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ON-LINE CAPILLARY ISOELECTRIC FOCUSING HYPHENATED TO NATIVE ELECTROSPRAY IONIZATION MASS SPECTROMETRY FOR THE CHARACTERIZATION OF INTERFERON-γ AND VARIANTS

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Abstract:

The on-line hyphenation of Capillary IsoElectric Focusing (CIEF) with ElectroSpray Ionization Mass Spectrometry (ESI/MS) has been carried out in a non-denaturing detection mode at the CIEF-MS interface. This CIEF-MS coupling methodology relied on the use of 40% glycerol-water medium as anti-convective agent in the CE capillary and the addition of 10 mM ammonium acetate buffer, pH 5, as a volatile aqueous sheath liquid. These CIEF-MS coupling conditions allowed the characterization of the highly basic cytokine human interferon-gamma (IFN-γ) and its detection as a non-covalent homodimer (33814.3 $g.mol^{-1}$) corresponding to the active form of this immune-regulatory protein. An experimental pI value of 9.95 was determined for the human IFN-γ homodimer in these conditions. The CIEF-MS analysis of several variants bearing punctual or deletion mutations within the two D1 and D2 basic clusters at the C-terminal end of IFN-γ revealed the different contribution of these domains to the charge properties of this heparan sulfate-binding protein.

1. Introduction

Capillary electrophoresis (CE) and its hyphenation to mass spectrometry (MS) offer powerful attributes for characterizing biomolecules and studying their bio-molecular interactions. As we previously reported, CE and CE-MS coupling are especially well suited for the characterization of basic protein cytokines and their non-covalent complexes with glycosaminoglycans (GAGs), a major and challenging topic in glycobiology.[1-3] Given that most of the GAG-binding cytokines are highly basic proteins and that the protein charges are major determinants in the interaction with these acidic polysaccharides, it is then of prime importance to assess their charge properties and to precisely determine their isoelectric point (pI). Capillary IsoElectric Focusing (CIEF) is a high-resolution electrophoretic technique for the separation and focusing of ampholytes, such as proteins, leading to the accurate determination of pI.[4, 5] While the hyphenation of CE with ElectroSpray Ionization Mass Spectrometry (ESI/MS) has been widely described either in zone or in frontal format, the on-line coupling of CIEF to an ESI interface has been rarely reported.[6-8] Indeed, CIEF is one of the most challenging CE technique and its on-line MS coupling faces up to several difficulties, among them (i) the maintenance of electrical continuity for electrophoretic separations without the catholyte reservoir, (ii) the compatibility of electrolyte composition with MS detector, and (iii) the chemical and thermal resistance of modified separation capillaries with the ESI interface. Nevertheless, CIEF coupled online with MS is regarded as a promising alternative to 2-D PAGE for fast proteome analysis with high-resolving capabilities. It enables access to enhanced structural information through accurate determination of pI and mass values, without the drawbacks of conventional slab-gel electrophoresis, *i.e.* rough determination of molecular weight, restrictions in sensitivity and tedious staining procedures. We have recently reported the on-line coupling between CIEF and MS that overcome the main difficulties by using a glycerol/water medium in unmodified bare fused-capillaries instead of an aqueous gel medium in coated capillaries.[9] Besides being an efficient anti-convective medium, glycerol/water strongly reduces electro-osmotic flux (EOF), minimizes protein adsorption, and is fully compatible with on-line MS detection. Furthermore the preservation of electric continuity during the whole analysis was achieved thanks to a discontinuous filling of the capillary with catholyte/proteins-ampholytes mixtures. This original methodology has been recently applied for the separation and quantification of milk whey proteins of close pI values in the context of milk allergens in biological samples[10], and with a flow-through

microvial, facilitating interface for model proteins.[11] However, this method was carried in denaturing conditions as regards the MS detection, thus preventing the CIEF-MS detection of quaternary or pseudo-native structures and of non-covalent complexes. In the study herein, we present the application of this new CIEF-ESI/MS coupling method using non-denaturing MS conditions for the pI determination and MS characterization of interferon-γ (IFN-γ), a highly basic and dimeric cytokine, and of various mutants of this heparan sulfate (HS)-binding protein.

IFN-γ is a key immune-regulatory protein involved in antiviral, anti-proliferative and immune-stimulating activities as well as in physio-pathological processes including autoimmune diseases and chronic inflammation.[12] The active form of human IFN-γ is a homodimer (33552.3 g.mol^{-1}) comprising 143 amino acids per subunit (Fig. 1). The C-terminal region is characterized by two clusters of basic residues referred as D1 and D2 (D1, residues 125-131 KTGKRKR; D2, residues 137-140 RGRR), which are involved in the binding to the membrane receptor (D1) and heparan sulfate (D1 and D2) on proteoglycans anchored to the membrane.[13-15] These two domains are key regulatory elements of both the biological activity of the cytokine and its tissue distribution.[16, 17] The impact of deletion and point mutations within D1 and D2 basic domains on electrophoretic mobility, pI values and MS detection has thus been investigated.

2. Experimental

2.1 Chemicals and reagents

Recombinant wild type (WT) IFN-γ as well as single and deletion mutants, expressed in *E. coli* (thus comprising an additional Met residue at the N-terminus) were available at 0.8-5.1 mg/mL (49-302 µM, according to molecular weight) prepared in TRIS buffer 10 mM pH 6.8 with 25 mg/mL mannitol as previously described.[15] Carrier ampholytes pH 9–11 and pH 3–10 were purchased from Servalyt (Serva Electrophoresis GmbH, Heidelberg, Germany). Peptide markers at pI 4.1, 5.5, 7.0, 9.5 and 10.0 (cIEF Peptide pI Marker Kit) were obtained from Beckman Instruments (Fullerton, CA, USA). Cytochrome C (CytC, equine heart, pI = 10.25, Mw = 12 384 g.mol^{-1}) and carbonic anhydrase I (CA I, pI = 6.6, Mw = 28 781 g.mol^{-1}), L-arginine ≥98.5%, dichloromethane, 3-(trimethoxysilyl)propyl methacrylate 98%, acrylamide ≥99%, N,N,N',N'-tetramethylethylendiamine (TEMED) 99%, ammonium persulfate ≥98%, glutamic acid ≥99.5%, formic acid ≥95%, acetic acid ≥99.7% and hydrochloric acid 37% were purchased from Sigma-Aldrich-Fluka (St.-Quentin Fallavier,

France). Methanol 99.8%, ammonia 32%, acetone 99.9% and sodium hydroxide 35% were from VWR (Fontenay-sous-Bois, France). Glycerol ≥99.5%, were purchased from Carlo Erba (Val de Reuil, France) and dichloromethane 99.99% from Fisher scientific (Illkirch, France). All buffers and solutions were prepared using Ultrapure water (Milli-Q, Millipore, Milford, MA) and degassed by filtration through 0.2 µm filter units before use.

2.2 Capillary Electrophoresis

CE experiments were performed with a Hewlett-Packard ^{3D}CE apparatus (Agilent Technologies, Massy, France), equipped with a built-in diode array detector and a power supply able to deliver up to 30 kV. Bare fused-silica capillaries of 50 µm i.d. (360 µm o.d.) were from Phymep (Paris, France). The temperature of the capillary cartridge was set at 25°C. Data were collected and analyzed using the HP Chemstation software. The separation electrolyte was made up of 40/60 (v/v) glycerol/water containing the carrier ampholytes pH 3–10 (1.5% v/v) and pH 9-11 (1% v/v) according to a slightly modified previously reported method.[9] The amount of glycerol content in water (v/v) was increased from 30% in the previously reported method to 40% in the present study so as to further minimize the adsorption of basic proteins. Theoretical pI values were determined using the Compute pI/Mw module available on Expasy website.

2.3 Capillary polyacrylamide wall coating

60 cm-length capillary was prepared without a detection window and was conditioned by successive flushes at 950 mbar with acetone, 1 M sodium hydroxide, 0.1 M hydrochloric acid and water for 10 min, 30 min, 10 min and 10 min, respectively. Polyacrylamide (PA) coating was next achieved following a previously described procedure[18]: about 6 µL of 3-(Trimethoxysilyl)propyl methacrylate with 5 µL acetic acid were mixed with 1 mL of dichloromethane. This silane solution was sucked up into the capillary by flush during 3 min at 950 mbar. After reaction at room temperature for at least 1 h the silane solution was withdrawn. The tubes were washed by successive flushes with methanol and water under a pressure of 950 mbar for 10 min. Capillary was filled with a 1.5% PA solution previously degassed during 30 min, and freshly added with 0.1% of both TEMED and ammonium persulfate by flushing at 950 mbar for 3 min. Extremities of the capillary was let in PA vial for 1 h, then the excess of PA was sucked away and the tubes were rinsed with water under a pressure of 950 mbar for 10 min. Finally, capillary was heated in a gas chromatography oven for 1 h at 100°C under nitrogen flow (1.5 bar).

2.4 Mass Spectrometry

Mass spectrometry was carried out with an Esquire 3000+ ESI source ion trap mass spectrometer, using Esquire Control software versions 5.1 (Bruker Daltonics, Wissembourg, France). Mass spectra were recorded and processed using Data Analysis software versions 5.1 (Bruker Daltonics). The drying gas (nitrogen) temperature was maintained at 150°C, with a flow rate of 5 L.min^{-1}. The nebulizing gas (nitrogen) pressure was optimized at 10 psi. The instrument was calibrated using ES tuning mix provided by the manufacturer. The mass spectra were obtained in the standard mode (*m/z* 140-3000) with a target mass set at *m/z* 900, and in the extended mode (*m/z* 500-4500) with a target mass set at *m/z* 2800 for denaturing and non denaturing conditions, respectively. Data acquisition was performed in the standard resolution mode with a scan speed of *m/z* 13 000 per second. The maximum numbers of accumulated ions and accumulation time were fixed at 1.10^5 and 500 ms, respectively, and the average scan number was 5. Mass spectra and extracted ion chromatograms (EIC) were obtained using Bruker Daltonics 5.1 Data Analysis software.

2.5 Capillary Electrophoresis-Mass Spectrometry

The CE capillary was hyphenated to mass spectrometer using a coaxial sheath-flow interface G1607A (Agilent Technologies). According to the 3-11 pH gradient formed by the ampholytes, the anolyte was 50 mM formic acid/1 mM glutamic acid, pH 1.9, and catholyte was 100 mM ammonia/1 mM arginine, pH 11.3, both in 40/60 (v/v) glycerol/water so that anolyte and catholyte viscosity was the same as in the separation electrolyte. The capillary was flushed for 3 min at 950 mbar with the catholyte and then 60 s at 950 mbar with the separation electrolyte containing the pI markers with or without the target cytokine (6.4 to 39.4 µM), so that 40 cm of capillary was filled with this latter solution (taking into account the viscosity of the mixture). The concentration in the separation electrolyte of each individual peptide standard from pI Marker Kit was 6% (v/v), 0.27 mg/mL for CytC and 0.45 mg/mL for CA I. The focusing step was achieved by applying a voltage of + 30 kV for 12 min. Mobilization to cathodic side was performed under a 50 mbar pressure, while maintaining the applied focusing voltage. The capillary effluent was mixed in the coaxial sheath-flow interface either with hydro-organic or aqueous sheath liquid delivered at a flow rate of 5 µL.min^{-1} using a syringe pump (74900 series, Cole-Palmer). The sheath liquid consisted of 60:40 (v/v) water/acetonitrile with 1% formic acid in denaturing conditions, and 10 mM ammonium acetate, pH 5, in non-denaturing conditions. Electrospray was achieved in positive ionization mode by applying a capillary voltage of -4 kV.

3. Results and discussion

The CIEF-MS protocol that we previously developed used 30% glycerol content in water (v/v) to take advantage of both the anti-convective effect of glycerol and a low electro-osmotic flow (EOF) while ensuring ionization efficiency. To further reduce the possible adsorption of basic proteins like IFN-γ, we adapted the concentration of glycerol medium to 40% while allowing a better focusing (results not shown). Glutamic acid (Mr 146.2) and arginine (Mr 174.2) were added to the anolyte and catolyte, respectively, in order to delineate the limits of the pH gradient zone. Carrier ampholytes concentration from 1 to 5% (v/v) is currently used in standard CIEF-UV experiments. The coupling between CIEF and MS requires low ampholyte concentrations to avoid ion suppression. Considering resolution, sensitivity and repeatability, an optimal final concentration of the pH 3-10 ampholytes mixture was set at 1.5% v/v in glycerol-water medium. In order to achieve a better separation of the basic proteins, this pH 3-10 ampholytes mixture was supplemented with a pH 9-11 ampholytes mixture at an optimized concentration of 1% (v/v).

The CIEF separation of various protein pI markers ranging from pI 4.1 to pI 10.25 coupled to on-line ESI/MS in positive ionization mode allowed their MS detection as depicted on the extract ion chromatogram (Fig. 2A). The distinct focalization of each pI markers allowed the establishment of a pI calibration curve that exhibited a slope break around pH 9 due to the use of two different carrier ampholytes at pH 3-10 and pH 9-11, respectively. Thanks to the smaller slope in the pH 9-11 range, an improved resolution of basic protein species could be expected (Fig. 2B).

The CIEF-ESI/MS analysis of recombinant IFN-γ in positive ionization mode was then carried out using the hydro-organic sheath liquid water/acetonitrile/formic acid 60/40/0.1 (v/v/v). In these conditions, IFN-γ focused close to the marker around pI 10. Based on the pI calibration curve, an experimental pI value of 9.95 was determined, *i.e.* a slightly higher value than the theoretical value of 9.70 determined for the IFN-γ monomer (Fig. 2B), thus confirming the highly basic nature of this cytokine. The resulting mass spectrum exhibited a well-resolved charge distribution from +28 (m/z 604.8) to +15 (m/z 1128.2) corresponding to a molecular mass of 16907.5 g.mol^{-1}, in agreement with the theoretical average molecular mass of 16907.4 g.mol^{-1} expected for the IFN-γ monomer (Fig. 3A), including the Met residue at position -1. IFN-γ being a homodimeric cytokine, this result indicated that the hydro-organic sheath liquid led to the disruption of the non-covalent association between monomers upon electrospray ionization. We have previously shown that zone and frontal

CE can be coupled to ESI/MS using an aqueous sheath liquid in order to maintain non-covalent complexes in the ion source and allow their on-line MS detection.[19] Such native conditions have never been described for the CIEF-MS coupling. It prompted us to carry out CIEF-MS coupling using a volatile aqueous sheath liquid composed of 10 mM ammonium acetate, pH 5.0. In these conditions, the spectrum showed fewer ions centered at a lower charge state (Fig. 3B), *i.e.* +14 (m/z 2416.5) (with a charge distribution from +12 to +21), than when using a denaturing hydro-organic sheath liquid. Consistently, the range of m/z ratio of detected ions was shifted from m/z 600-1130 to m/z 1500-3000, indicating that less protein sites were exposed for protonation in these aqueous conditions, as expected for a protein in a conformation closer to its native state. Accordingly, a molecular mass of 33814.3 g.mol^{-1} was determined, corresponding to the mass value of dimeric IFN-γ (theoretical molecular mass of 33814.7 g.mol^{-1}). This result indicates that IFN-γ migrates in CIEF under a dimer state and that the acetate buffer sheath liquid enabled to keep the non-covalent association of IFN-γ monomers at the capillary exit in the ion source and up to the MS detection. Furthermore, no ion corresponding to IFN-γ monomer was detected in the m/z 600-1200 range (data not shown). It confirmed that the pI value of 9.95 measured here upon capillary isoelectrofocusing of IFN-γ was for a homodimer structure that actually corresponds to the active form of this cytokine. The few pI values reported to date for IFN-γ were obtained by gel isoelectric focusing. They were spread over a wide range from 8.5 to 9.2, likely due to heterogeneity of IFN-γ preparations that were purified or partially purified from human lymphocytes.[20, 21] More recently, the use of recombinant form of human IFN-γ is widespread throughout many studies of this cytokine. The CIEF-MS analysis reported herein provides the first experimental determination of pI for the recombinant IFN-γ in its dimeric form.

It has been previously reported that the two C-terminal clusters D1 and D2 of basic amino acids greatly contributed to a highly marked positive electrostatic potential in the C-terminal ends of IFN-γ.[15] Various mutants of IFN-γ were produced through the substitution of a basic residue by a serine within D1 or D2, or the complete substitution of all basic residues within a domain (SD1 and SD2 mutants), or deletion of the D2 domain (Δ136 mutant)*. The optimized CIEF-MS was applied to the analysis of single amino acid mutation within D2. It showed a weak impact of point mutation of this domain on the pI value of IFN-γ (Table 1), except for the R137S mutant showing a larger decrease of the pI value.

* The mutant with deletion of both D1 and D2 domains described in [15] was not available for the study herein.

Consistently, the SD2 mutant exhibited almost the same experimental pI value than the wild type IFN-γ, indicating that the D2 basic cluster does not significantly contribute to the basic pI of IFN-γ. This weak impact of D2 was further confirmed by the CIEF/MS analysis of the deleted D2 mutant (Δ136), which even showed a slightly more basic pI (Table 1).

Unlike within D2, point mutations in the D1 domain lowered the pI value of the resulting IFN-γ mutants, ranging from a slight effect for the K130S mutant to a more pronounced decrease of pI for the K125S mutant. The CIEF/MS analysis of the SD1 mutant featured by the complete substitution of the five basic residues showed also a decrease of the pI value, highlighting the significant impact of the D1 domain on the strong basic pI of IFN-γ.

The net charge of a protein is determined by its ionizable groups, the pK values of which depend on their exposed or buried positions in the folded protein and their involvement in forming ion pairs and hydrogen bonds.[22-24] Its is worth noting that all reported crystallographic structures of IFN-γ for human[25, 26] and other mammalian species[27, 28] exhibited a disordered organization of the C-terminal end (amino acids 124-143), indicating that this region comprising the D1 and D2 domains was rather unstructured or flexible with possible multiple conformations. Hence, it is difficult to link the differential impact on the pI observed between D1 and D2 with a possible distinct exposition of their respective basic residues in the folded IFN-γ. Yet, the variable effect on the pI values observed according to the mutations within D1 or D2 likely reflects difference in their relative exposition in the native cytokine. In this regard, the CIEF-MS native spectra of the D2 mutants Δ136 and SD2 (Fig. 4A and 4B) and wild-type IFN-γ exhibited a charge distribution identically centered at charge state +14, supporting the above-mentioned weak impact of D2 on the global charge of IFN-γ. Conversely, CIEF-MS native spectrum of the SD1 mutant was centered at a lower charge state (Fig. 4C), confirming the effective contribution of D1 to the charge properties of IFN-γ. Besides, it is worth noting that the charge state distributions observed on mass spectra of the wild-type as well as the mutants IFN-γ showed a deviation from a Gaussian-type distribution suggesting multiple conformations of IFN-γ likely due to flexibility of the C-terminal end. Interestingly, these striking differences between the two D1 and D2 domains for their respective contribution to the pI value of IFN-γ may be related to their distinct involvement in the interaction with HS that has been recently reported.[15] Although both D1 and D2 exhibit favorable electrostatic charges to interact with this sulfated polysaccharide, only D1 was shown to mediate highly specific interaction, and actually to be the HS binding site. It has been assumed that D2 favor productive encounters

between IFN-γ and HS through non-specific electrostatic forces but was not involved in the resulting complex.

4. Conclusions

In this work, CIEF-MS coupling was carried out in non-denaturing conditions as regards the MS detection. For that purpose, we showed that the use of 40% glycerol-water medium as anti-convective agent added the ampholytes/sample mixture could be efficiently combined to a volatile aqueous sheath liquid at the CIEF-MS interface. It allowed the accurate electrophoretic focalization of proteins variants with close pI values and the MS detection of non-covalent assembly. It was successfully applied to the characterization of the non-covalent dimer IFN-γ, a highly basic cytokine featured by a marked protein dipole. The two contiguous D1 and D2 basic clusters within the flexible carboxy-terminal sequence of IFN-γ strongly contributed to the positive electrostatic potential of the protein dipole. A highly basic experimental pI value of 9.95 was measured for the first time for the recombinant form of the IFN-γ dimer. The CIEF-MS analysis in native conditions of several mutants pointed out the differential impact of D1 and D2 on the pI values, which may reflect a different exposition between D1 and D2 favored by the flexibility of the C-terminal domain of the protein. This result could be correlated to the proposed mechanism in which the two D1 and D2 basic clusters play different roles in the interaction and the binding to HS. The CIEF/MS hyphenation described here both in denaturing and non-denaturing conditions, constitutes a useful tool to determine pI of multi-protein non-covalent complexes such as several others chemokines known to be prone to oligomerization.[29]

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5. References

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Figure and table legends

Figure 1.

Structural model of dimeric wild-type IFN-γ (PDB entry 1HIG) including the D1 and D2 basic C-terminal domains. Glycosylation sites (Asn^{25} and Asn^{97}) are noted in red.[26]

Figure 2.

On-line CIEF–ESI/MS separation of standard pI markers in mixture and WT IFN-γ in positive ionization mode and denaturing hydro-organic sheath liquid. (A) Extract ion chromatograms (EIC) corresponding to cyt C pI 10.25 (m/z 765.4), pI marker 10.0 (m/z 624.8), pI marker 9.5 (m/z 475.8), pI marker 7.0 (m/z 627.7), CA I pI 6.6 (m/z 929.5), pI marker 5.5 (m/z 471.5), pI marker 4.1 (m/z 591.6), and WT IFN-γ (m/z 806.1). (B) Protein pI values as a function of the migration time using the two different carrier ampholyte compositions. Least-squares regression lines: y (pH 4.1–7) = -4.2991x + 146.92, R^2 = 0.98; y (pH 9.5–10.25) = -0.4567x + 23.38, R^2 = 0.99 (x referring to protein migration time and y to pI value). CE conditions: bare fused-silica capillary, 50 μm i.d., 60 cm length; separation electrolyte, glycerol/water 40/60; focusing achieved at + 30 kV for 12 min and mobilization at 50 mbar pressure at + 30 kV, while maintaining the same voltage; temperature, 25°C; sample injection, 950 mbar, 60 s. MS conditions: target mass 2800 m/z; sheath liquid, 10 mM ammonium acetate, pH 5, delivered at a flow rate of 5 $\mu L.min^{-1}$; the drying gas temperature was maintained at 150°C, with a flow rate of 5 $L.min^{-1}$; the nebulizing gas pressure was at 10 psi.

Figure 3.

On-line CIEF–ESI mass spectra of WT IFN-γ in (A) denaturing and (B) non-denaturing conditions. CE and MS conditions as in Figure 2 except that for MS in denaturing conditions: sheath liquid was 60:40 (v/v) water/acetonitrile with 1% formic acid, pH 5 and target mass was set at 900 m/z.

Figure 4.

On-line CIEF–ESI native mass spectra of IFN-γ mutants. (A) D2 domain mutant Δ136, (B) D2 domain mutant SD2, and (C) D1 domain mutant SD1. CE and MS conditions were similar to Figures 2 and 3 in non-denaturing conditions.

Table 1. Recapitulative of isolectric points and molecular weights values determined by on-line CIEF-ESI/MS on WT and partially deleted or mutated IFN-γ Carboxy-terminal sequences.

Modified or deleted domain	IFN-γ	Isoelectric point (pI)[a]	Molecular weight (Mw) (g.mol^{-1})	
			Theoretical	Experimental[b]
-	WT	9.95	16907.4	16907.5 ± 1.2
D2	$R^{140}S$	9.90	16838.2	16838.0 ± 0.7
	$R^{138}S$	9.92	16838.2	16838.0 ± 1.1
	$R^{137}S$	9.67	16838.2	16838.3 ± 1.0
	SD2	9.94	16700.0	16700.8 ± 1.3
	Δ136	9.99	16095.4	16095.8 ± 1.2
D1	$R^{131}S$	9.77	16838.2	16838.4 ± 1.2
	$K^{130}S$	9.90	16866.3	16866.2 ± 1.1
	$R^{129}S$	9.68	16838.2	16838.4 ± 1.2
	$K^{128}S$	9.70	16866.3	16866.2 ± 1.1
	$K^{125}S$	9.65	16866.3	16866.2 ± 1.3
	SD1	9.67	16645.8	16645.8 ± 2.9

[a] pI determined in non-denaturing conditions. [b]Mw values determined in denaturing conditions

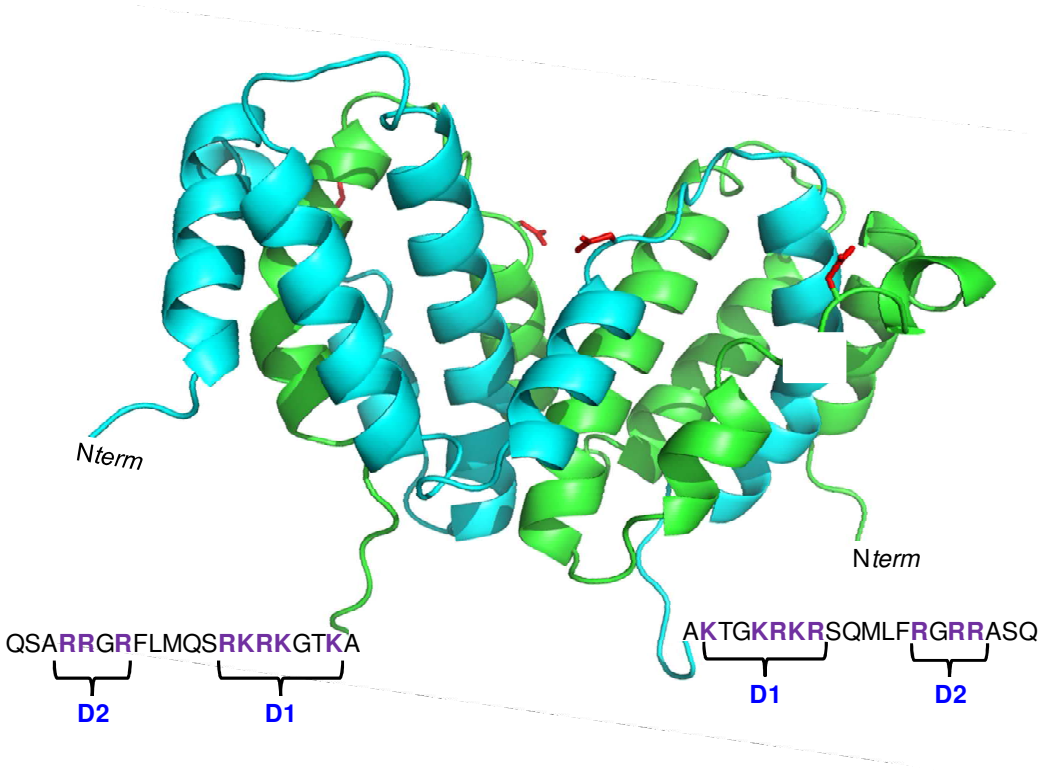
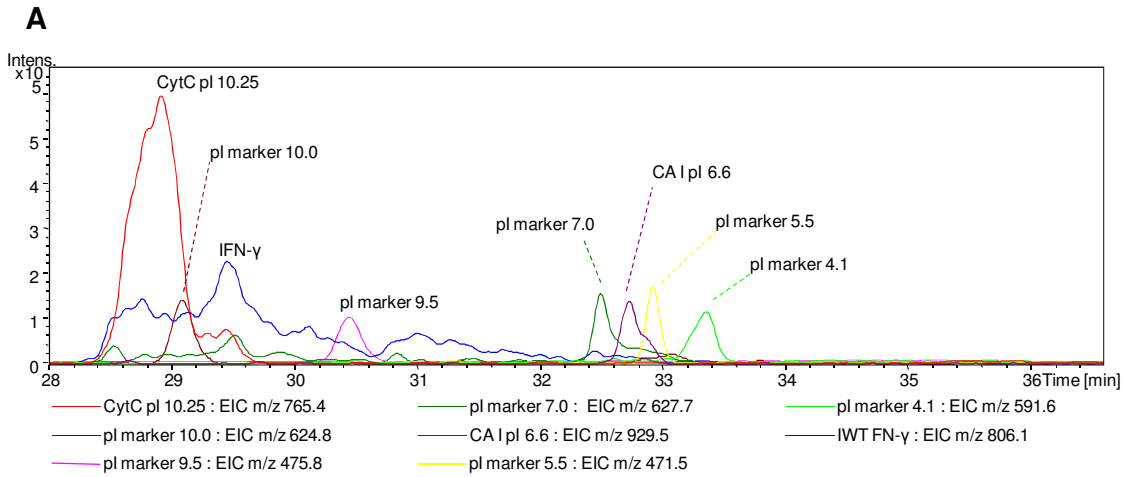


Figure 1.



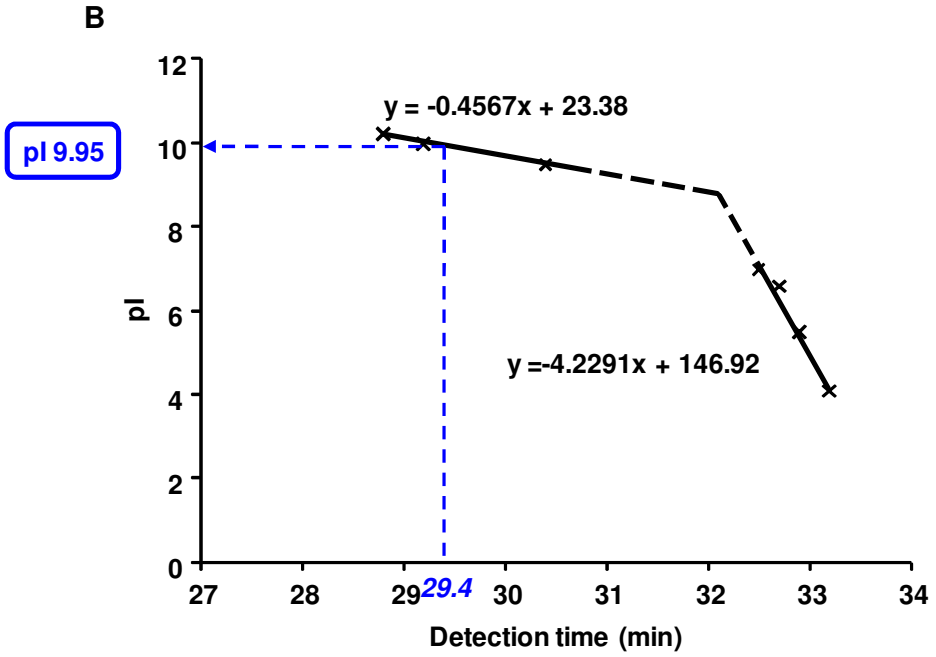


Figure 2.

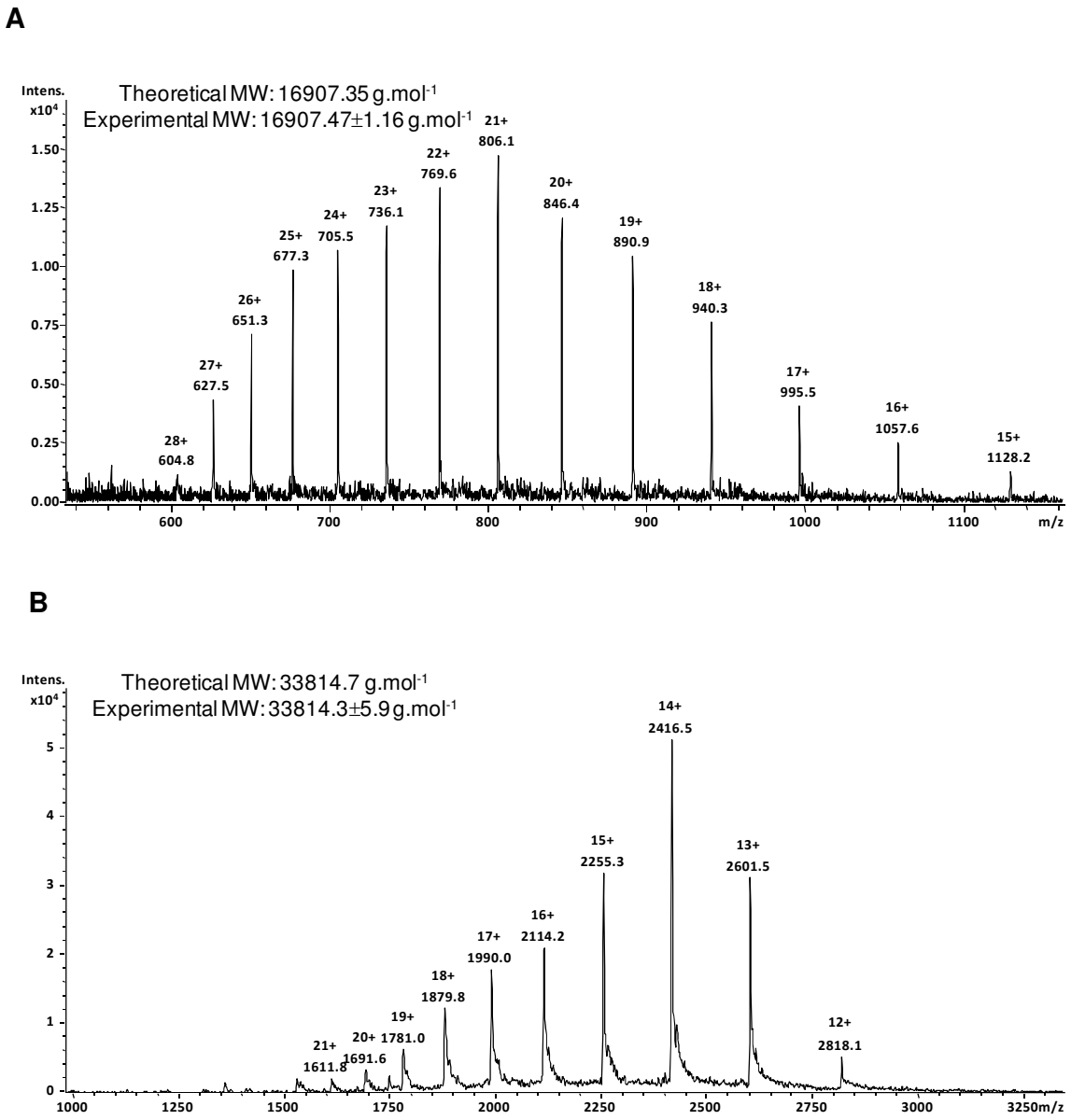


Figure 3.

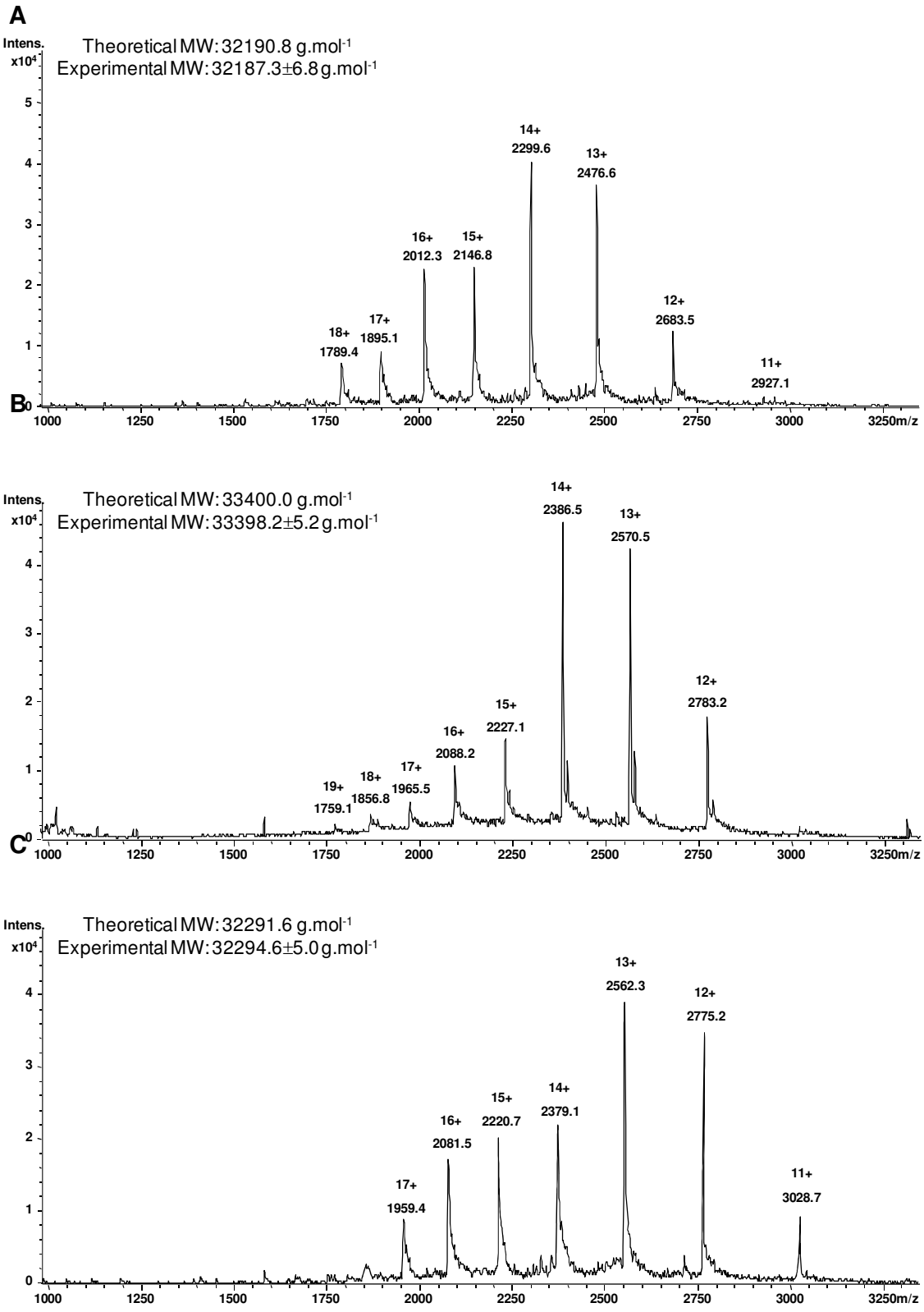
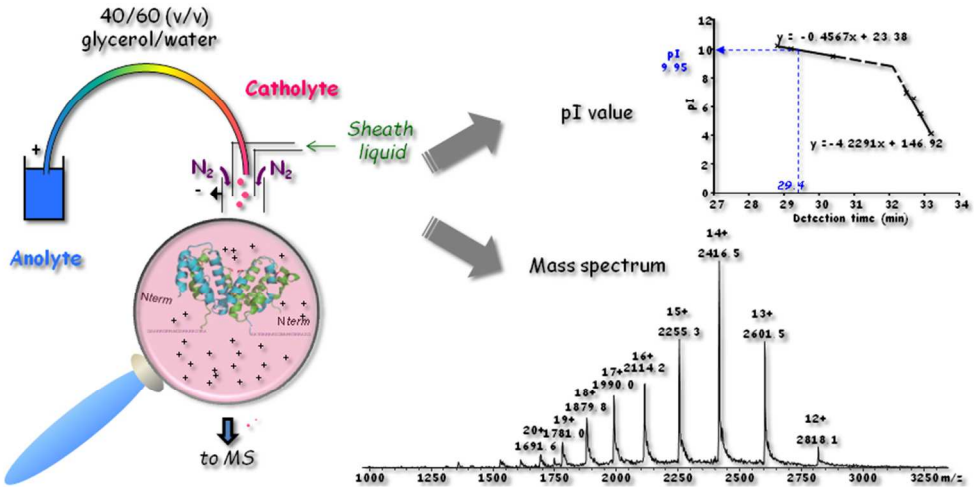


Figure 4.



252x121mm (96 x 96 DPI)