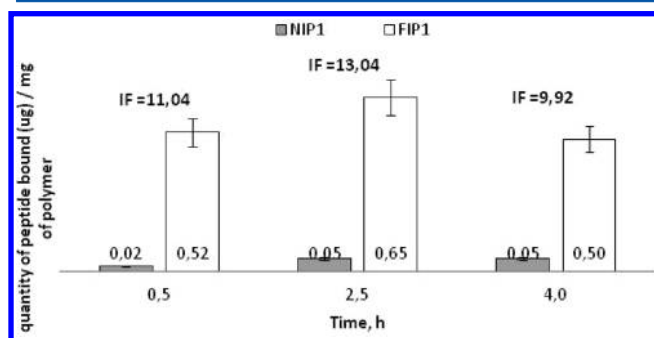


Figure 2. Capacity and IF values calculated for the rebinding of epitope LG9 to FIP1/NIP1, monitored at different time points.

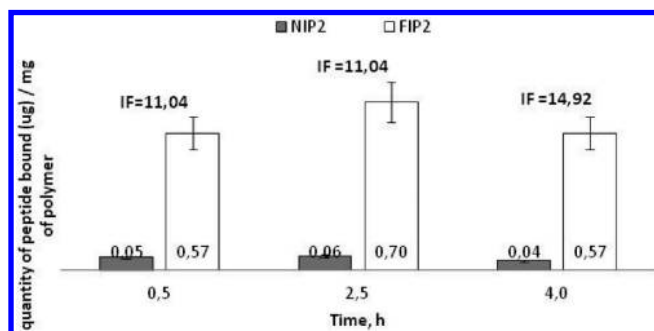


Figure 3. Capacity and IF values calculated for the rebinding of epitope LG9 to FIP2/NIP2, monitored at different time points.

exhibited by the FIPs and the NIPs. The binding capacity for FIPs was 0.6 mg/g; modest binding occurred to NIPs. IFs attained values around 10, which signify an excellent polymer performance. Most of the binding occurred within the first 30 min, while the maximum was binding was reached in 2.5 h. According to these results, 30 min of incubation with the FIP should be enough for practical purposes.

Binding Performance of FIP3/NIP3 to LG9. Polymer FIP3/NIP3 was prepared decreasing the molar ratio of the functional monomers/epitope and with decreased rigidity. FIP3

showed (Figure 4) the highest binding capacity (ca. 2 mg/g polymer) and steady increase in the binding capacity for high

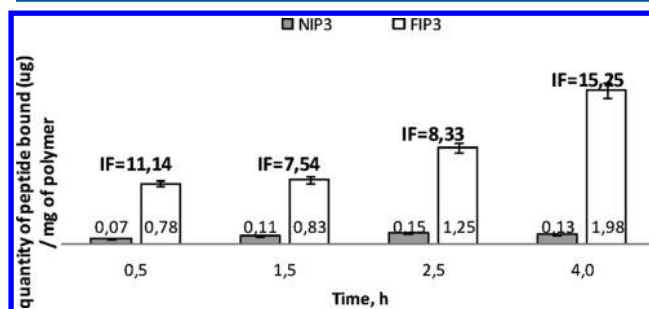


Figure 4. Capacity and IF values calculated for the rebinding of epitope LG9 to FIP3/NIP3, monitored at different time points.

incubation times, as expected by the lower degree of cross-linking; it had significant IF too.

Binding Performance of FIP4/NIP4 to LG9. Polymer FIP/NIP4 were prepared with a higher ratio of functional monomers/peptide epitope (T/M 1:10 mol/mol). The IF was modest (IF = 6 at 30 min, but IF = 1.6 at 4 h) due to relatively high unspecific binding, probably due to the overall increase in charges on the polymer. Because of the poor performance FIP4 was not considered further. Considering all the results, the best performance was obtained with FIP1 and FIP2, resulting in the highest IF and minimal time for an effective rebinding (30 min).

Binding of Peptide Fragment LG9 from the NT-proBNP Digest and from Serum to Its Specific FIP. The concept of using FIPs for detecting the presence of a protein in a sample by identifying a peptide fragment generated by specific digestion of the protein was examined by testing the ability of FIP1 and FIP2 to bind the peptide fragment LG9 (template) from the digest of NT-proBNP. The FIPs were prepared against the synthetic LG9 peptide fragment predicted by the in silico digestion of NT-proBNP, and these FIPs should rebinding the peptide fragment LG9, generated by actual digestion of the target protein by the same enzyme that was used for the in silico simulation. The complete in silico digestion of the NT-proBNP by thermolysin give rise to 17 peptide fragments (Supporting Information Table S1). The maximum amount of LG9 in the total NT-proBNP digest determined was 1.60 μ g. The binding of LG9 was carried out in the presence of all the other 16 fragments of NT-proBNP. Figure 5 shows that FIP1 captured the 33% of the total LG9 present in the digest, whereas NIP1 captured only 9%. FIP2 captured the 43% of LG9 in the digest, whereas NIP2 captured only the 16%. These results are a strong positive indication of the validity of the FIP concept, demonstrating that a rational selection of a peptide epitope fragment (LG9) leads to the preparation of an effective specific FIP that was able to recover the LG9 peptide fragment from a complex milieu as a whole digested peptide pool. These results showed that the FIPs can operate successfully in aqueous conditions that are similar to analysis of body fluids for clinical purposes. Finally, we attempted the rebinding of LG9 from digested serum. The results indicate FIP1 bound 0.11 μ g of LG9, with respect to the 0.05 μ g of NIP1; this indication permitted us to calculate an IF 2.2 for FIP1. No difference in binding was observed between the pair FIP2/NIP2. It should be noted all the polymers tested bound a number of other components that are not related with LG9, indicating high

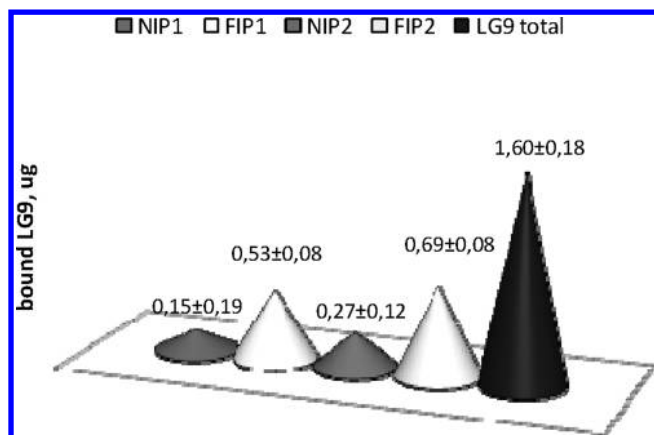


Figure 5. Quantity of the LG9 fragment from the whole digest mix of NT-proBNP bound to FIP1/NIP1/FIP2/NIP2. The total amount of LG9 fragment present at the beginning of the incubation in the digest mix was 1.60 μ g.

aspecific binding (especially marked for the FIP2/NIP2 composition). The binding conditions should be optimized further, and a sample pretreatment step might be introduced, prior to binding. Despite the need of optimization, the results are encouraging, considering the high complexity of real samples.

CONCLUSIONS

The present work was intended to propose a revolutionary rational approach to protein and peptide imprinting. In the case of a protein when its entire structure is difficult to imprint, short peptides exposed on the surface (epitopes) have been used to prepare a MIP that later is expected to bind the native protein. The choice of the epitope is crucial for the success or the failure of the imprinting process.¹¹ Until the present FIP approach was conceived, the choice of the epitope was limited to those peptides located at the surface of the protein and already identified by antibodies or X-ray crystallography. The FIP approach, which is characterized by the *in silico* method for a rational identification of suitable peptide epitopes (the templates) for imprinting, on widely known and freely available online protein databases and software tools, offers a general method applicable to all proteins whose sequences are available. The steps for identifying a peptide candidate for imprinting are (I) *in silico* cleavage of the sequence of the protein of interest by various cleaving agents; (II) selection of the resulting peptide fragments that possess the appropriate length and hydrophilicity; (III) screening of each peptide candidate against the entire protein databank (e.g., UniProtKB). This associates to each peptide candidate a score of probability of uniqueness and permits the selection of unique sequences to be used as templates for MIPs, with the ultimate goal to greatly favor the selective recognition of the protein on the imprinted polymer. The use of such approach circumvents the issue of the folding of the protein that might influence its interaction with the imprinted polymer. A key feature of the FIP approach is the precleavage of the target protein into small peptide fragments, virtually permanently available for the interaction with the capturing FIP. Moreover, the possibility to use internal epitopes has an additional potential in case of protein target belonging to pathogens; in fact, the internal sequences are more conserved with respect to the outer domains, subjected to the pressure of the immune system.

Since the target peptides are smaller than the intact protein, the equilibrium with the binding sites of the FIP occurs in relatively short times (e.g., 30 min). The feasibility of the FIP approach was verified by successful imprinting of peptides selected by our rational selection process from the NT-proBNP biomarker. All the FIPs that were synthesized showed significant imprinting factor over the control polymers (NIP), which is probably the result of choosing peptide with structural qualities highly suitable for imprinting. The most effective FIP in rebinding the target peptide was selected and was later shown to effectively recognize the same target peptide fragment from the whole pool of peptides generated by the enzymatic cleavage of native NT-proBNP in aqueous conditions, thus in the presence of many other nonrelated peptides that were also generated by the cleavage, and finally in presence of the whole serum peptides. The performance of the FIPs shown in the present work offers an indication to the valuable contribution that the FIP concept might have on the use of MIPs as a tool for detection of proteins.

ASSOCIATED CONTENT

Supporting Information

Details on the statistics criteria adopted for the rational selection of the epitope for imprinting, detailed results of the UniProtKB database queries with BLAST software, composition of the FIP polymers, peptides from digestion of NT-proBNP, calibration curves in CZE and in HPLC, and chromatograms. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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SUPPLEMENTARY INFORMATION

Content:

In silico selection of the epitope. Information on how the scores S and E are calculated (page 1)

Process of *in silico* cleavage of NT-proBNP (page 2)

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In silico selection of the epitope. Information on how the score S and E are calculated.

S: is the score of an alignment. It is calculated as the sum of substitution and gap scores. Substitution scores are given by a look-up table (see PAM, BLOSUM). Gap scores are typically calculated as the sum of G, the gap opening penalty and L, the gap extension penalty. For a gap of length n, the gap cost would be $G + Ln$. The choice of gap costs, G and L is empirical, but it is customary to choose a high value for G (10-15) and a low value for L (1-2). The value S' is derived from the raw alignment score S in which the statistical properties of the scoring system used have been taken into account.

Because bit scores have been normalized with respect to the scoring system, they can be used to compare alignment scores from different searches.

The Expect value (E) is a parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size. It decreases exponentially as the Score (S) of the match increases. Essentially, the E value describes the random background noise. It gives the number of different alignments with scores equivalent to or better than S that are expected to occur in a database search by chance. The lower the E-value, or the closer it is to zero, the more "significant" the match is. However, virtually identical short alignments have relatively high E values. This is because the calculation of the E value takes into account the length of the query sequence. These high E values make sense because shorter sequences have a higher probability of occurring in the database purely by chance. The Expect value can also be used as a convenient way to create a significance threshold for reporting results. When the Expect value is increased from the default value of 10, a larger list with more low-scoring hits can be reported.

Process of *in silico* cleavage of NT-proBNP

The sequence of NT-proBNP is: HPLGSPGSAS DLETSGLQEQ RNHLQGKLSE LQVEQTSLEP LQESPRPTGV WKSREVATEG IRGHRKMVLY TLRAPRS

Table S1 shows all the cleavage points on NT-proBNP of each the enzyme used for the query.

Table S1.

These enzymes cleave the sequence:

Name of enzyme	No. of cleavages	Positions of cleavage sites
Arg-C proteinase	7	21 46 54 62 65 73 76
Asp-N endopeptidase	1	10
Asp-N endopeptidase + N-terminal Glu	9	10 12 18 29 33 38 42 54 58
BNPS-Skatole	1	51
CNBr	1	67
Chymotrypsin-high specificity (C-term to [FYW], not before P)	2	51 70
Chymotrypsin-low specificity (C-term to [FYWML], not before P)	15	3 12 17 23 24 28 31 38 41 51 64 67 69 70 72
Clostripain	7	21 46 54 62 65 73 76
Formic acid	1	11
Glutamyl endopeptidase	8	13 19 30 34 39 43 55 59
Iodosobenzoic acid	1	51
LysC	3	27 52 66
LysN	3	26 51 65
Pepsin (pH1.3)	19	2 11 12 16 17 24 27 28 30 31 37 40 50 51 69 69 70 71 72
Pepsin (pH>2)	15	2 11 12 16 17 24 27 28 30 31 37 40 69 71 72
Proline-endopeptidase [*]	2	2 47
Proteinase K	25	3 9 12 14 17 24 28 31 33 36 38 41 48 50 51 56 57 58 61 68 69 70 71 72 74
Staphylococcal peptidase I	8	13 19 30 34 39 43 55 59
Thermolysin	15	2 8 16 23 27 32 37 40 49 56 60 66 67 68 71
Trypsin	9	21 27 52 54 62 65 66 73 76

Steps of the queries with BLAST software on UniProtKB and selection of the peptide candidate.

The peptide candidates of interest are reported in the text of the manuscript in Table 1.

The software **BLAST** was used and each sequence entered in the ‘query’ in raw format was screened against the entire protein databank to obtain the list of the potentially matching protein sequences from the Data Base UniProtKB.

BLAST results of peptide query sequence obtained after applying Trypsin protease EVATEGIR 8 AA

Date run: 2009-10-27 17:18:58 UTC+0100 on blast01.vital-it.ch

Program: NCBI BLASTP 2.2.17 [Aug-26-2007]

Database: UniProtKB

10,040,573 sequences; 3,265,158,447 total letters

UniProt Knowledgebase Release 15.9 consists of:

UniProtKB/Swiss-Prot Release 57.9 of 13-Oct-09: 510076 entries

UniProtKB/TrEMBL Release 40.9 of 13-Oct-09: 9501907 entries

List of potentially matching sequences

Db AC	Description	Score E-value
☐ sp P16860 ANFB_HUMAN	Natriuretic peptides B precursor (Gamma...	30 29
☐ tr Q92LJ9 _RHIME	Putative oxidoreductase (EC 1.-.-) [R03049...	30 29
☐ tr Q12GY8 _POLSJ	Short-chain dehydrogenase/reductase SDR prec...	30 29
☐ tr A4G369 _HERAR	Short-chain dehydrogenase/reductase [HEAR076...	30 29

Blast results of peptide query sequence MVLYTLR 7 AA

Date run: 2009-10-27 17:24:26 UTC+0100 on blast01.vital-it.ch

Program: NCBI BLASTP 2.2.17 [Aug-26-2007]

Database: UniProtKB

10,040,573 sequences; 3,265,158,447 total letters

UniProt Knowledgebase Release 15.9 consists of:

UniProtKB/Swiss-Prot Release 57.9 of 13-Oct-09: 510076 entries

UniProtKB/TrEMBL Release 40.9 of 13-Oct-09: 9501907 entries

List of potentially matching sequences

Db AC	Description	Score E-value
☐ sp P16860 ANFB_HUMAN	Natriuretic peptides B precursor (Gamma...	29 61
☐ tr B0W5Y5 _CULQU	Putative uncharacterized protein [CpipJ_CPIJ...	29 61
☐ tr Q8SUE4 _ENCCU	Putative uncharacterized protein ECU10_0900 ...	29 61

Blast results of peptide query sequence obtained after applying thermolysin ASDLETSG 8 AA

Date run: 2009-10-27 17:28:17 UTC+0100 on frt.prclst.vital-it.ch

Program: NCBI BLASTP 2.2.16 [Mar-25-2007]

Database: UniProtKB

10,040,573 sequences; 3,265,158,447 total letters

UniProt Knowledgebase Release 15.9 consists of:
 UniProtKB/Swiss-Prot Release 57.9 of 13-Oct-09: 510076 entries
 UniProtKB/TrEMBL Release 40.9 of 13-Oct-09: 9501907 entries

List of potentially matching sequences

Db AC	Description	Score	E-value
☐ sp P16860 ANFB_HUMAN	Natriuretic peptides B precursor (Gamma...	28	80
☐ tr A6T3P3 _JANMA	Transposition protein, TnsD-related protein ...	28	80
☐ tr Q9N2E8 _PANTR	Natriuretic protein (Fragment) [Pan troglody...	28	80
☐ tr Q9N2E7 _9PRIM	Natriuretic protein [Gorilla gorilla (Wester...	28	80
☐ tr Q9P2Q7 _HUMAN	Natriuretic protein (Fragment) [Homo sapiens...	28	80

Blast results of peptide query sequence LQESPRPTG 9 AA

Date run: 2009-10-27 17:34:59 UTC+0100 on blast01.vital-it.ch
 Program: NCBI BLASTP 2.2.17 [Aug-26-2007]
 Database: UniProtKB
 10,040,573 sequences; 3,265,158,447 total letters
 UniProt Knowledgebase Release 15.9 consists of:
 UniProtKB/Swiss-Prot Release 57.9 of 13-Oct-09: 510076 entries
 UniProtKB/TrEMBL Release 40.9 of 13-Oct-09: 9501907 entries

List of potentially matching sequences

Db AC	Description	Score	E-value
☐ sp P16860 ANFB_HUMAN	Natriuretic peptides B precursor (Gamma...	34	1.2
☐ tr B9SEJ3 _RICCO	Allene oxide cyclase 3, chloroplast, putativ...	31	9.0
☐ tr Q2ULD5 _ASPOR	Helicase-like transcription factor HLTF/DNA ...	31	9.0
☐ tr B8N443 _ASPFN	SWI/SNF family DNA-dependent ATPase Ris1, pu...	31	9.0
☐ tr Q1R0X3 _CHRS	D Transcriptional regulator, LacI family [Csa...	28	70
☐ tr Q1J372 _DEIG	D DNA damage repair protein PprA [Dgeo_2628] [...	28	99
☐ tr A9BZ71 _DELAS	Putative uncharacterized protein [Daci_2533]...	28	99
☐ tr C6K3U0 _9TRY	P Putative amino acid transporter [CDFL12A17_1...	28	99
☐ tr A8PI49 _BRUM	A Putative uncharacterized protein [Bm1_26210]...	28	99
☐ tr A4HSJ1 _LEIIN	I Putative uncharacterized protein [LinJ05.070...	28	99

Blast results of peptide query sequence obtained after applying pepsin pH>2 LGSPGSASD 9 AA

Date run: 2009-10-27 17:39:13 UTC+0100 on blast01.vital-it.ch
 Program: NCBI BLASTP 2.2.17 [Aug-26-2007]
 Database: UniProtKB
 10,040,573 sequences; 3,265,158,447 total letters
 UniProt Knowledgebase Release 15.9 consists of:
 UniProtKB/Swiss-Prot Release 57.9 of 13-Oct-09: 510076 entries
 UniProtKB/TrEMBL Release 40.9 of 13-Oct-09: 9501907 entries

List of potentially matching sequences

Db AC	Description	Score	E-value
☐ sp P16860 ANFB_HUMAN	Natriuretic peptides B precursor (Gamma...	32	5.8
☐ tr A1ZRW9 _9SPHI	PKD domain protein [M23134_04807] [Microscil...	32	5.8
☐ tr Q9N2E8 _PANTR	Natriuretic protein (Fragment) [Pan troglody...	32	5.8
☐ tr Q9N2E7 _9PRIM	Natriuretic protein [Gorilla gorilla (Wester...	32	5.8
☐ tr Q9P2Q7 _HUMAN	Natriuretic protein (Fragment) [Homo sapiens...	32	5.8
☐ tr C5BWD1 _BEUC1	Putative uncharacterized protein [Bcav_0325]...	28	70

Blast results of peptide query sequence obtained after applying: SPGSASDL 8 AA

Date run: 2009-10-27 17:42:01 UTC+0100 on blast01.vital-it.ch
Program: NCBI BLASTP 2.2.17 [Aug-26-2007]
Database: UniProtKB
10,040,573 sequences; 3,265,158,447 total letters
UniProt Knowledgebase Release 15.9 consists of:
UniProtKB/Swiss-Prot Release 57.9 of 13-Oct-09: 510076 entries
UniProtKB/TrEMBL Release 40.9 of 13-Oct-09: 9501907 entries

List of potentially matching sequences

Db AC	Description	Score	E-value
☐ sp P16860 ANFB_HUMAN	Natriuretic peptides B precursor (Gamma...	29	66
☐ tr Q9N2E8 _PANTR	Natriuretic protein (Fragment) [Pan troglody...	29	66
☐ tr Q9N2E7 _9PRIM	Natriuretic protein [Gorilla gorilla (Wester...	29	66
☐ tr Q9P2Q7 _HUMAN	Natriuretic protein (Fragment) [Homo sapiens...	29	66

Blast results of peptide query sequence QESPRPTGVW 10 AA

Date run: 2009-10-27 17:44:13 UTC+0100 on blast01.vital-it.ch
Program: NCBI BLASTP 2.2.17 [Aug-26-2007]
Database: UniProtKB
10,040,573 sequences; 3,265,158,447 total letters
UniProt Knowledgebase Release 15.9 consists of:
UniProtKB/Swiss-Prot Release 57.9 of 13-Oct-09: 510076 entries
UniProtKB/TrEMBL Release 40.9 of 13-Oct-09: 9501907 entries

List of potentially matching sequences

Db AC	Description	Score	E-value
☐ sp P16860 ANFB_HUMAN	Natriuretic peptides B precursor (Gamma...	41	0.014
☐ tr Q5NRC7 _ZYMMO	Putative uncharacterized protein [ZMO0103] [...	30	18
☐ tr Q1R0X3 _CHRS	D Transcriptional regulator, LacI family [Csa...	30	18
☐ tr C5TEZ2 _ZYMMO	Beta-lactamase precursor [ZmobDRAFT_0582] [Z...	30	18
☐ tr Q5DGC5 _SCHJA	SJCHGC04899 protein [Schistosoma japonicum (...	30	18

☐	tr Q7NER9 _GLOVI Glr3809 protein [glr3809] [Gloeobacter viola...	30	25
☐	tr B4HI51 _DROSE GM25509 [GM25509] [Drosophila sechellia (Fru...	30	25
☐	tr Q8WS98 _LEITR ABCA1.2 transporter (Fragment) [Leishmania t...	29	49
☐	tr B3MYV9 _DROAN GF21949 [GF21949] [Drosophila ananassae (Fru...	29	49
☐	tr A4HV32 _LEIIN ABC transporter, putative [LinJ11.1230] [Lei...	29	49
☐	tr A4HV29 _LEIIN ABC transporter, putative [LinJ11.1200] [Lei...	29	49
☐	tr C0U079 _9ACTO DNA polymerase I family protein with 3'-5'-e...	28	69
☐	tr B4CZQ7 _9BACT Putative uncharacterized protein precursor [...	28	69
☐	sp P98201 NFAC1_BOVIN Nuclear factor of activated T-cells, c...	28	97
☐	tr B9SEJ3 _RICCO Allene oxide cyclase 3, chloroplast, putativ...	28	97
☐	tr B2CWA1 _TRITU Plasma membrane Na ⁺ /H ⁺ antiporter [SOS1] [Tr...	28	97
☐	tr A8XF46 _CAEBR Putative uncharacterized protein [CBG12265] ...	28	97
☐	tr A4HSJ1 _LEIIN Putative uncharacterized protein [LinJ05.070...	28	97
☐	tr Q2ULD5 _ASPOR Helicase-like transcription factor HLTF/DNA ...	28	97
☐	tr B8N443 _ASPFN SWI/SNF family DNA-dependent ATPase Ris1, pu...	28	97

Blast results of peptide query sequence PTGVWKS R 8 AA

Date run: 2009-10-27 17:46:55 UTC+0100 on blast01.vital-it.ch

Program: NCBI BLASTP 2.2.17 [Aug-26-2007]

Database: UniProtKB

10,040,573 sequences; 3,265,158,447 total letters

UniProt Knowledgebase Release 15.9 consists of:

UniProtKB/Swiss-Prot Release 57.9 of 13-Oct-09: 510076 entries

UniProtKB/TrEMBL Release 40.9 of 13-Oct-09: 9501907 entries

List of potentially matching sequences

Db AC	Description	Score	E-value
☐	sp P16860 ANFB_HUMAN Natriuretic peptides B precursor (Gamma...	33	2.6
☐	tr Q01PB7 _SOLUE Beta-galactosidase precursor (EC 3.2.1.23) [...	29	42
☐	tr A1B0Q5 _PARDP Phage Terminase [Pden_0988] [Paracoccus deni...	29	42
☐	tr A1AYX9 _PARDP Phage Terminase [Pden_0359] [Paracoccus deni...	29	42
☐	tr A8SE92 _9FIRM Putative uncharacterized protein [FAEPRAM212...	29	42
☐	tr A7SA94 _NEMVE Predicted protein [v1g235180] [Nematostella ...	29	42

Blast results of peptide query sequence EVATEGRG 8 AA

Date run: 2009-10-27 17:54:56 UTC+0100 on frt.prdclst.vital-it.ch

Program: NCBI BLASTP 2.2.16 [Mar-25-2007]

Database: UniProtKB

10,040,573 sequences; 3,265,158,447 total letters
UniProt Knowledgebase Release 15.9 consists of:
UniProtKB/Swiss-Prot Release 57.9 of 13-Oct-09: 510076 entries
UniProtKB/TrEMBL Release 40.9 of 13-Oct-09: 9501907 entries

List of potentially matching sequences

	Db AC	Description	Score	E-value
☐	tr B2AS56	_PODAN Predicted CDS Pa_1_22400 [Podospora anserina]	30	34

Blast results of peptide query sequence KMVLYTLR 8 AA

Date run: 2009-10-27 17:58:07 UTC+0100 on blast01.vital-it.ch
Program: NCBI BLASTP 2.2.17 [Aug-26-2007]
Database: UniProtKB

10,040,573 sequences; 3,265,158,447 total letters
UniProt Knowledgebase Release 15.9 consists of:
UniProtKB/Swiss-Prot Release 57.9 of 13-Oct-09: 510076 entries
UniProtKB/TrEMBL Release 40.9 of 13-Oct-09: 9501907 entries

List of potentially matching sequences

	Db AC	Description	Score	E-value
☐	sp P16860	ANFB_HUMAN Natriuretic peptides B precursor (Gamma...	32	4.6
☐	tr B0W5Y5	_CULQU Putative uncharacterized protein [CpipJ_CPIJ...	29	47
☐	tr Q8SUE4	_ENCCU Putative uncharacterized protein ECU10_0900 ...	29	47
☐	tr A1VT56	_POLNA Extracellular solute-binding protein, family...	29	65

Composition of the polymer synthesised.

Table S.2. Composition of the polymers synthesized (initiator 1% w/w).

Polymer	Monomer (μL)	Cross-linker (μL)	Template (mg)	Solvent	Molar ratio T : M ; M : C
FIP AA	DEAEM (4.66) 20.0 μmol	TEGMA (148) 400 μmol	EVATEGIR (5) 5 μmol	DMF (200 μL)	1 : 4 ; 4 : 20
FIP A	DEAEM(4.66) 20.0 μmol	TEGMA (148) 400 μmol	EVATEGIR (0.5) 0.5 μmol	DMF (200 μL)	1 : 40 ; 40 : 200
NIP A	DEAEM(4.66)	TEGMA (148)	-----	DMF (200 μL)	- ; 4 : 20
FIP1	DEAEM (4.0) 20.0 μmol	TEGMA (127.8) 400 μmol	LQESPRPTG (5.0) 5 μmol	DMF (200 μL)	1 : 4 ; 4 : 20
NIP1	DEAEM (4.0) 20.0 μmol	TEGMA (127.8) 400 μmol	-----	DMF (200 μL)	- ; 4 : 20
FIP2	MAA(1.7) 20.0 μmol	TEGMA (127.8) 400 μmol	LQESPRPTG (5.0) 5.0 μmol	DMF (200 μL)	1 : 4 ; 4 : 20
NIP2	MAA(1.7) 20.0 μmol	TEGMA (127.8) 400 μmol	-----	DMF (200 μL)	- ; 4 : 20
FIP3	MAA (0.68) 8.0 μmol	EGDMA (14) 72 μmol	LQESPRPTG (4.0) 4 μmol	Isopropanol:water 170 μL : 20 μL	1 : 2 ; 2 : 9
NIP3	MAA (0.68) 8.0 μmol	EGDMA (14) 72 μmol	-----	Isopropanol:water 170 μL :20 μL	- ; 2 : 9
FIP4	MAA(3.4) 40 μmol	EGDMA (75.4) 400 μmol	LQESPRPTG (4.0) 4.0 μmol	DMF (100 μL)	1 : 10 ; 10 : 100
NIP4	MAA(3.4) 40 μmol	EGDMA (75.4) 400 μmol	-----	DMF (100 μL)	- ; 10 : 100

T = template; M = functional monomer; C = crosslinker

Calibration curve of the epitope peptide EVATEGIR in CZE

Running conditions in CZE were: buffer aspartic acid 40 mM, 10 kV applied voltage. Samples at different concentrations are added to 0.3 mg/mL internal standard (di-peptide glycyl-aspartate, GD). Retention time for ER8 is 9.8 min, for biotinylated-ER8 18.6 min, GD 14 min. The area of peaks were integrated, normalized with the internal standard and plotted in the calibration curve. Range of sensitivity of the CZE method results in the interval 5–100 µg/mL peptide. The equation for the calibration curve was: y (normalized area of the peak) = $2.72 \times (\text{mg/ml of ER8}) + 0.0032$ and $R^2 = 0.9933$.

Conditions of HPLC measurements and calibration curve of LG9 in HPLC

The calibration curve for peptide LG9 in HPLC was obtained in the range 0.5-200 µg/mL of LG9; the equation was: y (normalized area of the peak) = $0.0023 \times (\text{ug/ml LG9}) + 0.293$ and $R^2 = 0.9961$.

Digestion of NT-proBNP and rebinding of LG9 peptide from the pool of digest peptides

The NTproBNP has been digested. The internal standard was added and samples injected in HPLC. A representative chromatograms of the digested NT-proBNP is shown in Figure 1S. To identify the peak of LG9, a known concentration of LG9 was added to the digested peptide pool.

Figure 1S Panel A: shows the HPLC elution profile of the NT-proBNP after digestion with the enzyme thermolysin. The presence of a number of peaks on the chromatogram indicates the digestion of NT-proBNP has occurred. The arrows indicate the peak of the internal standard and the peak of the peptide LG9.

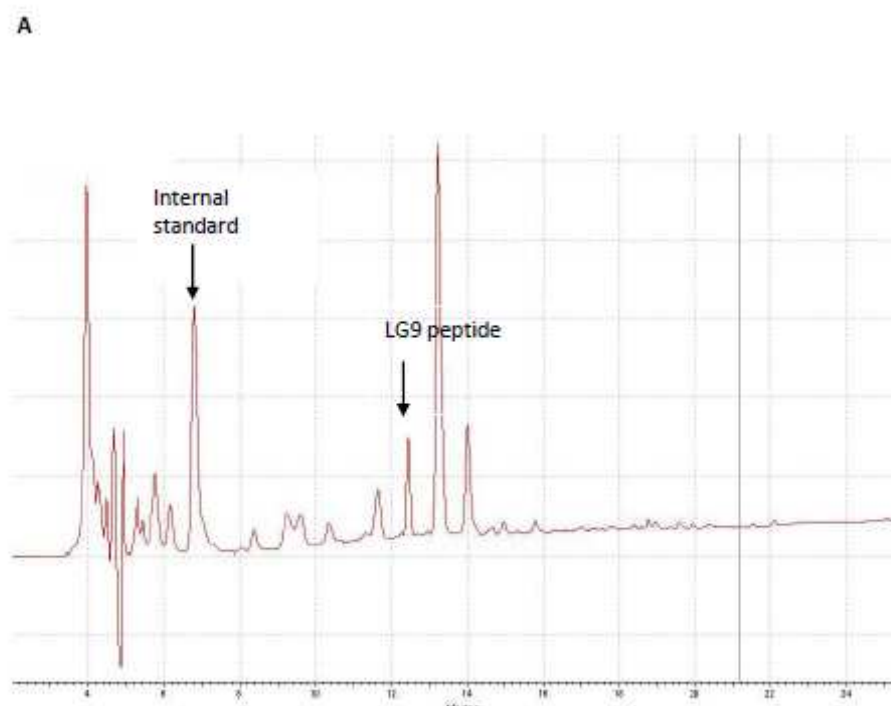
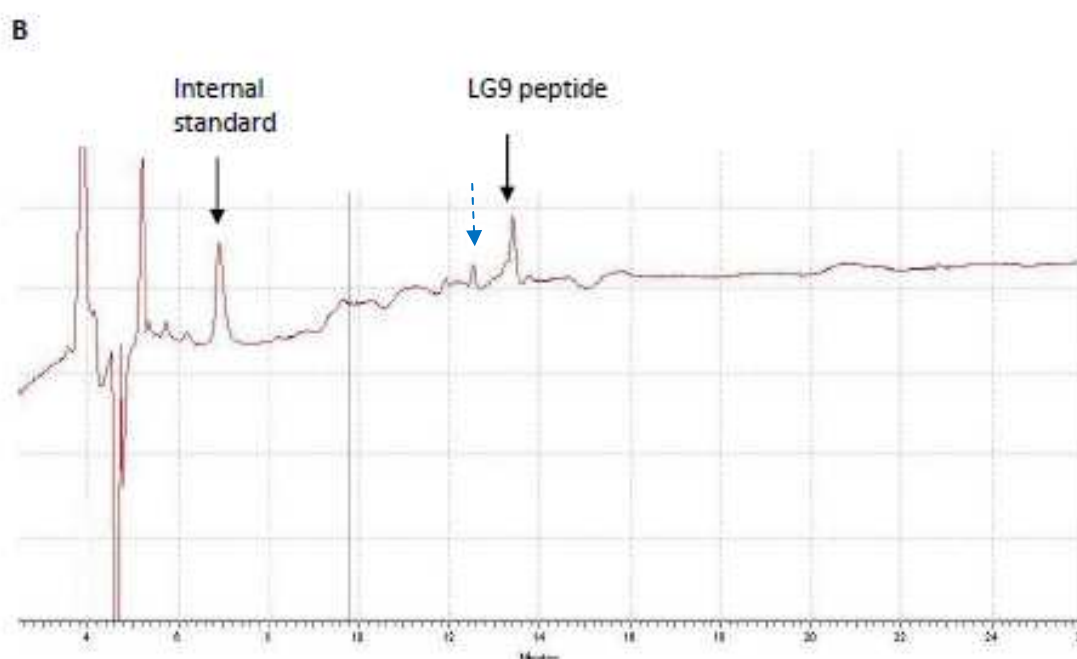


Figure 1S Panel B: shows the HPLC elution profile of the binding experiment. The digested NT-proBNP was incubated with FIP as indicated in the Materials and Method section. At the end of the incubation, FIP polymer was washed, the bound peptides eluted in acetonitrile. The solvent was evaporated; the peptide reconstituted in buffer and injected in HPLC.

The HPLC elution graph here reported shows that FIP binds the LG9 peptide quite specifically from the pool of peptides of digested NT-proBNP. A small peak is visible around 13 min (blue dotted arrow), which is probably due to the unspecific binding to FIP of another peptide of the pool. This experiments accounted for a reasonable specific recognition of FIP versus his template LG9.



Evaluation of the selectivity of FIP in competitive experiment.

Method:

The competitor peptide of sequence LQRSPRPTG was synthesized. It differs from the template peptide LG9, this latter of sequence LQESPRPTG, by one aminoacid substitution. Twenty mg of polymers (FIP1, FIP2, FIP3, NIP1, NIP2, NIP3) were suspended in 600 μ L NaP 50mM pH 7.0 containing a 1:1 ratio of LQRSPRPTG and orbitally shaken at T amb. At the time point 4.0 hours, the solution was withdrawn and microfuged with 0.2 μ m Vivaspin (Sartorius, Italy) for 2 min. Each polymer was incubated for 10 min with pure ACN (400 μ L), then centrifuged and ACN was evaporated under N₂. The recovered peptides were re-dissolved in 100 μ L sodium phosphate buffer 50 mM pH 7.0 and analyzed by HPLC.

Results, summarized in Table S.3, indicate the polymers bind with strong preference the template peptide, LG9, whereas only a small part of the competitor peptide is bound.

Table S.3

polymer	template peptide peak area	competitor peptide peak area
FIP1	22125	1560
FIP2	32600	2004
FIP3	24002	3012