THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

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Sonolysis of High Macroviscosity Systems: Hen Albumen Hydrogel

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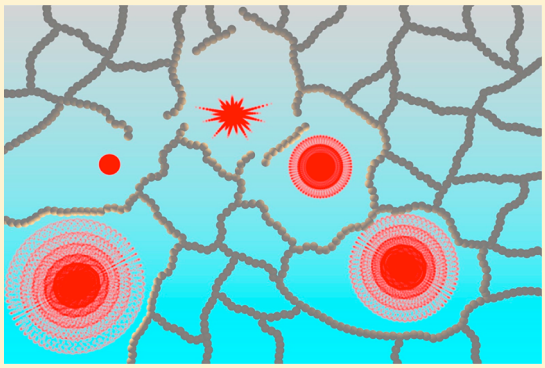
ABSTRACT: To understand molecular effects of ultrasound on protein gels (cross-linked, hydrated macromolecular systems of immeasurably high macroviscosity, but low microviscosity), the thick fraction of hen albumen was sonicated. The immeasurably high viscosity of the intact thick fraction decreased to 2.5−4.0 mPa·s (depending on the sample) after a 12 min sonication (0.14 mM of radicals were produced and 19 J g^{-1} of thermal energy absorbed) indicating that the 3D protein network was degraded. SDS-PAGE analysis indicated the breaking of intermolecular S−S bridges holding together the protein network rather than the primary structure of constituent proteins. Despite the relatively large concentration of OH radical produced in the sonication time range applied, no protein cross-linking was observed which can be attributed to the high degree of protein glycosylation and protein immobility. Differential scanning calorimetry (DSC) showed that both the amount of bound water and the enthalpy of denaturation of the constituent proteins are not affected by sonication, which is consistent with the SDS-PAGE results. A small increase in sample turbidity can be attributed to the small extent of thermal denaturation occurring in the vicinity of cavitation sites.



INTRODUCTION

Despite the extensive use of ultrasound in medicine, a comprehensive understanding of the effects of ultrasound on biological macromolecules in cells and tissues in treatment areas remains elusive. Thermal effects, hydrodynamic sheer stress, and free radical reactions are possible mechanisms by which sonication may alter inter- and intramolecular covalent and noncovalent interactions.[1−3] It is well-established that sonolysis of aqueous homogeneous solutions, including polymeric solutions, depend on their viscosities;[4] that is, the higher the viscosity, the less pronounced the effect of ultrasound.[5−7] If extrapolated to systems of infinitely high viscosities (like gels), then it could be inferred that no effects of sonication should be expected, which contradicts experimental observations. In our previous study of the radiation chemistry of hydrogels, we showed that the concept of microviscosity must be included to correctly interpret experimental data. Namely, we characterized the thick fraction of hen albumen and found that although it has an immeasurably high macroviscosity, its microviscosity is similar to that of bulk water,[8] which proved to have dramatic effects on the radiation chemistry.[9]

Sonochemistry results from ultrasonically induced acoustic cavitation, which is the formation, expansion, and implosive collapse of bubbles within a liquid containing dissolved gas.[10−13] For cavitation to occur, enough gas must be dissolved, and the liquid medium in which the sonication occurs must be sufficiently large. It was shown before that the solubility of air in the thick fraction of hen albumen is the same as in bulk water[8,14] and DSC measurements indicated that some of the water pools may be large enough to allow cavitation.[8] Therefore, despite an immeasurably high macroviscosity, there is a potential for some sonochemical effects to be observed upon exposure of the thick fraction to ultrasound.

In this work, we used the thick fraction of hen albumen to assess the molecular level effects of ultrasound on proteins and the mechanism(s) responsible for ultrasound-induced degradation of biomolecular gels and other organized macromolecular biological structures. In addition to the mechanistic component of this work, the data obtained should also provide insight on the effects of ultrasound on luminal mammalian mucous membrane, for which the thick fraction is an excellent model system.[8,15]

EXPERIMENTAL SECTION

Materials. Chemicals for SDS-PAGE were of electrophoresis grade purity and were used as received from Sigma-Aldrich Chemical Co. Prior to use, all chemicals for Fricke dosimetry passed spectrophotometric tests and therefore were used as received. Fresh hen eggs were obtained from a local farm. The yolk was mechanically separated from the albumen. The thin and the thick fraction of egg white were separated by filtration on a Büchner funnel. The thin fraction, being a true solution, flows through the pores, whereas the thick fraction remains in the funnel.[15]

Sonication. Samples to be sonicated were placed into a 25 × 150 mm Pyrex test tube that was centered in a Cole-Parmer ultrasonic cleaner (Model B3-R; frequency = 55 kHz; nominal

Received: December 28, 2012
Revised: May 26, 2013

   dx.doi.org/10.1021/jp312818f | J. Phys. Chem. B XXXX, XXX, XXX−XXX

power = 17 W). The bath intensity was selected such that the sonolysis effects become measurable on the time scale from 1 min to 1 h. The shorter lower limit would make the time/intensity measurement imprecise (0.5 s variations in 60 s sonication time results in ~1% error), whereas the longer upper limit might create different problems, such as sample overheating, a nonlinear temperature gradient within the sample, ultrasound intensity variations, and so forth. More specifically, at temperatures above 55 °C, thermal denaturation may occur either in the entire sample or in localized areas. In addition, with prolonged use, the sonication bath itself may overheat and result in a decrease in intensity which is difficult to account for.

Fricke Dosimetery. Ferrous-sulfate (Fricke) solution was prepared according to Law[16] and Scharf.[17] A 20 mL aliquot of Fricke solution was sonicated for a given amount of time and equilibrated to 20 °C before UV/vis measurement. Optical absorption spectra were obtained using a Cary 4000 spectrophotometer. The absorbance at 305 nm and the extinction coefficient (2090 $M^{-1}cm^{-1}$)[17] were used to determine the ferric ion concentration.

Viscosity Measurements. Viscosities were measured at 20 °C using several Ostwald viscometers (Cannon Instrument Co.) with various capillary sizes. Measurements were performed in a 17 L custom-made thermostat (the temperature was measured with 0.05 °C precision using a mercury thermometer).

Turbidity Measurements. Optical absorption spectra of intact and sonicated hen albumen samples (thin and thick fractions) were obtained using the aforementioned spectrophotometer.

SDS-PAGE. Analysis of unsonicated (intact) and sonicated thick and thin fractions of hen albumen was done using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli.[18] Samples were boiled for 2 min at 100 °C in sample buffer with or without 5% β-mercaptoethanol. SDS-PAGE was done using a Bio-Rad Mini-Protein Tetra Cell System (model 165−8001) and Bio-Rad Power-Pac Basic (Bio-Rad Laboratories). Proteins were visualized by Coomassie Blue staining, and Un-Scan It Gel (Silk Scientific, Inc.) was used for gel analysis.

Differential Scanning Calorimetry. A TA Instruments, Inc. differential scanning calorimeter (model DSC Q200) was used. Egg albumen samples were hermetically sealed in Tzero aluminum sample pans (Thermal Analysis Instrument, Inc.). To assess bound water, samples were cooled from room temperature to −50 °C and subsequently heated to +50 °C at a heating rate of 10 °C/min. To assess the extent of denaturation, samples were heated from room temperature to 105 °C at a heating rate of 10 °C/min.

RESULTS AND DISCUSSION

Characterization of the Ultrasonic Bath. Prior to investigating the effects of ultrasound on the thick fraction of hen albumen, the ultrasonic bath to be used to sonicate samples was characterized (1) to confirm that acoustic cavitation occurs in the bath and (2) to determine the amount of thermal energy absorbed by water in both the bath and sample.

Fricke Dosimetry. Fricke dosimetry was used to confirm that acoustic cavitation occurs in the ultrasonic bath and to quantify the concentration of radicals formed per unit time. The optical absorption in the wavelength range of 200 to 350 nm after the sonication of an aerated solution of 1 mM ammonium iron(II) sulfate hexahydrate in 0.8 N sulfuric acid was monitored (Figure 1 inset). The change in the optical

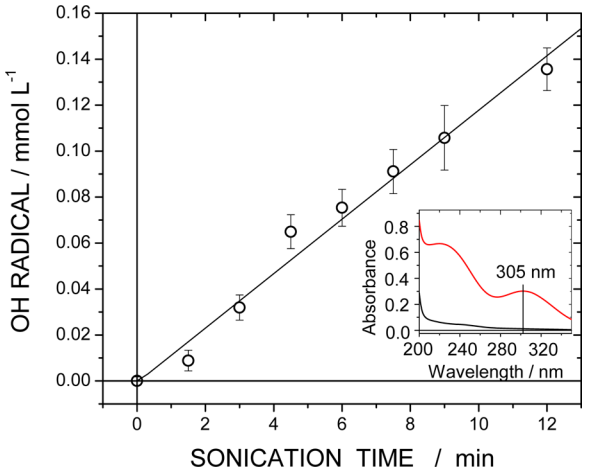


Figure 1. Concentration of hydroxide radicals as a function of sonication time. Inset shows the absorption spectra of Fricke solution prior to sonication (black) and after 12 min (red).

absorption of this solution after sonication for a given amount of time indicated that hydroxyl radicals (and hydrogen radicals) formed via the thermally induced homolysis of water caused by acoustic cavitation. The hydroxyl radicals generated oxidized ferrous ions to ferric ions which absorbs maximally at 305 nm. Using the Beer−Lambert law, the concentration of ferric ion at each sonication time was determined and, as shown in Figure 1, increased linearly as a function of sonication time. Approximately 10 μM of ferric ion were generated per minute of sonication, which corresponds to a concentration of 10 μM hydroxyl radicals (the hydrogen radicals are converted to hydroxyl radicals via reactions with water molecules). The microviscosity of the thick fraction is very similar to that of bulk water,[8] therefore, it is expected that the concentration of radicals generated by acoustic cavitation in water within the thick fraction (where cavitation can occur) at a given sonication time will be comparable to a corresponding amount of bulk water.

Calorimetry. The thermal energy absorbed per unit time by water in both the ultrasonic bath and the sample test tube was determined calorimetrically. The bath and sample test tube containing 300 and 20 mL of deionized water, respectively, were thermally insulated. Thermocouples placed in both the bath and test tube water measured the temperature prior to and immediately after a 3 min sonication. The total heat added to the water in both the bath and the test tube was determined to be 1400 ± 13 J, which means that the ultrasonic bath delivers 8.1 ± 0.7 J s^{-1} with an efficiency of 48%. From this, it was found that samples in the test tube absorb 19 J g^{-1} of thermal energy within a 12 min sonication. The corresponding temperature rise is insufficient to cause thermal denaturation due to heating of the bulk.

Rheological Properties of Sonicated Thick Fraction of Hen Albumen. To assess the effect of ultrasound on the protein hydrogel of the thick fraction, dynamic viscosities of sonicated samples were measured. After the initial sonication, a compact gel was broken; fragments of various sizes of thick fraction were dispersed in a rather fluid protein solution. The fragments were too large to be analyzed by SDS-PAGE, size-

dx.doi.org/10.1021/jp312818f | J. Phys. Chem. B XXXX, XXX, XXX−XXX

exclusion chromatography, or other methods for determining molar mass. In our previous research, we showed that there is a correlation between the molar mass of cross-linked protein and solution viscosity.[19] Therefore, viscometry seemed to be the analytical method of choice, as it allowed for the determination of the fragment size distribution beyond 10^6 kDa.[19] An Ostwald viscometer operative in the range 4000−20 000 mPa·s was initially used. However, flow time measurements for the same sonicated sample were highly variable, which indicated that the flow was non-Newtonian; that is, some of the fragments were too large. Since the direct measurement of the sonicated sample flow time failed, we opted to separate the largest fragments from the fluidized fraction by applying the sonicated sample to mesh. Various sizes of mesh were assessed and a mesh with a 0.035 in. diameter (a diameter slightly smaller than that of the capillary of the Ostwald viscometer used to measure viscosity) proved effective. Within a 12 min sonication, the majority (67%) of thick fraction samples were more than 50% fluidized.

Figure 2 shows the viscosity of the fluidized thick fraction as a function of percent protein content and compares these to

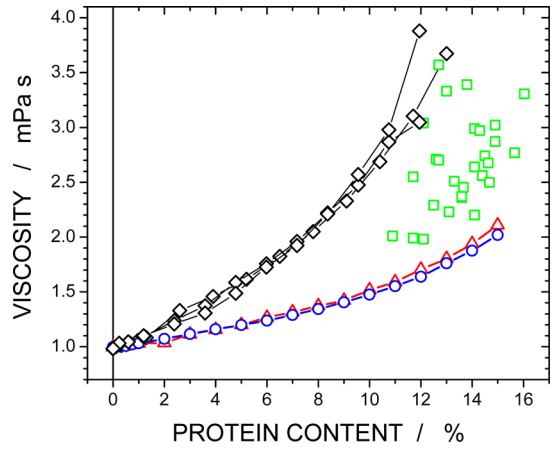


Figure 2. Viscosity versus protein concentration for fluidized thick fraction with a 12 min sonication (green, □) and dilutions of ovalbumin (blue, ○), bovine serum albumin (red, △), and intact thin fraction of hen albumen (black, ◊).

the viscosities of dilutions of ovalbumin (45 kDa), bovine serum albumin (66 kDa), and thin fraction of hen albumen; the thin fraction is a true protein solution consisting of protein dimers and trimers.[20] The viscosity of fluidized thick fraction was found to be ∼2.5 to 4 mPa·s when sonicated for 12 min. The notable change in the dynamic viscosity suggests that the three-dimensional protein network of the thick fraction held by intermolecular S−S bridges was degraded. The viscosities indicate that the fluidized fraction consists primarily of protein monomers and dimers comparable to the intact thin fraction.

When the unfluidized fractions were sonicated for an additional 12 min, a similar distribution was observed, namely two-thirds of samples were more than 50% fluidized. The presence of unfluidized thick fraction (even within a 24 min sonication) is likely a result of structural variability within regions of the biological sample; namely, due to the nonuniform water pool sizes, some portions of the thick fraction may contain water pools too small to allow gas bubbles to form and, therefore, prevent cavitation. However, it is reasonable to assume that the distribution of water pool sizes changes during the sonication-induced protein network degradation; the number of water pools large enough for cavitation increases, eventually leading to the complete degradation of the hydrogel. The aforementioned results suggest that the microviscosity of the water in the thick fraction, which is very similar to bulk water, governs how ultrasound impacts the protein hydrogel.

Hydrogel Structure Degradation Mechanism. The observed degradation of the hydrogel may be the result of free radical action (which can result in both scission and cross-linking[20]) and/or the hydrodynamic sheer that may break the primary structure and S−S bridges. To elucidate the mechanism, SDS-PAGE analyses were performed. Nonreducing SDS-PAGE (without β-mercaptoethanol treatment, which reduces disulfide bonds) and corresponding electrophoretograms for thin fraction of hen albumen and thick fraction sonicated for 12 min are nearly identical (Figure 3). Sonication

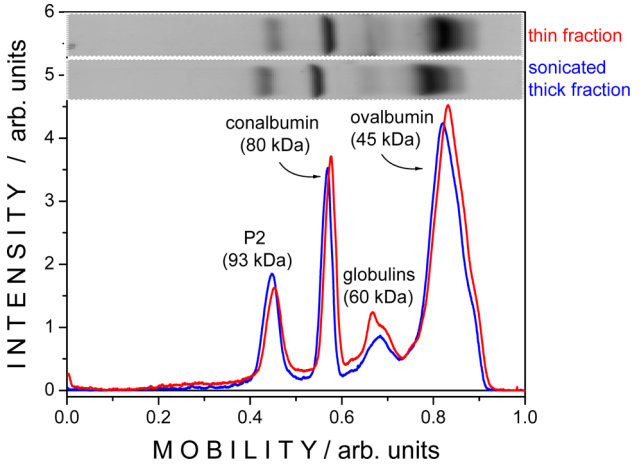


Figure 3. SDS-PAGE and corresponding electrophoretograms of unsonicated thin fraction (red) and sonicated thick fraction (blue) for 12 min (without β-mercaptoethanol).

did not result in a molecular mass change, even with extended sonication times (Figure 4A); that is, protein scission and/or

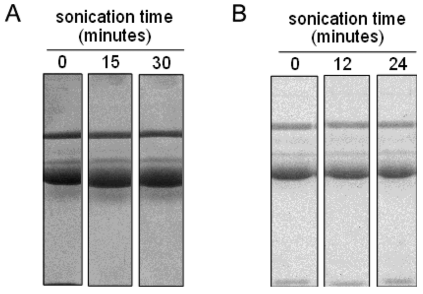


Figure 4. SDS-PAGEs of (A) intact thick fraction (0 min) and thick fraction sonicated (15 and 30 min) and (B) intact (0 min) and sonicated dialyzed thin fraction (12 min, and 24 min). Intact thick fractions were treated with (with β-mercaptoethanol).

cross-linking were not observed, and, thus, primary structures of constituent proteins and peptide bonds were conserved during sonication. The concentration of radicals generated by sonication (0.14 mM) is 5% of the total protein concentration (2 mM), which should result in a ∼10% decrease in protein monomer concentration from the formation of protein dimers.

dx.doi.org/10.1021/jp312818f | J. Phys. Chem. B XXXX, XXX, XXX−XXX

Explanations for the observed absence of scission and/or cross-linking in sonicated hen albumen thick fraction samples follow.

We previously showed that that hen albumen radiation stability was due in part to the presence of glucose (22 mM), which has antioxidant properties.[20,21] Similarly, glucose and glycosylated proteins in the thick fraction are expected to react with hydroxyl radicals generated by sonication before the radicals can react with protein molecules to an extent that would result in scission and/or cross-linking. To further investigate the role of the antioxidant properties of glucose in preserving the primary structure of hen albumen proteins with sonication, we diluted thin fraction ten times in an aqueous salt solution with a concentration of ions (namely sodium, potassium, and magnesium) comparable to that of the typical thick fraction of hen albumen (~0.1 M).[22] A 50 mL aliquot of the dilute thin fraction sample was dialyzed in 1 L of the same aqueous salt solution with several solution changes over 72 h to remove glucose from the dilute thin fraction. After dialyzing the dilute thin fraction, samples were sonicated for 12 and 24 min and SDS-PAGE analysis showed that, even in the absence of glucose, hydroxyl radical reactions that can cause protein molecules to undergo agglomeration and scission did not occur (Figure 4B). These results indicate that the radical reactions with glycosylated proteins in the hen albumen may shield protein molecules from detectable effects of radical reactions. We studied in detail the OH radical reaction mechanism with ovalbumin using purified ovalbumin.[23] The G-value for cross-linking was found to be five times larger than that reported in ref 9 (dealing with the intact thick fraction radiolysis), so one can argue that the native conformation may be responsible (in part) for the results observed. However, the enthalpy of denaturation of the native ovalbumin and purified one are the same which means that the purification did not affect the tertiary and quaternary structure which in turn makes the conformation hypothesis rather improbable. However, mobility may be responsible for the relatively inefficient cross-linking. Namely, our study also shows that in aqueous solutions (where ovalbumin molecules are free to diffuse) there is competition between ovalbumin agglomeration and some intramolecular reactions which did not lead to the change in the molecular mass.[23] Therefore, the ovalbumin molecules immobilized in the 3D gel structure can show no cross-linking when the OH concentration involved is relatively low (0.14 mM as in our sonolysis study).

Sonication disrupts S−S bridges of the protein hydrogel network as evidenced by the change in dynamic viscosity and by the fact that protein fragments were less than 1 MDa; that is, all proteins entered the 7% separating gel, as indicated by that absence of protein bands or smears in the 4.5% stacking gel. After 12 min, the energy transferred to the water in both the bath and thick fraction sample is 19 J g^{-1}, which our results suggest is an adequate amount of energy to disrupt the S−S bonds (226 kJ mol^{-1}) which have a lower bond enthalpy than the C−C (348 kJ mol^{-1}) and C−N (285 kJ mol^{-1}) bonds that make up the amino acid chain of the primary structure.[24] Therefore, the hydrodynamic sheer from the collapse of bubbles is the predominate mechanism for the degradation of the protein hydrogel.

Ultrasound-Induced Protein Denaturation. Differential scanning calorimetry was used to compare the enthalpy of melting and denaturation in both intact and sonicated thick fraction. We previously showed that 16% of water in intact thick fraction is bound (nonfreezable).[8] The enthalpies of melting of the intact and sonicated thick fraction are nearly identical; therefore, the amount of bound water is not significantly altered by the degradation of the thick fraction protein network. The thermograms of intact and sonicated thick fraction are very similar in the temperature range of 60 to 100 °C, where protein denaturation occurs (Figure 5). The

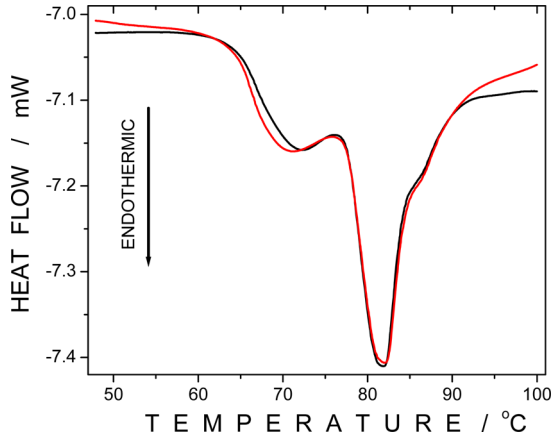


Figure 5. DSC thermograms of intact thick fraction (red) and sonicated (12 min) thick fraction (black).

enthalpies of denaturation for intact thick fraction and sonicated thick fraction (12 min sonication time) were determined by integrating the denaturation peak in this temperature range, and were found to be 16.4 ± 0.6 J g^{-1} and 15.7 ± 1.1 J g^{-1} for intact and sonicated thick fraction, respectively.

Thermal Denaturation and Turbidity. To further assess the ultrasound-induced degradation, the turbidity was measured. The native proteins are globular and upon thermal denaturation, assume a random coil conformation. This transition is accompanied by an increase in turbidity and is used to evaluate whether any thermally induced denaturation of proteins occurs during the sonolysis process. The turbidity of the thick fraction of hen albumen as a function of sonication time was measured (at 384 nm) and was found to increase from 0.36 ± 0.06 for intact thick fraction to 0.48 ± 0.08 after a 12 min sonication. The small increase in turbidity could be attributed to thermal denaturation of protein molecules near sites where high temperature bubbles, formed by acoustic cavitation, collapse. To quantify this, a solution of 0.5 g L^{-1} thin fraction of hen albumen (prepared in the aforementioned salt solution) was heated for 15 min at 90 °C (corresponding to the denaturation temperature from the DSC thermogram). After heating, the increase in the turbidity of the dilute thin fraction solution was 0.055 ± 0.008. Since the thin fraction is an ~10% protein solution, this means that the denaturation of 0.05 g L^{-1} ovalbumin would result in an increase in turbidity of 0.05. Therefore, since the thick fraction is ~100 g L^{-1} solution, a turbidity increase of 0.1 measured in the sonicated thick fraction corresponds to the thermal denaturation of ~0.1% of the protein molecules during sonication. The number found is too small to be detected with DSC technique, the relative error of which is ~4%.

CONCLUSIONS

As determined by the decrease in viscosity, the 3D protein hydrogel structure of the thick fraction of hen albumen was

dx.doi.org/10.1021/jp312818f | J. Phys. Chem. B XXXX, XXX, XXX−XXX

efficiently degraded; fragments of various sizes of thick fraction were dispersed in a rather fluid protein solution. SDS-PAGE showed that the interprotein S−S bonds, which hold the protein network together, were broken while the primary structure of the proteins was not affected, thus, indicating that hydrodynamic sheer is the predominate mechanism in the 3D network degradation (bond enthalpies for S−S, C−N, and C−C are 226, 285, and 348 kJ mol^{-1}, respectively). Neither cross-linking nor scission of the protein constituents were observed in the sonication time applied, which is likely a consequence of the antioxidant properties of the glucose (22 mM) present in the gel and the highly glycosylated proteins in the thick fraction, as well as protein immobility. The percent of bound water did not decrease or increase as measured using DSC, which is consistent with the observation that the primary protein structure was not affected. The enthalpy of denaturation of proteins in the thick fraction of egg was determined by differential scanning calorimetry to be the same for intact and sonicated samples. A small increase in sample turbidity can be attributed to the small extent of thermal denaturation occurring in the vicinity of cavitation sites.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank the Pennsylvania State University Department of Chemistry for support.

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