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Hydrogen/deuterium exchange on protein solutions containing nucleic acids: utility of protamine sulfate

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Obtaining global hydrogen/deuterium (H/D) exchange data on proteins is an important first step in amide proton exchange experiments. Important information such as the mode of exchange, the cooperativity of folding/unfolding reactions, and the effects of ligand binding can be readily obtained in global exchange experiments. Many interesting biological systems are complexes containing both proteins and nucleic acids. The low pH conditions required to quench H/D exchange reactions result in the formation of stable protein/nucleic acid precipitates which interfere with the liquid chromatography step of the experiment and preclude obtaining mass spectrometric data. In this work we show that the precipitation of proteins and nucleic acids is electrostatic in nature and can be prevented by high ionic strength and by removing nucleic acids by protamine sulfate. Using protamine sulfate in quenching solution, we were able to obtain global H/D data with protein samples containing large amounts of DNA or RNA. Copyright © 2008 John Wiley & Sons, Ltd.

Hydrogen/deuterium (H/D) exchange (aka amide proton exchange) coupled to mass spectrometry (MS) is widely used to study protein folding/unfolding reactions, protein-ligand interactions, as well as conformational changes and structural aspects of individual proteins and protein complexes.[1–3] The exchange of amide protons for deuterons results in a mass increase that is detectable by MS. Unlike nuclear magnetic resonance (NMR), following H/D exchange by MS does not require high protein concentration, and is not significantly restricted by the solubility or mass of the protein. It is therefore capable of obtaining information on multiprotein complexes.[4–6]

Two kinds of H/D exchange measurements are possible: global and local.[1–3] In the global exchange experiment the mass of the intact protein is measured. In local exchange experiments the protein is digested with an acid-resistant protease (usually pepsin) and the masses of individual peptides are measured. Local exchange experiments provide more detailed information as they allow estimation of the H/D exchange rates of different regions of the protein. However, they are significantly more laborious than global exchange experiments (both in terms of sample handling and analysis) and, optimally, require a high-resolution mass spectrometer. Global exchange, on the other hand, can provide very useful information at a fraction of the cost of a local H/D experiment. For example, global exchange can be used to estimate the overall protein stability, determine the mode of exchange (EX1 or EX2) and to see if any particular ligand has an effect on the protein.[7–10] Thus global H/D exchange is often the first step of an H/D exchange experiment.

Both matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) MS have been used for the two types of H/D exchange experiments, global and local, but the latter is used more frequently. Typically, an H/D exchange experiment is initiated by diluting the protein(s) of interest into a D_2O-containing buffer.[11] At different time points the reaction is quenched by rapid acidification of the sample to pH 2.6–2.8 (which is a global minima for the H/D exchange reaction[12]) and rapid cooling. These steps are designed to minimize the back exchange. Quenching and cooling is often done by diluting the sample with an ice-cold acidic buffer and flash-freezing it in liquid nitrogen. Flash-freezing is convenient because it uncouples the exchange reaction from the MS analysis. The sample can then be thawed (and if desired digested) just prior to measurement and injected onto an ice-submerged reversed-phase column for desalting. After all the non-volatile contaminants (salts, urea, etc.) have been washed away the protein is eluted by a gradient of organic solvent directly into the ESI-MS system.

Global H/D exchange experiments are usually very straightforward. However, we have experienced significant detection problems while trying to perform global H/D exchange studies with protein/nucleic acid complexes. The complexes included the dodecameric portal protein/pRNA from bacteriophage Phi-29,[13,14] DNA packaging reactions containing the Phi-29 procapsid and its cognate DNA,[14,15] and the gp16 terminase.[16] In all cases we were unable to obtain any protein signal.

In this work we show that under the low pH quenching conditions the proteins form strong electrostatic precipitates

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with nucleic acids, which preclude obtaining any global H/D exchange information. We found that a mixture of very basic polypeptides collectively called protamine sulfate is an excellent reagent for removing nucleic acids out of protein/nucleic acid mixtures under quenching conditions and does not interfere with protein chromatography and H/D exchange. It is therefore well suited for enabling global H/D exchange experiments on protein/nucleic acids mixtures.

EXPERIMENTAL

Materials

Protamine sulfate and D_2O were from Sigma. Phi29 portal oligomer in complex with pRNA, Phi29 proheads with Phi29 genomic DNA and Phi29 gp16 protein were produced as published previously.[14] All samples were in TMS buffer: 50 mM Tris, pH 7.4, 100 mM NaCl and 10 mM $MgCl_2$.

Studying solubility under H/D exchange quenching conditions

The samples were diluted 1:3 with ice-cold 1% formic acid with or without 8 M urea, 6 M guanidinium chloride, NaCl and different amounts of protamine sulfate. For studying precipitation 1 μg of Phi29 proheads and 1 μg of Phi29 DNA of Phi29 proheads were taken. The ratio of Phi29 portal and pRNA is around 1:1 by weight. The quantities and nature of nucleotides contaminating gp16 protein were not known.

Light absorption was measured on a Beckman DU640 spectrophotometer.

Liquid chromatography (LC) was performed on an Ultimate Nano HPLC system (Dionex). A C4 reversed-phase microtrap (Michrom) with all tubing was submerged into an ice-bath. Protein elution was achieved by applying a gradient of 100% acetonitrile, 0.1% formic acid. The column output was directly connected to the microspray source of an LCT mass spectrometer (Micromass).

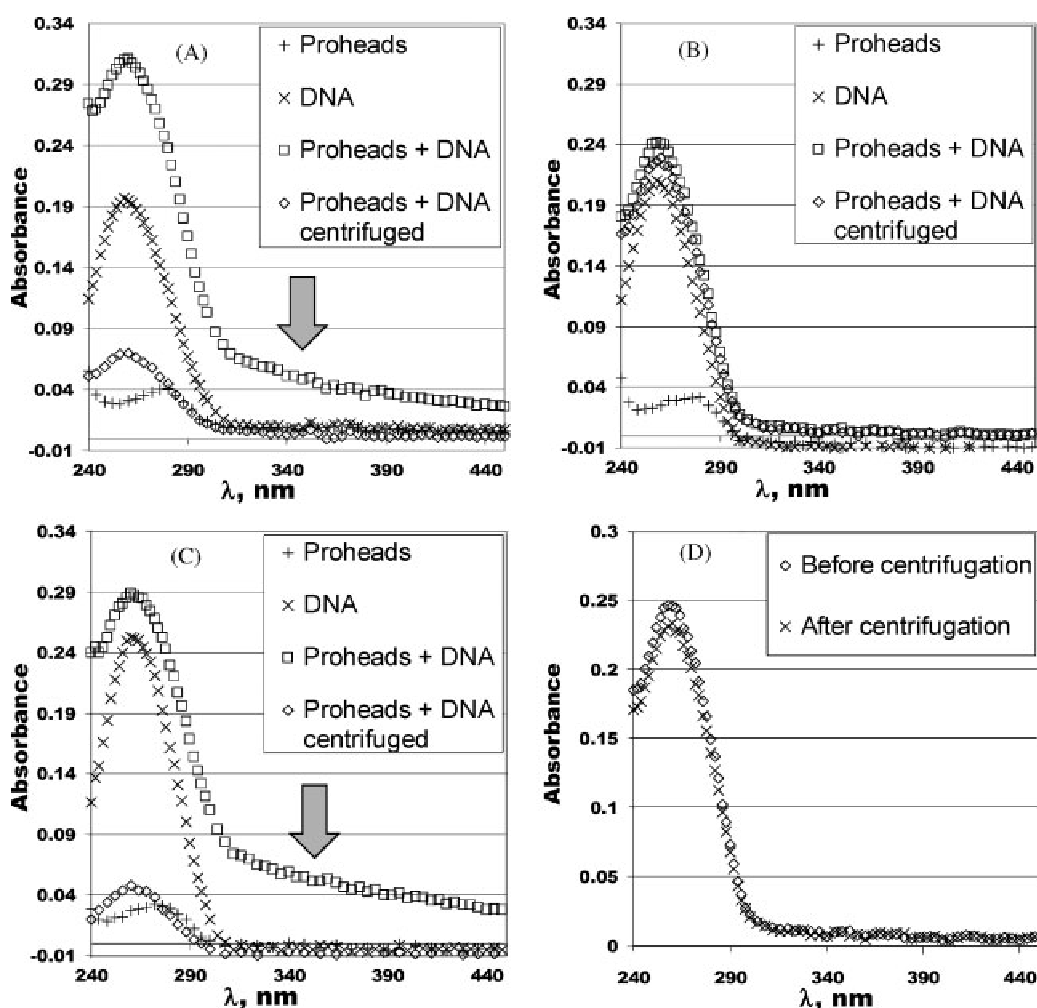

Figure 1. Precipitation of proteins and nucleic acid at low pH. Gray arrow indicates light scattering in the region where neither protein nor DNA absorb light. (A) UV/Vis spectra of Phi29 proheads, Phi29 DNA and their mixture before and after centrifugation at pH 2.8. (B) UV/Vis spectra of Phi29 proheads, Phi29 DNA and their mixtures before and after centrifugation at pH 7.4. (C) UV/Vis spectra of Phi29 proheads, Phi29 DNA and their mixture before and after centrifugation at pH 2.8 in 8 M urea. (D) UV/Vis spectra of Phi29 proheads/Phi29 DNA mixture before and after centrifugation at pH 2.8 in 6 M guanidinium chloride.

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To study the effect of protamine sulfate on H/D exchange we diluted Phi29 proheads 10× with D_2O-based TMS buffer and left it at room temperature for 10 min. The H/D exchange reaction was quenched by diluting the sample 1:3 with an ice-cold 1% formic acid solution containing 8 M urea with or without 1 mg/mL protamine sulfate as indicated and immediately frozen in liquid nitrogen. The samples were analyzed as described above.

RESULTS

Proteins and nucleic acids form precipitates at pH 2.7

Initial attempts to obtain global H/D exchange data on protein samples containing nucleic acids by reversed-phase chromatography and ESI-MS were unsuccessful due to the lack of protein signal. Three systems were examined: (a) a mixture of Phi29 procapsids (empty virion shell consisting of capsid, scaffolding and portal proteins) and Phi29 genomic dsDNA, (b) the Phi29 portal protein complex with bound pRNA molecules, and (c) Phi29 gp16 terminase with extensive DNA and RNA contamination.[13–16] In the first two cases the ratio protein/nucleic acid was close to 1:1 by weight.

When the Phi29 portal protein or the Phi29 prohead under quenching conditions in the absence of nucleic acids was injected onto the C4 reversed-phase microtrap and eluted into the mass spectrometer a good signal was obtained, suggesting that the presence of nucleic acids is the reason for the lack of signals in protein/nucleic acid mixtures. To test this hypothesis bovine serum albumin (BSA) was mixed with Phi29 DNA at an approximately 1:1mass ratio and treated with quenching solution. A sample of BSA without DNA was prepared in parallel. No LC/MS signal was obtained for the DNA-containing sample although there was a good BSA signal when the DNA was absent.

We hypothesized that the reason for the lack of the signal is formation of protein/nucleic acid precipitates. In order to test that hypothesis we recorded the UV/Vis spectrum of the mixture of Phi29 proheads and DNA under quenching and non-quenching conditions. As is evident in Fig. 1(A) under quenching conditions neither the DNA alone nor the procapsids alone displayed turbidity, as evidenced by the lack of absorbance at 340 nm. In contrast, the mixture displayed significant turbidity. The turbidity could be cleared by centrifugation at 10 000 *g* for 1 min. However, this occurred at the expense of the protein and DNA as witnessed by the decreased absorbance between 260–280 nm. In contrast, there was no detectable turbidity for either of the components at pH 7.4 (Fig. 1(B)). These results demonstrate that there are no pre-existing aggregates which, in turn, suggest that aggregation only occurs at low pH.

Including 8 M urea in the quenching buffer, which should destroy any residual protein structure, did not prevent precipitation suggesting the interaction was not structure dependent (Fig. 1(C)). In contrast, inclusion of 6 M guanidinium chloride prevented precipitation (Fig. 1(D)). This strongly suggested that the nature of the protein/DNA precipitation is electrostatic, since guanidinium chloride, unlike urea, has high ionic strength. Supporting this hypothesis is the observation that NaCl prevented precipitation in a concentration-dependent manner, with some precipitation observed at 0.5 M NaCl and no precipitation at 1 M (Figs. 2(A) and 2(B)).

We assessed the possibility of using either 6 M GuCl or 1 M NaCl in the quenching buffer. GuCl was unsuitable as it produced a very strong signal by itself (not shown). On the other hand we were able to obtain a signal from samples quenched in the presence of 1 M NaCl but doing so required extensively washing the C4 microtrap (up to 30 min). This washing step would be unsuitable for H/D exchange experiments where the time between loading the sample on the column and its elution must be kept to a minimum to reduce back-exchange.

Based on the hypothesis that aggregation was due to non-specific electrostatic interactions we decided to assess the possibility of using protamine sulfate, a mixture of highly basic polypeptides with masses between 4 and 4.5 kDa, to

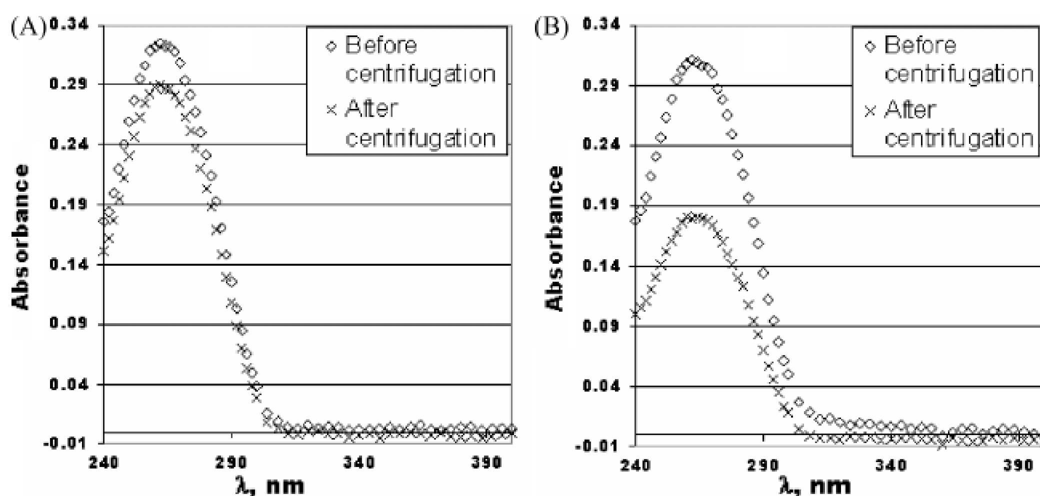

Figure 2. Precipitation of proteins and nucleic acid at low pH and high ionic strength. (A) UV/Vis spectra of Phi29 proheads/Phi29 DNA mixture before and after centrifugation at pH 2.8 in 0.5 M NaCl. (B) UV/Vis spectra of Phi29 proheads/Phi29 DNA mixture before and after centrifugation at pH 2.8 in 1 M NaCl.

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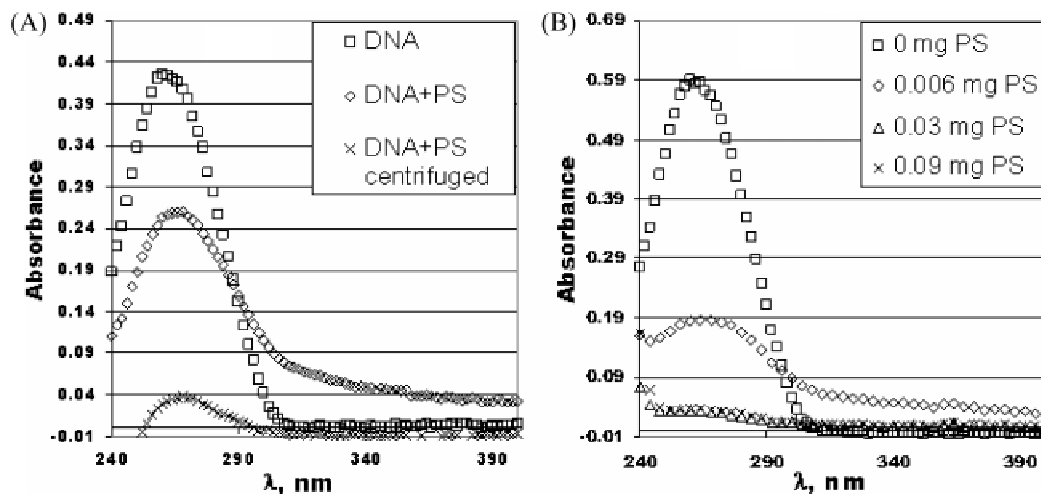

Figure 3. Precipitation of DNA by protamine sulfate. (A) UV/Vis spectra of Phi29 DNA without protamine sulfate and with it before and after centrifugation. (B) Dependence of the precipitation reaction on protamine sulfate concentration.

compete with the protein for the nucleic acid. Protamine sulfate was fully soluble in 0.1% formic acid at concentrations as high as 10 mg/mL. The ability of protamine sulfate to precipitate DNA under quenching conditions was determined by titrating increasing amounts of protamine sulfate in solutions of DNA, centrifuging, and recording the absorption spectra. The results indicate that protamine sulfate is capable of capturing and precipitating nucleic acids under quenching conditions in a concentration-dependent manner (Fig. 3(B)), and that complete precipitation of the DNA occurred at 30:1 weight ratio of protamine sulfate/DNA.

To determine if protamine sulfate could outcompete the proteins for DNA binding the same experiment was performed with Phi29 DNA and Phi29 proheads at a final protamine/DNA weight ratio of 60:1. The precipitate which formed was cleared by centrifugation and the supernatant analyzed by MS. Using these conditions we were able to observe multiple protein peaks eluting from the LC column (Figs. 4(A)–4(D)) which demonstrates that protamine sulfate efficiently and selectively removes DNA from the DNA/protein mixture under quenching conditions, and does not interfere with LC. All the major proteins constituting the Phi29 prohead were observed: scaffolding protein (Fig. 4(A), theoretical mass 11135.3 Da), portal protein (Fig. 4(B), theoretical mass 35747.2 Da), and the major head protein (Fig. 4(C), theoretical mass with first Met retained 49844.1 Da). To determine the general applicability of this approach we performed the same procedure with DNA-contaminated Phi29 gp16 protein (Fig. 4(E), theoretical mass with first Met retained 38964.5 Da) and Phi29 portal protein with the bound pRNA (not shown but spectra similar to that shown in Fig. 4(C)). In all cases we were able to obtain protein signals and measure the masses for the first time under H/D exchange quenching conditions. Protamine sulfate itself did not interfere with MS since we found that it binds to C4 trap loosely and elutes at very low acetonitrile concentrations.

We further examined the effect of protamine sulfate on the rate of back-exchange. We have initiated H/D exchange on Phi29 proheads (without DNA) by diluting them ten-fold into D_2O buffer. After 10 min the reaction was split, quenched with solution with and without a final concentration of 0.75 mg/mL protamine sulfate, and immediately frozen in liquid nitrogen. The masses of the proteins were subsequently determined (Table 1). Depending on the protein the extent of exchange ranged from ~30 to 70% (63 to 124 deuterons incorporated and the difference between samples with and without protamine sulfate was within experimental error (less than 2 Da)). This clearly showed that protamine sulfate does not influence the rate of the H/D exchange reaction.

DISCUSSION

We have shown that at low pH (quenching conditions for amide proton exchange reaction) proteins and nucleic acids form electrostatic precipitates, which preclude global H/D exchange measurements. The main indication that the precipitation is electrostatic is that it is prevented by high ionic strength (guanidinium chloride and NaCl) but not by urea. At pH 2.8 the only charge that is left on any protein should be positive as all carboxylic groups ($pK_a \approx 4.6$) will be effectively protonated, leaving only basic amino acids being charged. DNA and RNA, on the other hand, will still have a substantially negative net charge due to the lower pK_a of phosphoric acid (=2.12), making protein/DNA (or RNA) interaction under these conditions similar to cation exchange. While the interaction of the individual charge pairs may be transient and weak, the large number of them distributed along the polymers will ensure tight interaction. Protamine sulfate is a mixture of several highly basic polypeptides, consisting of 65% arginine. It is naturally found in sperm of fish where it acts in a histone-like manner and is essential for sperm head condensation, i.e. tight packing of the DNA.[17] It has been used for precipitating and removing nucleic acids and heparin from solutions. The useful properties of protamine sulfate that make it very

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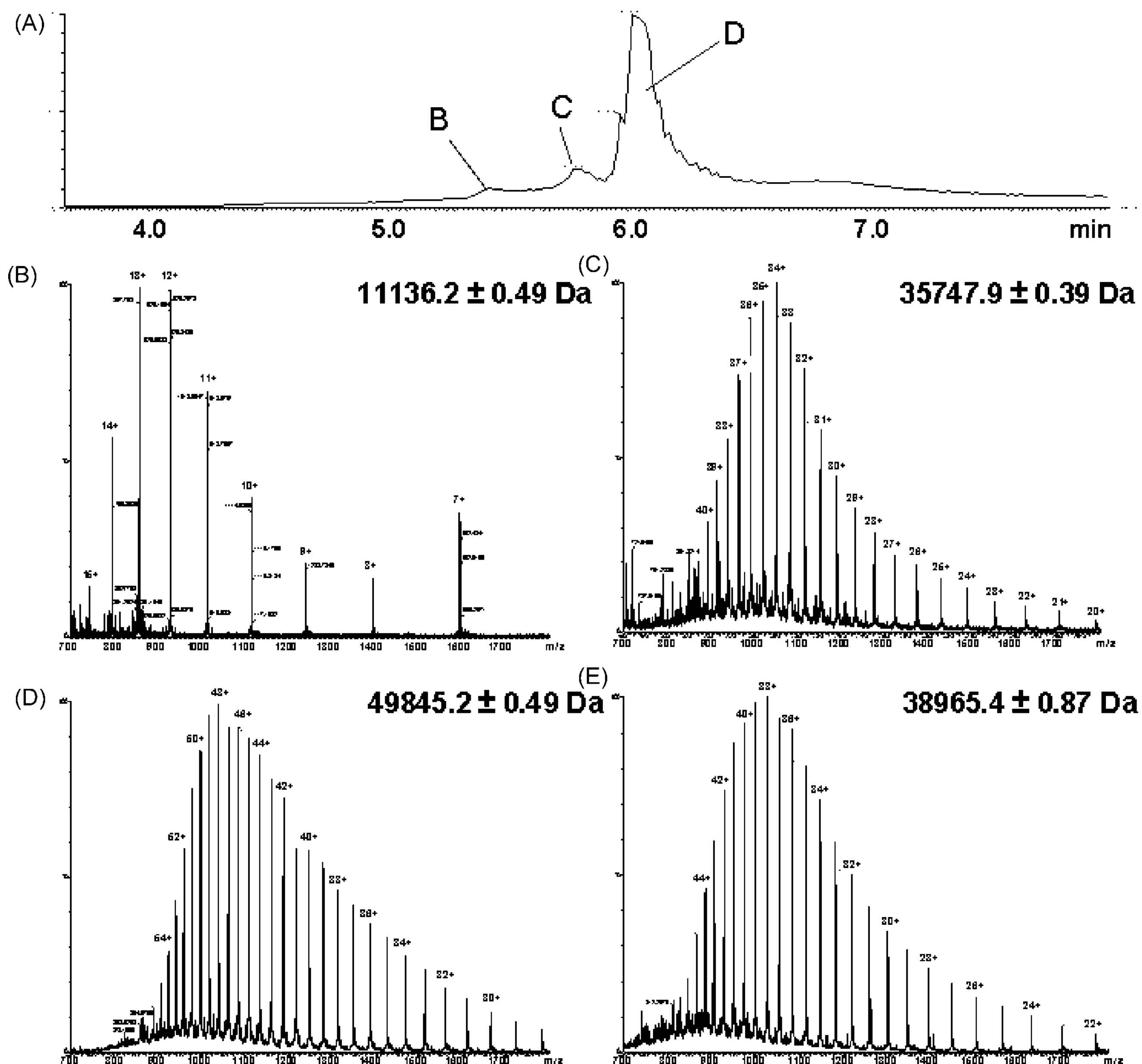

Figure 4. Determination of masses of proteins in mixture with nucleic acids using quenching buffer with protamine sulfate. (A) LC/MS of Phi29 proheads monitored by total ion current. Letters correspond to the mass spectra in (B)–(D). (B) Scaffolding protein; (C) portal protein; D) major head protein; and (E) mass spectrum of Phi29 gp16 protein, the chromatogram is not shown.

suitable for DNA/RNA immobilization under H/D exchange quenching conditions are its high solubility and hydrophilicity: it elutes from a C4 reversed-phase column at low acetonitrile concentration and therefore does not interfere with the proteins signals. The amount of protamine sulfate in the quenching buffer can be adjusted for the amount of nucleic acids present in the studied solution. When a very large amount of nucleic acids is present a brief centrifugation of the sample is advisable since large precipitates may clog the column.

Table 1. Effect of protamine sulfate on H/D exchange

	Phi29 scaffolding protein, Da	Phi29 portal protein, Da	Phi29 capsid protein mass, Da
–	11198.66	35859.14	49968.16
	11197.95	35859.75	49968.94
+	11199.08	35860.54	49970.46
	11198.29	35861.33	49969.97

CONCLUSIONS

Quenching of an H/D exchange reaction of a mixture of protein and nucleic acid results in their electrostatic precipitation, which precludes measurement of the mass of the protein. Protamine sulfate in the quenching solution selectively sequesters nucleic acids, which allows the study of H/D exchange in protein/nucleic acid mixtures where nucleic acid is either an unavoidable contaminant or a ligand of interest like whole viruses, nucleosomes and DNA or RNA polymerases in complex with their substrates.

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Note added in press

While this manuscript was in press Sperry *et al*. described an alternative method for removing nucleic acid from H/D exchange samples using in-line ion-exchange chromatography (Sperry JB, Wilcox JM, Gross ML. *J. Am. Soc. Mass Spectrom*. 2008; **19**: 887).

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