A novel quantitative proteomics reagent based on soluble nanopolymers†

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Bi-functionalized dendrimers leads to highly efficient quantitative proteomics and the determination of protease activities in snake venoms.

Quantitative proteomics holds significant promise for the discovery of diagnostic or prognostic protein markers and for the detection of new therapeutic targets.[1] The isotope-coded affinity tagging (ICAT) method is perhaps the best characterized approach for quantitative proteomics that combines stable isotope labeling with affinity purification.[2] Among other popular methods[3,4] developed since then, the adaptation of solid-phase capture and release process is an important improvement for isotope tagging and selective peptide isolation.[5,6] However, the most notable liability of solid phase capture is the heterogeneous reaction conditions, which can exhibit several of the following problems. Due to extreme complexity and proteins in low-abundance, the heterogeneous nature of solid-phase reaction presents a significant issue for proteomic sample recovery.

Here we devise a new strategy, termed Soluble Polymer-based Isotopic Labeling (SoPIL). The design was based on the concept that the derivatization with proteomic samples in limited amount was carried out in the solution phase for maximum yield and in the second step, samples tagged with the nanopolymer were isolated on a solid phase by choosing a highly efficient reaction between a pair of bioorthogonal groups on the soluble polymer and on the solid phase (Fig. 1A). The cornerstone of SoPIL is a soluble, globular nanopolymer (*e.g.* dendrimers) which was functionalized with reactive groups for site-specific, stable isotopic labeling of proteins/peptides of interest. Unmodified dendrimer was recently used for phosphoproteomics.[7] Here, a 'handle' group on the polymer facilitates the isolation of tagged samples through highly efficient bio-conjugations. The method takes advantage of the homogeneity of solution-phase reaction, convenience of solid-phase capture and release process, and characteristics of cell-permeable nanoparticles.[8] High concentration ratio of the reactive group to the 'handle' group facilitates the completion of solution phase reaction while eliminating extra steps to remove excess reagents which is the case for small chemical reagents such as ICAT reagents.[2] In this initial report, bromoaceto group was employed to selectively capture cysteine-containing peptides/proteins for a direct comparison with solid-phase reagents. In order to isotopically tag and then recover tagged samples for mass spectrometry (MS) analysis, we introduced an acid-cleavable linker, 4-(4-formyl-3,5-dimethoxyphenoxy)butyric acid, and an isotope tag (aniline-$^{12}C_6$ or -$^{13}C_6$) between dendrimer and the reactive group (Fig. 1B). The dendrimer surface was also functionalized with the terminal alkyne group as the 'handle' to achieve efficient isolation through the click chemistry,[9] a highly potent bioconjugation between terminal alkyne group and azide group. Selective isolation, identification and quantification of Cys-containing peptides is illustrated in Fig. 1C.

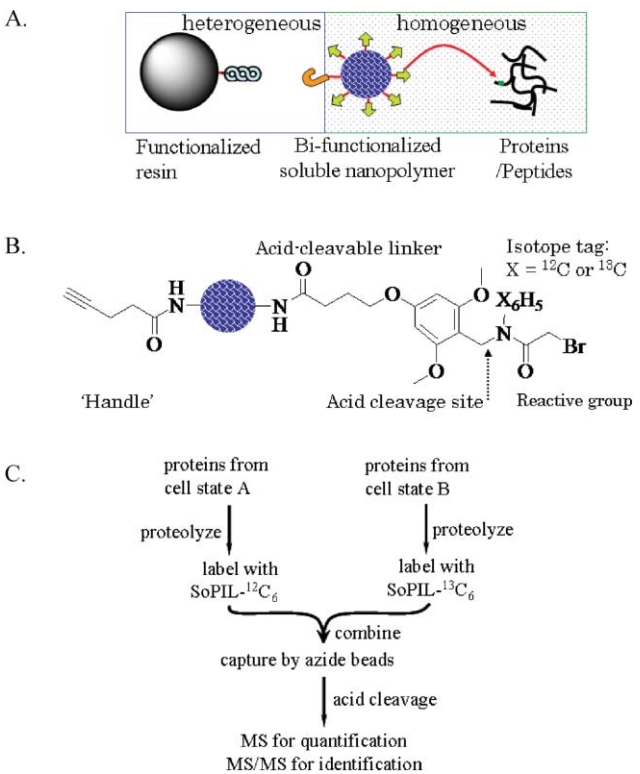

Fig. 1 Schematic representation of the SoPIL method. A: Modular composition of the SoPIL strategy which consists of two step reactions: a homogeneous reaction between proteins/peptides and the SoPIL reagent and a heterogeneous reaction between the solid-phase and the SoPIL reagent. B: Chemical composition of the SH-reactive solid-phase. C: Strategy for quantitative proteome analysis. Two protein samples to be comparatively analyzed were proteolyzed. The Cys-containing peptides were reduced and captured on SoPIL reagents carrying either the light or heavy isotope tag. The samples were then combined and immobilized by the azide beads through the efficient click chemistry. After stringent washing of the beads, the peptides were released by acid-cleavage and analyzed by μLC-MS/MS.

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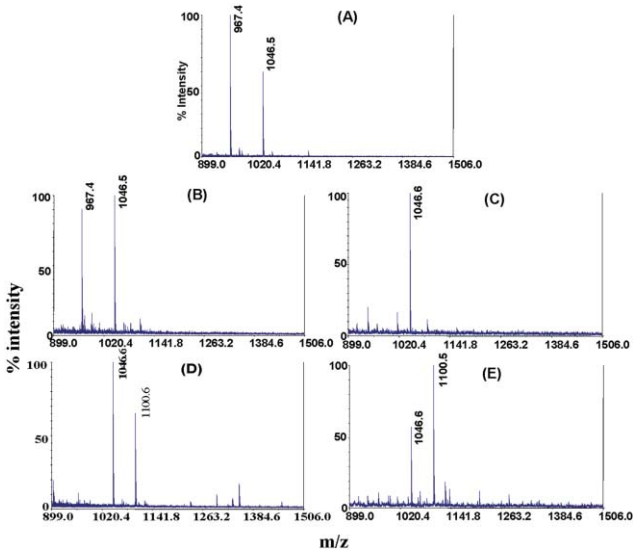

Fig. 2 Comparison between the one-step solid phase method and the SoPIL method. MALDI-TOF MS was used to analyze a peptide mixture consisting of a Cys-containing peptide laminin B (*m*/*z* 967.4) and a non Cys-containing peptide angiotensin II (*m*/*z* 1046.6). (A) Prior to the reaction; (B) right after the addition of solid phase beads (1 min); (C) right after the addition of the SoPIL reagent (1 min); (D) acid cleaved product from the one-step solid phase method; (E) acid cleaved product from the SoPIL method. The ion of *m*/*z* 1100.6 is the product after the modification on the cysteine residue. The *m*/*z* 1046.6 ion was added for comparison.

To demonstrate the whole strategy and also compare it to the direct solid-phase isotope tagging method, a standard peptide mixture consisting of cysteine-containing peptide laminin B (*m*/*z* 967) and non-cysteine-containing angiotensin II (*m*/*z* 1046) was used (Fig. 2). The solid phase reagent was synthesized in a similar fashion to directly incorporate the acid-cleavable linker, the isotope tag aniline, and bromoacetyl group as the thiol-specific group on the aminopropyl controlled pore glass beads (See ESI for detailed synthesis†). Laminin B was attached to the polymer in less than 1 min after the SoPIL reagent was added into the peptide mixture, and in contrast it took over 30 min for the solid phase reagent to completely capture the peptide in the solution. Both reactions were allowed to go to completion. The SoPIL reagent was then captured on the azide-functionalized beads through click chemistry. After 1 h of acid treatment, the tagged peptide was cleaved off the SoPIL reagent and recovered into the solution efficiently, while the recovery yield was much lower with the same treatment of the solid phase reagent (compare Fig. 2D, 2E). Stable isotope labeling was also used to accurately compare the yield between two methods on the same spectrum (ESI Fig. S1†). The yield using the SoPIL method was over 80% while the solid phase method was less than 40%. Therefore, the data demonstrated that the capture and release of laminin B using the SoPIL reagent was specific and more efficient than the one-step solid phase isotopic labeling reagent.

The SoPIL reagents for quantitative analysis were first demonstrated with a standard protein mixture. Two mixtures containing the same four proteins (bovine serum albumin, α-lactalbumin, lysozyme C, and β-lactoglobulin) at different concentration ratios were prepared and analyzed as illustrated in Fig. 1C. The isolated labeled cysteine-containing peptides were quantified and sequenced by μLC-MS/MS experiments. Using a limited amount of proteins (1–400 fmols), all four proteins were unambiguously identified and accurately quantified (ESI Table S1†). Multiple tagged peptides were encountered for each protein. The mean differences between the observed and expected quantities for the four proteins ranged 2–6%.

The SoPIL strategy was applied to study differences in protein abundance in two snake venoms. Snake venom contains complex mixtures of pharmacologically active molecules including small peptides and proteins. The biological effects of venom are complex because different components have distinct but sometimes synergistic actions.[10] The fact that members of the same protein family show remarkable structure similarity but diverge in their biological targeting makes them valuable biotechnological tools for studying physiological processes and for drug discovery. In addition, a number of snake venom proteins are extremely cysteine-rich, which makes a perfect paradigm for us to study using cysteine-specific SoPIL reagents.

The same amount of two characteristically different snake venoms A and B from *Crotalus scutulatus scutulatus* (Mohave rattlesnake) were labeled on light and heavy SoPIL reagents, respectively, combined and processed as described in Fig. 1C. Although the snake genome has not been sequenced, the analysis identified and quantified over 250 unique peptides representing 51 snake toxins in Swiss Protein Database, by far the largest presentation of snake venoms (Selected cysteine-containing peptides from snake venoms in Table 1 and a complete list is provided in ESI Table S2†). Consistent with previous reports, quantitative measurements indicated that several classes of cysteine-rich proteins dominantly exist in venom A but not in venom B. A few proteins, such as disintegrin, were only observed in venom B. Fig. 3 illustrates the identification of one peptide from L-amino acid oxidase and its relatively abundance in two snake venoms. We also observed for the first time extensive cleavage products by proteases in snake venoms (two examples were shown in ESI S3†). Quantitative analysis by SoPIL reagents allowed us to measure difference of protease activities in two snake venoms (Table 1 and S2†).

Dendrimers have been shown to cross cell membranes at sufficient rates to act as potential carrier/delivery systems.[8,11] Therefore, SoPIL reagents have the potential to directly tag and label proteins in living cells and *in vivo*. We examined the efficiency of SoPIL reagents to cross cell membranes. To facilitate the observation of the delivery efficiency, SoPIL reagents were functionalized with fluorescence groups. The delivery of SoPIL reagents into HeLa cells was monitored directly under a fluorescence microscope as a function of SoPIL concentration and incubation time with the living cells (ESI Fig. S2†). Using a concentration of 5 μM for four hour treatment, a maximal of 80% of cells displayed bright fluorescence signals. After 4 h incubation of specific SoPIL reagents with cells, cells were lysed and protein samples labeled by SoPIL reagents were recovered and analyzed by mass spectrometry. Only a few cysteine-containing proteins were identified (data not shown). We reason that, although we used Cys- and Met-free media to culture the cells, an intracellular reducing environment was still present and the SoPIL reagents predominantly reacted with glutathione in living cells. We are currently synthesizing SoPIL reagents with more specificity to target a specific class of proteins.

Table 1 A partial list of snake venom peptides analyzed

Prob	Peptide sequence[a]	Protein description	ASAP ratio[b]
1.00	D.VVVGGDECNINEH.R	Fibrinogen-clotting enzyme TL-BJ	2.56 (0.26)
0.99	A.TLCAGILEGGKDTCK.E	Ancrod	9.68 (0.48)
0.92	A.DTCVGDSGGPLICNGQF.V	Pallabin-2	1.08 (0.18)
0.99	C.DCADIVINDLSLIHELPK.E	L-amino-acid oxidase	2.12 0.22)
0.99	C.CFVHDCCYGK.V	Phospholipase A2, acidic	3.54 (0.24)
1.00	D.C*TGQSADC*PR.N	Disintegrin ussuristatin-1	0.11 (0.06)
1.00	D.PCGTQICECDK.A	Crotoxin acid chain	0.54 (0.06)
0.93	E.FIMNQKPQCILK.K	Hemorrhagic metalloproteinase HT-E	0.00
0.94	E.HIAPLSLPSSPPSVGSACR.V	Venom serine proteinase 3	1.75 (0.545)
1.00	K.CGENIYMSPVPIK.W	Catrin-1\2	2.34 (0.28)
0.99	K.FFCLSSK.N	Calobin	4.42 (0.20)
0.98	K.FFCLSSR.N	Venom serine proteinase A	5.30 (0.51)

[a] C and C* refer to cysteine residues labeled with light or heavy SoPIL reagents, respectively. [b] Ratio of intensities of peptides from snake venom A and B, respectively. In parentheses are measurement errors.

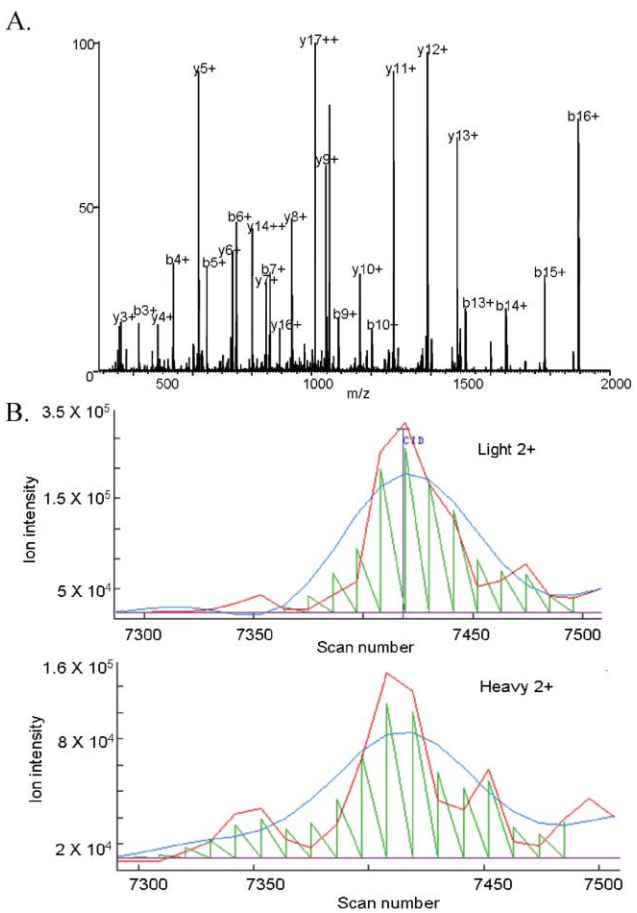

Fig. 3 (A) MS/MS of the peptide DC*ADIVINDLSLIHELPK derived from L-amino acid oxidase (LAO; * refers the cysteine residues labeled by the heavy isotope tag). Its characteristic peptide bond fragment ions, type b and type y ions, are labeled. (B) Reconstructed ion chromatogram of the precursor and its heavy version using ASAPRatio software.[13] The program draws a smoothed chromatogram based on the ion signal and then calculates the ratio of the peak areas. Raw chromatograms are plotted in red, smoothed chromatograms in blue and areas used for calculating abundance ratio of the charge state in green.

Overall, the promising data presented here clearly demonstrate that the novel SoPIL method is simple and efficient for quantitative proteomics. In the experiments using snake venoms the SoPIL reagents efficiently labeled multiple close-spaced cysteine residues, a feature the solid phase method cannot achieve due to steric hindrance. Using SoPIL reagents as the carrier of functional groups, we demonstrated the possibility to deliver the reagents into living cells to react with potential targets. Targeted proteins were isolated and identified by MS *in vitro*. Compared to current strategies using small chemical reagents,[12] conjugating functional groups to nanopolymers may improve their bioavailability in living systems, particularly for hydrophobic molecules, and also increases transmembrane permeation by bypassing the cells' efflux transporters. We expect the SoPIL strategy can be used to discover various enzyme activities and screen specific inhibitors for these enzymes using different reactive groups. It is also reasonable to assume that the reagent can be used to study non-covalent interactions such as drug/ligand-protein interactions.

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