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# Adverse Interfacial Effects upon Protein Stability: Implications in Developing Emulsion-Based Protein Delivery Systems

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## Abstract

The objective of this study was to investigate the behavior of ribonuclease A (RNase) at the water/ methylene chloride' interface. It was aimed at better understanding the denaturation of proteins upon emulsification. RNase was vulnerable to the interface-induced aggregation reactions that led to formation of water-insoluble aggregates upon emulsification. Biochemical analyses demonstrated that intermolecular covalent linkages might have been involved in the aggregation reactions. The protein instability observed with emulsification was traced to consequences of protein adsorption and confor-mational rearrangements at the interface. These results indicated that emulsifying aqueous protein solu-tions in organic solvents should be handled with care, since emulsification could bring denaturation and aggregation to proteins.

## 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, there are increasing concerns about the issues of protein instability during manufacturing processes, especially the step of emulsification (1-4). As a starting point to tackle this important issue, this study sought to investigate the behavior of a model protein RNase at the water/methylene chloride interface.

## Experimental methods

Three milliliters of aqueous RNase solutions at 0.2 to 1.5 mg/ml concentrations were emulsified in 12 ml of methylene chloride with the aid of a Virtishear Tempest IQ2 homogenizer. Emulsification was performed to generate the water/methylene chloride interface. After emulsification, the amount of protein present in the interface and the aqueous phase was determined with a native SEC-HPLC. The amount of interfacially aggregated RNase was equal to that of RNase disappearing from the aqueous phase after emulsification. The characteristics of water-insoluble RNase aggregates obtained from the interface were assessed by ultraviolet spectral (HP 8453 UV-Visible spectrophotometer), dynamic light scattering (DynaPro-801TC), and SDS-PAGE experiments. Protein adsorption at the W/MC interfaces was investigated by monitoring changes in the dynamic interfacial tension. A drop volume tensiometer (Model DVT-10/Krüss USA, Charlotte, NC) was used for this experiment.

## Results and Discussion

Emulsification led to the formation of water-insoluble aggregates residing in the W/MC interface. This caused a considerable loss in its aqueous content. The extent of RNase recovery was affected by

changes in its aqueous concentration prior to emulsification. 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. Fig. 1 illustrates the SEC-HPLC chromatograms of fresh RNase and the RNase aggregates dissolved in a 0.5% SDS. 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 1). 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. In particular, the hydrodynamic radius of RNase samples in 6 M urea was overestimated. The results may have arisen from the fact that RNase species in 6 M urea exist in unfolded states. The UV spectra of monomeric RNase were also different from those of water-insoluble RNase aggregates. It can be inferred from the result that RNase aggregation changed the local environment of the chromophores-e.g., peptide groups, aromatic amino acids, or disulfide bonds-in RNase molecules. Finally, 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. The presence of RNase in water led to a considerable reduction in the interfacial tension, and the degree of its reduction was proportional to the duration of RNase exposure to the interface.

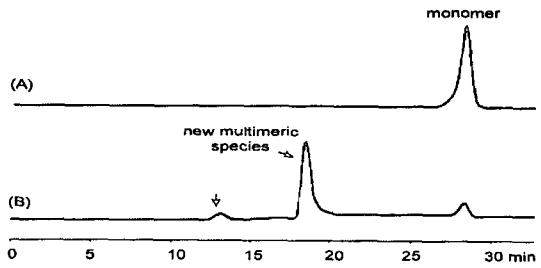


**Fig. 1.** SEC-HPLC chromatograms of (A) standard RNase before emulsification and (B) water-insoluble aggregates collected from the water/methylene chloride interface. The RNase aggregates were dissolved in a 0.5% SDS aqueous solution before injection to the SEC-HPLC.

**Table 1.** Comparisons of monomeric RNase and water-insoluble RNase aggregates. Prior to analysis, the water-insoluble ones obtained from the interface were dissolved in 0.5% SDS or 6 M urea.

| | RNase monomer dissolvedin | | RNase aggregates dissoloved in | |
|---|---|---|---|---|
| | 0.5% SDS | 6 M urea | 0.5% SDS | 6 M urea |
| 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 |

## Conclusion

Protein adsorption at the interface and its interfacial effect upon protein integrity have been corroborated to explain protein instabilization observed with emulsification. Exposure of RNase molecules to the interface resulted in inducing considerable conformational changes leading to

aggregation reactions and a subsequent reduction in its monomer content. The interface-triggered aggregation reactions were essentially irreversible. Information reported in this study should be considered in developing protein delivery systems such as emulsions and PLGA microspheres.

## References


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