# Protein Behavior at the Water/Methylene Chloride Interface

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**Abstract** □ The objective of this study was to investigate the behaviors of proteins at the water/methylene chloride interface to better understand denaturing effects of emulsification upon proteins. Ribonuclease A (RNase) and human serum albumin (HSA) were used as model proteins throughout this study. Their behaviors at the interface were studied in terms of protein recovery after emulsification, interfacial protein aggregation, and dynamic interfacial tension. This study demonstrated that protein instability during emulsification was traced to consequences of the adsorption and conformational rearrangements of proteins at the water/methylene chloride interface. Compared to HSA, RNase was much more vulnerable to the interface-induced aggregation reactions that led to formation of water-insoluble aggregates upon emulsification. Even though HSA was almost completely recovered from the emulsified aqueous phase, the protein underwent dimerization and oligomerization reactions to some extent. The results also demonstrated that the extent of interfacial RNase aggregation was affected by its aqueous concentration and the presence of HSA. Interestingly, RNase stability during emulsification was almost achieved by dissolving an adequate quantity of HSA in the RNase solution. HSA seemed to compete for the interface site and to effectively keep RNase out the interface, minimizing the likelihood of the interface-induced RNase aggregation. These results indicated that competitive adsorption modes of proteins could be used to stabilize a protein of interest against the denaturing effects of emulsification.

## Introduction

The field of protein and vaccine delivery systems based on emulsions and biodegradable poly-*d*,*l*-lactide-*co*-glycolide (PLGA) microspheres has grown exponentially. However, increasing concerns about the issues of protein instability during manufacturing processes exist. Maintaining protein stability is of paramount importance, because destabilized proteins lose biological efficacy and aggregate to increase the possibility of an unwanted immune response. Among a variety of manufacturing processes, emulsification of an aqueous protein solution in an organic solvent is considered a major risk factor that presents an obstacle to the successful development of emulsion- and microsphere-based protein delivery systems.[1−4] It is generally agreed that the observed instability stems from exposure of proteins to cavitation, heat, or shear produced during emulsification.[5,6] Protein inactivation is also speculated to arise from protein denaturation at the aqueous solution−organic solvent interface and protein interaction with polymer residuals dissolved in the organic solvent.[7−9] Another possible mechanism suggested elsewhere is that an organic solvent diffuses to an aqueous protein solution and interacts with hydrophobic domains of the protein, eventually disrupting its structural integrity.[10−12]

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The current study suggests that protein instability during emulsification is traced to consequences of protein contact with the water/organic solvent interface around emulsion droplets. Because of amphipathicity and flexible conformation, proteins are surface active so that they tend to adsorb at water/organic solvent interfaces.[13] This event seems not only to disturb the delicate conformation of proteins but also to trigger various physicochemical transformations, leading to protein destabilization reactions. Currently, however, many questions remain unanswered with regard to the origins of protein instability against the interfaces, mechanisms that govern protein destabilization at water/organic solvent interfaces, and competitive protein behavior at the interfaces. As a starting point to tackle these important issues, this study sought to investigate the behavior of model proteins ribonuclease A (RNase) and human serum albumin (HSA) at the water/methylene chloride (W/MC) interface.

## Materials and Methods

**Materials**—Ribonuclease A (RNase; type III-A from bovine pancreas; R5125) and human serum albumin (HSA; A3782) were obtained from Sigma Chemical Company (St. Louis, MO). Major physicochemical properties of the two proteins are compared in Table 1. A precast, 1.5-mm thick Tris-glycine gel (8−16%) was purchased from Novex (San Diego, CA). Methylene chloride of HPLC grade was used throughout this study.

**Emulsification to Generate the Water/Methylene Chloride (W/MC) Interface**—A protein sample was dissolved in distilled water to make 0.2−1.5 mg/mL concentrations. Aliquots (3 mL) of the solution were placed inside a 20-mL vial containing methylene chloride (12 mL). The mixture was emulsified for 1 min at room temperature by a rotor/stator-type VirtiShear Tempest IQ2 homogenizer (The VirTis Co., Gardiner, NY) equipped with a 10-mm shaft. During emulsification, the rotational speed of the homogenizer rotor was set at 16 000 rpm.

**Quantitation of Protein Distribution between Water and the W/MC Interface**—Emulsification led to the formation of water-insoluble RNase aggregates that resided in the interface. To determine the aqueous protein concentration and the amount of the aggregates, the aqueous phase was separated from the methylene chloride phase by centrifugation at 3500 rpm for 10 min (IEC Centrifuge/International Centrifuge Company, Needham Heights, MA). A protein sample collected from the aqueous phase was subjected to a native size exclusion chromatography (SEC)-HPLC experiment which was described later. Equation 1 was used to determine the percentage of a protein recovered in the aqueous phase after emulsification. The amount of interfacially aggregated protein was equal to that of protein disappearing from the aqueous phase after emulsification.

$$\text{protein recovered (\%)} = \frac{\text{measured aqueous protein concn after emulsification}}{\text{known aqueous protein concn before emulsification}} \times 100 \quad (1)$$

**Assay for Water-Insoluble RNase Aggregates Obtained from the W/MC Interface**—Ultraviolet spectral and dynamic light scattering experiments were performed to assess whether


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**Table 1—Major Properties of Ribonuclease A (RNase) and Human Serum Albumin (HSA)**

| protein | RNase[a] | HSA[b] |
|---|---|---|
| amino acids | 124 | 585 |
| mass (D) | 13 680 | 69 000 |
| dimensions ($nm^3$) | $3.8 \times 2.8 \times 2.2$ | $12 \times 2.7 \times 2.7$ |
| diffusion coefficient ($m^2\ s^{-1}$) | $1.26 \times 10^{-10}$ | $0.70 \times 10^{-10}$ |
| number of disulfide bonds | 4 | 17 |
| number of free thiol group | 0 | 1 |
| isoelectric point | 9.4–9.6 | 4.6–4.9 |

[a] Data from Scheraga and Rupley[14] and Haynes et al.[15] [b] Data from Haynes et al.[15] and Peters, Jr.[16]

the aggregates differed from native RNase. Before the experiments, the water-insoluble aggregates were dissolved in a 0.5% SDS aqueous solution or 6 M urea. For the UV spectral experiment, a HP 8453 UV−visible spectrophotometer (Hewlett-Packard Company, Palo Alto, CA) was used to monitor the spectra of native and aggregated RNase molecules over the wavelength of 200 to 320 nm. In addition, their first derivative spectra were also obtained using a Savitsky−Golay algorithm offered by the spectrophotometer software.

For the dynamic light-scattering experiment, RNase sample solutions in 0.5% SDS or 6 M urea were filtered through a Whatman Anotop Plus filter with 0.1 $\mu$m porosity. The sample solutions were placed inside the microsample chamber of a DynaPro-801TC (Protein Solutions, Inc., Charlottesville, VA) and were illuminated by the laser. The instrument determined the hydrodynamic radius by analyzing the scattered light (the angle of detection was 90°).

**Homogenization Effects on Protein Recovery**—A 0.5 mg/mL RNase or HSA solution (15 mL) was put into a 20-mL size vial and was homogenized in the absence of methylene chloride under the same experimental conditions described earlier. Later, protein concentration was determined by SEC−HPLC analysis. The protein sample was also subjected to SDS−PAGE experiments.

**Dissolved Methylene Chloride Effect on Protein Recovery**—To prepare an aqueous solution saturated with methylene chloride, 4 mL of methylene chloride was added to 16 mL of water. The mixture was then vigorously shaken for 1 h at room temperature with an Eberbach shaker (Eberbach Co., Ann Arbor, MI) and was centrifuged to obtain the methylene chloride−saturated aqueous phase. RNase was then dissolved in the solution phase at a 0.5 mg/mL concentration. After the methylene chloride−saturated aqueous RNase solution (15 mL) was homogenized as described earlier, its concentration was determined by SEC−HPLC analysis and was compared with that of unhomogenized RNase solution.

**SEC−HPLC**—This experiment was performed to measure the total protein content and to assess the composition of water-soluble protein species before and after emulsification. When water-insoluble protein aggregates were analyzed, they were dissolved in a 0.5% SDS aqueous solution before the SEC−HPLC experiment. Protein samples were eluted from a TSK Gel G3000 SW analytical column (7.5-mm inner diameter × 60-cm length) by a mobile phase consisting of 50 mM phosphate buffer solution containing 0.1 M NaCl (pH 7.0). The flow rate was maintained at 0.8 mL/min, and the elution of protein samples was monitored by a UV detector set at 280 nm. The concentration of each protein species was determined by a calibration curve constructed by peak area integration of protein standards with known concentrations. Throughout this study, the average of at least three experimental determinations was reported with a standard deviation.

**SDS−PAGE**—A gradient 8−16% Tris-Glycine gel was placed inside Novex Xcell II Mini-Cell. RNase samples were mixed well with a Laemmli Tris-Glycine loading buffer and were housed inside the gel wells. The running buffer consisted of 25 mM Tris, 192 mM glycine, and 0.1% SDS at pH 8.3. After being run at a 125 V constant voltage (Power PAC300/Bio-Rad Laboratories, Hercules, CA) for 125 min, the gel was stained for 30 min with a 0.1% Coomassie Blue solution containing 10% glacial acetic acid and 50% methanol. Protein bands were visualized after the gel was destained overnight with an aqueous solution containing 20% methanol and 10% glacial acetic acid.

**Inhibition of RNase Interfacial Aggregation**—To investigate the effect of HSA upon the degree of RNase recovery, 0.2−5.0 mg/mL HSA was dissolved in a 0.35 mg/mL RNase solution before emulsification. After the aqueous protein mixture solution was emulsified in methylene chloride, the degree of RNase recovery was determined following the SEC−HPLC analysis mentioned earlier.

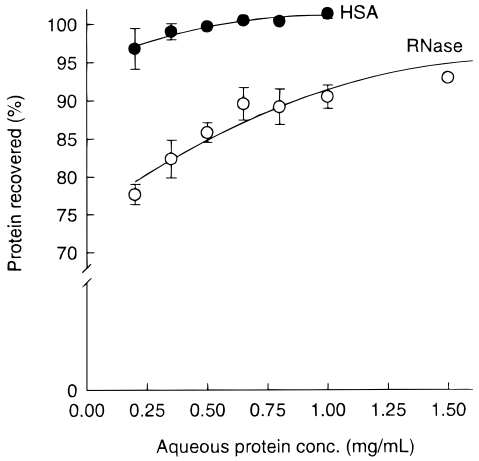


**Figure 1**—The percentage of protein recovery after emulsification. After an aqueous protein solution was emulsified in methylene chloride, the degree of protein recovery was determined by eq 1.

**Measurement of Dynamic Interfacial Tension**—Protein adsorption at the W/MC interfaces was investigated by a drop volume tensiometer (Model DVT-10/Krüss USA, Charlotte, NC). The tensiometer was considered suitable for studying the adsorption of surface active materials at interfaces, when they slowly attained equilibrium interfacial tension.[17,18] In this experiment, an aqueous 0.5 mg/mL RNase or HSA solution was placed in a 100-$\mu$L size gastight Hamilton syringe. A pump (Model 44/Harvard Apparatus, South Natick, MA) was used to deliver the protein solution at a constant rate to methylene chloride phase via a bore capillary (0.254-mm inner diameter) with a specially tapered tip. The flow rate ranged from 15 to 0.5 mL/h. As the protein solution was pumped into the methylene chloride phase, a drop grew and detached off the tip of the bore capillary. The time required for detachment of each droplet was defined as interface development time. Combined knowledge on the interface development time and the flow rate allowed us to calculate the volume of each droplet. Interfacial tension was then determined by eq 2.[19]

$$\sigma = \frac{V_{\text{drop}}\,(\rho_{\text{m}} - \rho_{\text{w}})g}{\pi d} \tag{2}$$

where $\sigma$ was interfacial tension; $V_{\text{drop}}$, the volume of an aqueous droplet; $g$, the acceleration due to gravity; $d$, diameter of the bore capillary; $\rho_{\text{m}}$, methylene chloride density; and $\rho_{\text{w}}$, the density of water phase. The interfacial tension measurement was repeated five times, and their mean ± standard deviation was reported.

## Results

Under our SEC−HPLC experimental conditions, only one peak representing monomeric RNase species was observed when the protein solution was not subject to emulsification. The same monomeric peak was detected with RNase solutions emulsified in methylene chloride, but there was a substantial reduction in the content of RNase monomer. A loss in its aqueous content was found to be caused by the formation of water-insoluble aggregates residing in the W/MC interface. Besides, the extent of RNase recovery was affected by changes in its aqueous concentration prior to emulsification (Figure 1). For instance, 77.7 ± 1.4% (mean ± standard deviation) of RNase was recovered after a 0.2 mg/mL RNase solution was emulsified in methylene chloride. An increase in its concentration to 1.5 mg/mL before emulsification enhanced its

**Table 2—Changes in the Composition of Water-Soluble HSA Species Triggered by Emulsification. After Emulsifying 0.2–1.0 mg/mL HSA Solutions in Methylene Chloride, the Compositions of HSA in the Aqueous Phase Were Determined by HPLC Analysis[a]**

| protein concn (mg/mL) | monomer % | dimer % | oligomer % (total $\mu$g)[b] |
|---|---|---|---|
| 0.20 | 71.2 ± 0.3 | 24.3 ± 0.2 | 4.6 ± 0.1 (27.3 ± 0.4) |
| 0.35 | 75.8 ± 0.2 | 21.2 ± 0.1 | 3.1 ± 0.2 (32.0 ± 2.2) |
| 0.50 | 78.5 ± 0.4 | 19.2 ± 0.4 | 2.4 ± 0.1 (36.0 ± 0.1) |
| 0.65 | 79.8 ± 0.2 | 18.1 ± 0.1 | 2.2 ± 0.1 (42.0 ± 1.4) |
| 0.80 | 80.8 ± 0.2 | 17.3 ± 0.3 | 1.9 ± 0.1 (45.6 ± 0.1) |
| 1.00 | 80.8 ± 0.2 | 17.2 ± 0.4 | 2.1 ± 0.1 (63.0 ± 4.2) |

[a] All determinations are performed at least three times, and mean ± standard deviation is reported. Before emulsification, HSA consisted of 86.7 ± 0.5% monomer and 13.3 ± 0.5% dimer. [b] The actual amount of a new water-soluble HSA oligomer is in parentheses.

degree of recovery up to 92.9 ± 0.2%. The data in Figure 1 were likely to give a false impression that fewer RNase molecules became aggregated at high concentrations. The amount of RNase aggregates in fact increased at high concentrations: emulsifying 0.2 and 1.5 mg/mL RNase solutions resulted in the formation of 133.9 ± 8.2 and 317.4 ± 10.8 $\mu$g of water-insoluble RNase aggregates, respectively.

Similar experiments were repeated to investigate the behavior of HSA at the W/MC interface developed during emulsification. From the viewpoint of percentage of protein recovery, HSA exhibited stronger resistance toward emulsification-induced aggregation reactions than RNase did (Figure 1). HSA was almost completely recovered in the aqueous phase. For instance, when a 0.2 mg/mL HSA solution was emulsified, protein recovery was 96.8 ± 2.7%. Moreover, at ≥0.35 mg/mL concentrations, at least 99.1% of HSA was recovered from the aqueous phase emulsified. However, it was found that some proportion of HSA molecules underwent dimerization and oligomerization reactions on emulsification (Table 2). Unemulsified HSA contained 86.7 ± 0.5% monomer and 13.3 ± 0.5% dimer, and homogenization of an aqueous HSA solution alone did not cause any change in the protein composition. On the contrary, after a 0.2 mg/mL HSA solution was emulsified in methylene chloride, the protein consisted of 71.2 ± 0.3% monomer, 24.3 ± 0.2% dimer, and 4.6 ± 0.1% oligomer. It should also be mentioned that the extent of HSA oligomerization also was concentration-dependent, such that the actual amount of the new water-soluble HSA oligomer species increased at high concentrations (Table 2).

Before carrying out the SEC−HPLC experiment, water-insoluble RNase aggregates obtained from the W/MC interface were dissolved using SDS which is known to disrupt the quaternary structures of most proteins and dissociate them into monomeric species. Figure 2 illustrates the SEC−HPLC chromatograms. The appearance of new RNase aggregates, despite the SDS treatment, indicated that the emulsification-associated RNase aggregation was an irreversible process, and the aggregates were not easily dissociable. To further back up this supposition, the hydrodynamic radius of monomeric RNase was compared with that of the water-insoluble RNase aggregates (Table 3). RNase monomer in 0.5% SDS solution was estimated to have the hydrodynamic radius of 2.0 nm. In contrast, the mean hydrodynamic radius of the water-insoluble RNase aggregates was determined to be 12.7 nm. In addition, an increase in the polydispersity observed with water-insoluble RNase aggregates represented that the aggregates were polydisperse in size and consisted of species with different hydrodynamic radii (the polydispersity in Table 3 indicates the standard deviation of the spread of sample sizes about the reported mean hydrodynamic radius). In particular, the DynaPro-801TC seemed to overestimate the hydrodynamic radius of RNase samples in 6 M urea. This result may have arisen from the fact that RNase species in 6 M urea exist in unfolded states.

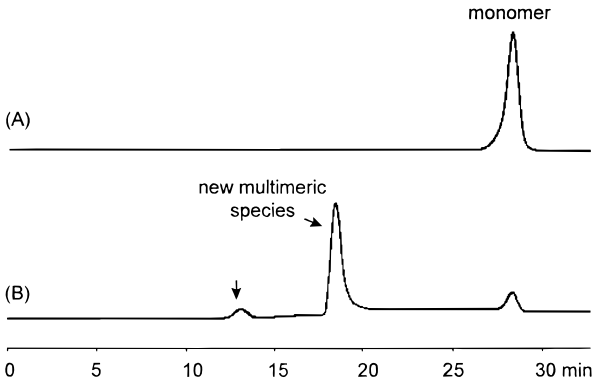


**Figure 2**—SEC–HPLC chromatograms of (A) standard RNase before emulsification and (B) water-insoluble RNase aggregates collected from the W/MC interface after emulsification. RNase aggregates were dissolved in a 0.5% SDS aqueous solution before injection to the SEC–HPLC.

Figure 3 illustrates the UV spectra of monomeric RNase and water-insoluble RNase aggregates. The spectrum and its first derivative for RNase aggregates were different from those of monomeric RNase (these differences were confirmed by a linear regression-based spectral match function provided by the HP 8453 UV−Visible spectrophotometer). Especially, the broadening of bands and shifts in wavelength were observed with RNase aggregates in comparison to RNase monomer. It can be inferred from the result that RNase aggregation might have changed the local environment of the chromophores, e.g., peptide groups, aromatic amino acids, and disulfide bonds, in RNase molecules.

To test whether the observed RNase aggregation might be caused by mechanical shear stress and/or the continual creation of a new air/water interface, a 0.5 mg/mL RNase solution (15 mL) was homogenized in the absence of methylene chloride. Slight foaming was observed after the treatment, but the foam dissipated rapidly. No considerable change in the content of RNase monomer was observed after homogenization (the aqueous solution homogenized was found to contain 99.67 ± 0.72% of the initially used amount of RNase). In addition, the SDS−PAGE results shown in Figure 4 demonstrated that RNase samples before and after homogenization exhibited the same gel patterns. A similar trend was observed when homogenization was performed on the methylene chloride−saturated aqueous RNase solution. Foaming also occurred during homogenization, but the foam disappeared when the solution was kept still. In this case, 99.0 ± 0.1% of the initial RNase amount was found in the solution.

So far, it has been suggested in this study that exposure of proteins at the W/MC interface initiated protein destabilization reactions. To prove this supposition, protein adsorption and conformational changes at the interface were demonstrated by analyzing the W/MC interfacial tension. In our study, the Krüss DVT-10 tensiometer determined the interfacial tension to be 27.84 ± 0.38 mN/m when the aqueous phase did not contain any protein molecules. At the same time, the volume of a single water droplet was found to be 7.17 ± 0.10 $\mu$L. The presence of either RNase or HSA at a 0.5 mg/mL concentration in water led to a considerable reduction in the interfacial tension, as well as the volume of an aqueous droplet (Table 4). Besides, changes in the interfacial tension were affected by interface development time that was manipulated by the flow rate of pumping a protein solution into methylene chloride. A lower flow rate, which provided a longer

**Table 3—Comparison of the Properties of Monomeric RNase and Water-Insoluble RNase Aggregates. Prior to Analysis, the Water-Insoluble Aggregates Obtained from the W/MC Interface Were Dissolved in 0.5% SDS Aqueous Solution or 6 M Urea[a]**

| | RNase monomer dissolved in | | RNase aggregates dissolved in | |
|---|---|---|---|---|
| | 0.5% SDS solution | 6 M urea | 0.5% SDS solution | 6 M urea |
| diffusion coefficient ($10^{-13}$ $m^2$ $s^{-1}$) | 1229 ± 40 | 985 ± 44 | 197.4 ± 9.9 | 149.1 ± 4.7 |
| hydrodynamic radius (nm) | 2.0 ± 0.1 | 2.3 ± 0.2 | 12.7 ± 0.7 | 16.9 ± 0.6 |
| polydispersity (nm) | 0.9 ± 0.1 | 1.3 ± 0.1 | 7.1 ± 0.4 | 16.3 ± 0.6 |

[a] Data were reported as mean ± standard deviation ($n = 8$).

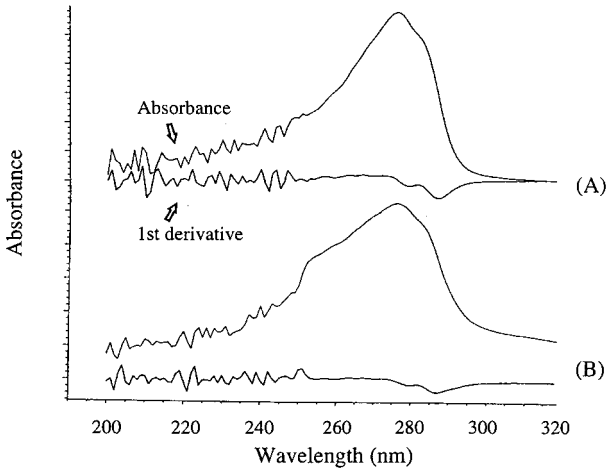


**Figure 3**—Absorption and first derivative spectra of (A) RNase monomer and (B) water-insoluble RNase aggregates. The protein samples were in 0.5% SDS aqueous solution.

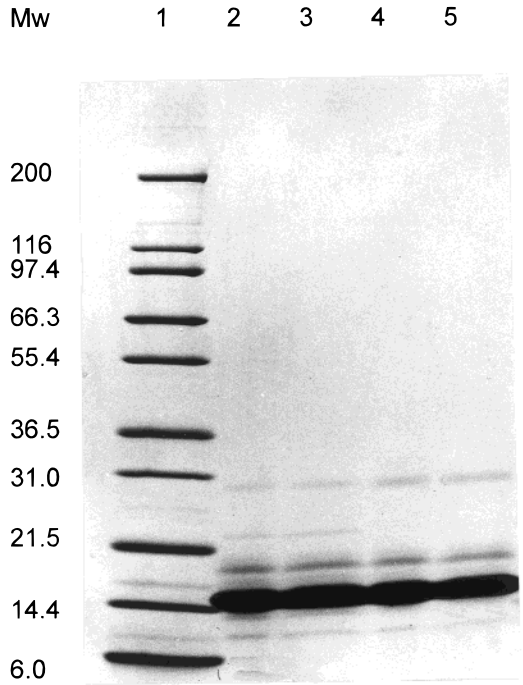


**Figure 4**—SDS–PAGE gel patterns of RNase samples before (lanes 2, 3) and after (lanes 4, 5) homogenization. Molecular markers (kDa) are shown in lane 1.

interface development time, led to a greater reduction in the aqueous droplet volume and the interfacial tension (a linear relationship between the volume of an aqueous droplet and the W/MC interfacial tension is well depicted by eq 2). A longer interface development time—more prolonged exposure of proteins to the interface—seemed to permit more protein molecules to adsorb and undergo conformational rearrangements at the interface. An additional interesting point observed in Table 4 was that, at relatively higher flow rates, the surface activity of RNase was greater than that of HSA. By contrast, the same conclusion did not remain valid when the flow rate was decreased below 1 mL/h. HSA was found to be more surface active when exposed to the interface for longer periods of time.

In terms of the degree of protein recovery, HSA exhibited superior stability over RNase against the W/MC interface-induced aggregation reactions (Figure 1). It was also speculated that owing to their surface activity, proteins would compete for adsorption sites at the W/MC interface. On the basis of these considerations, different levels of HSA (0.2 ~ 5.0 mg/mL) were codissolved in a 0.35 mg/mL RNase solution before emulsification to minimize RNase instability toward the W/MC interface. When RNase was the only protein species present in the aqueous phase, 82.4 ± 2.5% of RNase was recovered from the emulsified aqueous phase. When a sufficient quantity of HSA was added into the aqueous phase, the degree of RNase recovery was increased considerably (Figure 5). At ≥0.8 mg/mL HSA concentrations, more than 96.6 ± 1.2% of RNase was recovered from the emulsified aqueous phase. These data indicated that the use of HSA contributed to stabilizing RNase against the interface-triggered destabilizing reactions.

## Discussion

Water/oil interface is ascribed to be highly mobile in comparison to water/air and water/solid interfaces so that proteins are less likely to adsorb irreversibly and destabilize at the water/oil interface.[20] However, our experimental results demonstrate that the W/MC interface developed during emulsification is detrimental to the stability of RNase molecules. Our control experiments also demonstrated that homogenization-associated effects (e.g., cavitation, shear, and continual creation of a new air/water interface) and methylene chloride molecules dissolved in water are not the main causes of RNase instability observed with emulsification. Exposure of RNase molecules to the W/MC interface is held accountable for inducing considerable conformational changes leading to aggregation reactions and a subsequent reduction in its monomer content. In addition, a series of experiments provides evidence that the interface-triggered aggregation reactions are essentially irreversible (Figure 2 and Table 3). It remains to be investigated whether the formation of these RNase aggregates is induced by noncovalent, physical associations or by intermolecular covalent linkages.

It was previously reported that, although some proteins and vaccines were stable in organic solvents, they became inactivated either upon exposure to water—organic solvent mixture or during the emulsion-based microencapsulation process.[21,22] Most reports ascribed that organic solvent molecules dissolved in an aqueous phase could interact with hydrophobic domains of proteins, thereby triggering structural rearrangements detrimental to protein integrity. On the basis of this consideration, a rapid microsphere hardening process is recommended to maintain protein

**Table 4—Effects of the Flow Rate on Dynamic Changes in the W/MC Interfacial Tension (mN/m) and the Volume (μL) of an Aqueous Droplet. To Manipulate Interface Development Time (IDT in seconds), the Flow Rate of Pumping a 0.5 mg/mL Protein Solution into the Methylene Chloride Phase Was Changed from 15 mL/h to 0.5 mL/h**

| | when the aqueous phase contained RNase | | | when the aqueous phase contained HSA | | |
|---|---|---|---|---|---|---|
| flow rate (mg/mL) | IDT | droplet volume | interfacial tension | IDT | droplet volume | interfacial tension |
| 15 | 1.45 | 6.03 ± 0.08 | 23.42 ± 0.31 | 1.70 | 7.07 ± 0.16 | 27.43 ± 0.06 |
| 10 | 2.12 | 5.89 ± 0.09 | 22.87 ± 0.34 | 2.50 | 6.95 ± 0.11 | 26.95 ± 0.43 |
| 7.0 | 2.99 | 5.80 ± 0.09 | 22.52 ± 0.34 | 3.51 | 6.83 ± 0.08 | 26.49 ± 0.33 |
| 5.0 | 4.00 | 5.55 ± 0.06 | 21.54 ± 0.24 | 4.81 | 6.68 ± 0.13 | 25.92 ± 0.52 |
| 3.0 | 6.56 | 5.47 ± 0.08 | 21.23 ± 0.31 | 7.40 | 6.16 ± 0.11 | 23.92 ± 0.30 |
| 1.0 | 18.06 | 5.02 ± 0.05 | 19.46 ± 0.19 | 17.24 | 4.79 ± 0.07 | 18.59 ± 0.27 |
| 0.5 | 33.52 | 4.65 ± 0.01 | 18.07 ± 0.04 | 30.88 | 4.29 ± 0.01 | 16.64 ± 0.05 |

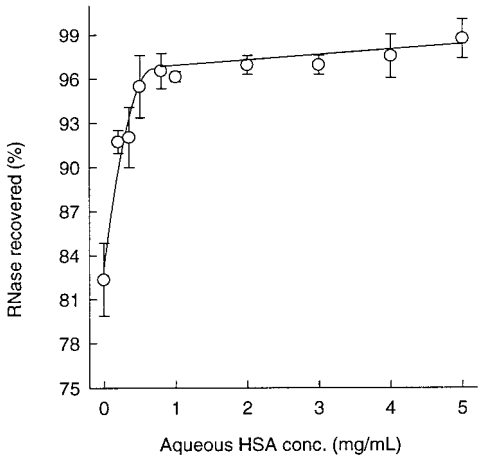


**Figure 5**—The effect of HSA on RNase recovery. Prior to emulsification, HSA (0.2 to 5.0 mg/mL) was codissolved in a 0.35 mg/mL RNase solution. The solution was emulsified in methylene chloride, and the percentage of RNase recovery was determined.

stability by minimizing exposure time of a protein to methylene chloride.[23] However, our results clearly substantiate that protein destabilization reactions occur primarily over a very short emulsification time scale that lasted for only 1 min in our experiment. This conclusion is in line with other reports suggesting that emulsification of preparing a primary w/o emulsion is the main risk factor responsible for protein inactivation when microspheres are prepared by the w/o/w microencapsulation process.[1–3,7]

In the current study, it has also been shown that the degree of the interfacial RNase aggregation is influenced by aqueous protein concentrations and the presence of another protein, HSA. With regard to the effect of protein concentration upon its stability, two hypotheses have been proposed to explain that a smaller fraction of proteins becomes inactivated at high concentrations. The first one takes into consideration that only a limited amount of a protein could interact at water/oil interfaces.[3] Under this condition, the protein is suggested to act as a self-protectant at high concentrations, thereby inhibiting its interaction with the interface. The other hypothesis elaborates on the role of energy barrier on protein adsorption.[24] At low protein concentrations, there is no barrier to adsorption and subsequent interfacial reactions. By contrast, at high protein concentrations, an energy barrier comes into play to prohibit further protein adsorption. At 0.2 to 1.5 mg/mL RNase concentrations used in our study, the protein aggregation increased with its aqueous concentration increasing. This result suggests that, in dilute concentrations, protein adsorption at the interface is enhanced by an increase in its aqueous concentration, because a great concentration gradient facilitates the mass transfer of RNase to the interface. As a consequence, collision frequencies and reactions among RNase molecules are increased. This contention agrees well with a widespread assertion that the extent of protein aggregation is kinetically controlled and the rate of aggregation is proportional to protein concentration.[25]

Serum albumins, particularly bovine serum albumin, have been popularly coencapsulated with therapeutic proteins into PLGA microspheres via the w/o/w emulsion and the spray-drying processes.[1,7,26–28] In these studies, it was reported that serum albumins improved the encapsulation efficiency of proteins and vaccines and contributed to preserving their activities. Currently, little information is available with regard to stabilization mechanisms of serum albumins for therapeutic proteins and their behaviors at the W/MC interface. From the data on the effect of HSA concentration upon the degree of RNase recovery (Figure 5), it can be inferred that the mass transfer phenomenon influences the kinetics of protein adsorption. According to this theory, a protein present in excess should occupy an interface first, because a steeper concentration gradient provides a greater diffusion rate to the protein. As a result of this, HSA at higher concentrations seems to effectively keep RNase molecules out of the W/MC interface, minimizing the likelihood of the interface-induced RNase aggregation. The other possible stabilizing function of HSA is that the protein molecules may adsorb preferentially at the interface and strip the adsorbed RNase molecules off the interface. In studies of protein behavior at solid/water interfaces, it has been demonstrated that HSA displaces IgG and fibrinogen adsorbed at solid surfaces.[29–31] The so-called "Vroman effect" describes this phenomenon that proteins can replace one another at biomaterial surfaces.[32,33]

Another important discussion should be made with regard to the quantity of serum albumins to be used for protecting therapeutic proteins. In most studies, a large quantity of serum albumins—their aqueous concentrations range from 50 to 400 mg/mL—has been used to enhance the stability of therapeutic proteins and vaccines during the emulsion-based microencapsulation procedure.[1,7,26–28] The data in Figure 5 indicate that the denaturing effects of the W/MC interface upon RNase can almost be avoided at HSA concentrations as low as 0.5 mg/mL. One should caution that the use of an excess amount of HSA leads to the generation of more HSA oligomer, as evidenced in Table 2. Therefore, a minimal quantity of HSA should be used to rule out the possibility of an untoward immune response toward the new HSA oligomer.

Finally, the tensiometer data in Table 4 provide direct evidence on the adsorption of proteins at the W/MC interface. Dynamic changes in the interfacial tension as a function of the interface development time also indicate that adsorbed protein molecules undergo conformational rearrangements in a way to minimize the free energy of the system. During the initial development of the W/MC interface, RNase seems to diffuse more rapidly to the

interface than HSA does, as indicated by their respective diffusion coefficients (Table 1). This is the reason that a lower W/MC interfacial tension is seen with an aqueous RNase solution over relatively short interface development times. Once adsorbed in the interface, HSA displays a much faster rate of the interfacial tension decrease. These results indicate that HSA possesses quite a flexible conformation and undergoes a fast conformational rearrangement at the interface, whereas rigid RNase undergoes a slow conformational rearrangement process. As a result, with the aging of the interface, an aqueous HSA solution provides a lower W/MC interfacial tension than an aqueous RNase solution does. This conclusion is consistent with a previous assertion that RNase has a relatively greater conformational stability than HSA.[34] On the basis of the results of the recovery of these two proteins after emulsification (Figure 1), however, it can be suggested that the conformational flexibility and the W/MC interface-induced aggregation are not closely related. Further investigations need to be performed to unravel their different behaviors at the interface.

In summary, protein adsorption at the W/MC interface and its effect upon protein integrity have been corroborated to explain protein instabilization observed with emulsification. Competitive protein adsorption modes have been used to stabilize a protein of interest—RNase in this study—against the denaturing effects of the W/MC interface. Information reported in this study might aid in the successful development of protein delivery systems such as emulsions and PLGA microspheres.

## References and Notes


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