New Insight into Cataract Formation: Enhanced Stability through Mutual Attraction

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Small-angle neutron scattering experiments and molecular dynamics simulations combined with an application of concepts from soft matter physics to complex protein mixtures provide new insight into the stability of eye lens protein mixtures. Exploring this colloid-protein analogy we demonstrate that weak attractions between unlike proteins help to maintain lens transparency in an extremely sensitive and nonmonotonic manner. These results not only represent an important step towards a better understanding of protein condensation diseases such as cataract formation, but provide general guidelines for tuning the stability of colloid mixtures, a topic relevant for soft matter physics and industrial applications.

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Cataract, sickle-cell, and Alzheimer's represent a broad class of "protein condensation diseases" in which intermolecular attractions lead to dense phases that can compromise cell and organ function [1]. Statistical and colloid physics have led to considerable progress in understanding the molecular origins of these diseases, in which a subtle interplay between protein attractions, repulsions, and entropy governs condensation, and health or disease can hinge on intermolecular interaction changes of just thermal energy, k_BT. In complementary fashion, proteins have proven useful as model colloids, for studying how short-range attractions and hard and/or soft repulsions combine to affect phase transitions, aggregation, and cluster formation [2–4].

Cataract, eye lens clouding due to light scattering, is a leading cause of blindness and can result from protein condensation [1,5–8]. Statistical and colloid physics have helped rationalize eye lens transparency, liquid structure, and thermodynamics [7–11]. Mammalian eye lens cells contain concentrated solutions of proteins called crystallins, of three major classes: α, β, and γ [9,10]. The lens is normally highly transparent and refractive, but loss of transparency due to protein aggregation or phase separation can lead to cataract. Quantifying and understanding crystallin interactions and their impact on lens transparency is therefore a first step towards cataract prevention [6].

The α-crystallins are globular, polydisperse, multisubunit, 800 kDa proteins with a diameter of about 18 nm, whose interactions are well described with a simple hard-sphere colloid model [12,13]. The γ-crystallins are monomeric, with a molecular weight of 21 kDa and a diameter between 3 and 4 nm for γB-crystallin. The discovery of a metastable liquid-liquid phase separation provided evidence for short-range attractions between γ-crystallins, and use of the corresponding colloid model has led to a quantitative description of the phase behavior [11,14–18].

Although the chaperone activity of α-crystallin can suppress aggregation of β- and γ-crystallin [19,20], little is known about interactions between unlike crystallins at high concentrations. Recently, α-crystallin has been found to enhance phase separation of γB-crystallin and lead to partial segregation of proteins by type in the separated phases [21]. Therefore, we have started a systematic study of α- and γB-crystallin mixtures up to concentrations found within the eye lens that combines small-angle neutron scattering (SANS) and molecular dynamics (MD) simulations of a coarse-grained colloidal model, appropriate given the hard cores of these folded, globular proteins. This approach allows for complementary experimental and computational investigation at the required large length scales and high number of proteins ($\sim$30 000–60 000).

We prepared different mixing ratios from stock solutions of 230 mg/mL α-crystallin (denoted as $C_\alpha = 100$) and 260 mg/mL γB-crystallin (denoted as $C_\alpha = 0$) in 0.1 M sodium phosphate buffer in D_2O at a pH of 7.1, with 20 mM dithiothreitol to inhibit protein oxidation and oligomerization [21]. With these precautions, γB-crystallin remained monomeric under all conditions used. We performed SANS using 1 and 2 mm Hellma quartz cells and varied wavelengths, sample-to-detector distances, and collimation lengths to cover a q range of 0.02–3 nm^{-1}. All experiments were performed at a temperature of 25 °C, i.e., 10 °C above the critical temperature T_c of γB-crystallin in the present buffer. The choice of an experimental temperature close to T_c of γB-crystallin amplifies thermodynamic stability variations due to the presence of alpha crystallin, and thereby enhances sensitivity for discerning effective protein interaction potentials.

We established a model for the protein interaction potentials by comparing MD computer simulations with the experimental scattering intensity $I(q)$ as a function of the scattering vector q. We performed MD simulations in periodic cubic boxes with $N = 32\,000$ particles for the pure

α-crystallin solution and the mixtures, and $N = 64\,000$ particles for the pure γB-crystallin solution. The scattering intensities were calculated directly from many independent MD configurations using the experimental form factors and a properly derived general resolution function to account for the experimental smearing [22]. From the three different mixing ratios ($C_\alpha = 50$, $C_\alpha = 25$, and $C_\alpha = 12.5$, where C_α is defined as $100 \times$ [volume of the $C_\alpha = 100$ solution]/[total volume of the mixture]) investigated, $C_\alpha = 50$ closely resembles the natural α- and γ-crystallin concentrations found in the lens nucleus [9]. Therefore, the model development is demonstrated on this sample, and its validity is tested by comparison with the remaining samples.

First we determined parameters that provide a quantitative description of $I(q)$ for the individual components. For α-crystallin we used a purely repulsive hard-sphere model and a diameter of $d_\alpha = 16.3$ nm [13], which results in perfect agreement between experimental and simulated $I(q)$ [Fig. 1(a)]. For $C_\alpha = 0$ the strongly enhanced intensity at low q indicates that a hard-sphere model is not sufficient to describe the interactions between γB-crystallins (diameter $d_\gamma = 3.6$ nm) [Fig. 1(b)]. This

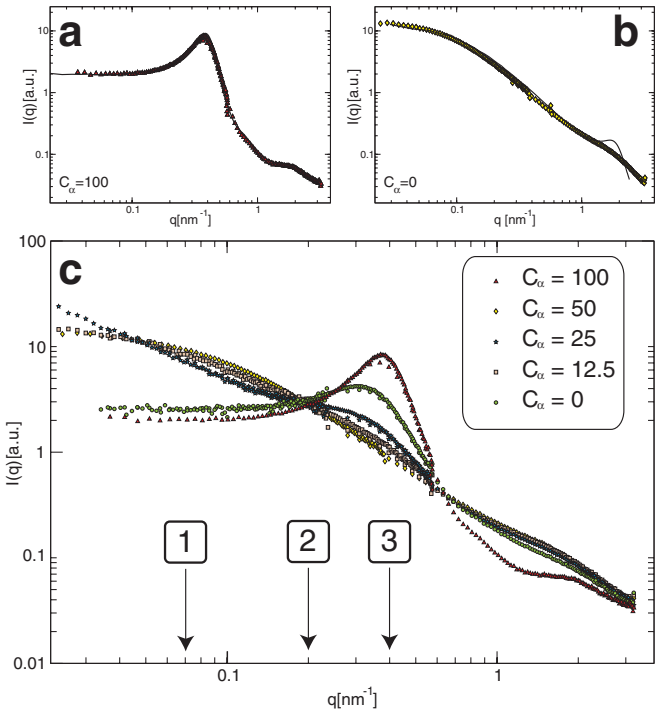

FIG. 1 (color online). Scattered intensities $I(q)$ of a 230 mg/ml ($C_\alpha = 100$) and a 260 mg/mL ($C_\alpha = 0$) solution and their mixtures. (a) Experimental $I(q)$ of $C_\alpha = 100$ (open symbols) together with MD computer simulations (full curve). (b) Experimental $I(q)$ of $C_\alpha = 0$ (open symbols) together with MD computer simulations (full curve). (c) $I(q)$ of mixtures containing different amounts of the crystallin solutions shown in (a) and (b). Shown are $C_\alpha = 100$ (▲), $C_\alpha = 50$ (◆), $C_\alpha = 25$ (★), $C_\alpha = 12.5$ (■), and $C_\alpha = 0$ (●). Numbers indicate q values for composition dependence in Fig. 4.

is a direct consequence of interprotein attraction and proximity to the critical concentration C_c [14,15,17], which leads to long-wavelength concentration fluctuations that are a potential source of lens turbidity in cataract [5]. To account for this we added a square-well attractive potential with a range of 0.25 d_γ [16] and a depth $u_{\gamma\gamma} = 1 k_B T$, where the temperature T is set to $T = 0.7875$ (for $k_B = 1$) for all simulated mixtures. This temperature T has been fixed to reproduce the γ-pure case and results in good agreement between simulated and experimental $I(q)$ in the low-q regime ($q \lesssim 0.047$ nm^{-1}) relevant for lens transparency [Fig. 1(b)]. While square-well potentials have unphysical shape, they have been widely used to study colloids with interaction ranges shorter than particle diameter, since the physics then depends only weakly on the shape of the potential [23]. Figure 1 indeed demonstrates that simple colloid models are capable of reproducing $I(q)$ for individual α- and γB-crystallin solutions.

In a next step we investigated the mixtures. Figure 1(c) demonstrates the effect of adding alpha crystallin, from $C_\alpha = 0$ to $C_\alpha = 100$. The forward scattering first increases and reaches a maximum for $C_\alpha = 25$. For $C_\alpha = 50$ it becomes highly suppressed, similar to the situation for pure α-crystallin. In MD simulations we first assumed hard-sphere α/γB interactions. The striking disagreement between the forward scattering of the corresponding simulation with experimental data for $C_\alpha = 50$ is shown in Fig. 2. The low-q increase of simulated intensity likely arises from depletion attractions known in hard-sphere mixtures [24]. Such an effect would increase T_c for liquid-liquid phase separation and result in segregation of the proteins into large domains of α-crystallin-rich and γB-crystallin-rich regions (Fig. 2). As a consequence, light scattering would have increased and transparency would have been lost, in contrast to the experimental data and the visual appearance of the sample. Such effects are in fact manifest in α-γB mixtures at temperatures lower than those investigated here [21]. However, the low forward scattering intensity $I(0)$ of the present SANS data indicates that they are suppressed under the present conditions, closer to body temperature. Nature must have circumvented long-wavelength fluctuations and eye lens clouding from hard-sphere α-γB interactions, and we can speculate that α and γB could have additional attraction.

In a second attempt we thus added an attractive interaction between α- and γB-crystallins, assuming that the attractive part of the potential has the same range as that used for the attraction between γB-crystallins. The depth of this interspecies attractive potential, $u_{\alpha\gamma}$, was chosen to reproduce $I(q)$ and is roughly one half of the γB-γB attraction. The simulated $I(q)$ (Fig. 3) indeed perfectly reproduces the SANS data throughout the entire q range. Use of the novel attractive term leads to good agreement between simulations and SANS for all mixing ratios (Fig. 4). A snapshot of the $C_\alpha = 50$ simulation (Fig. 3) suggests a qualitative explanation: attractions between un-

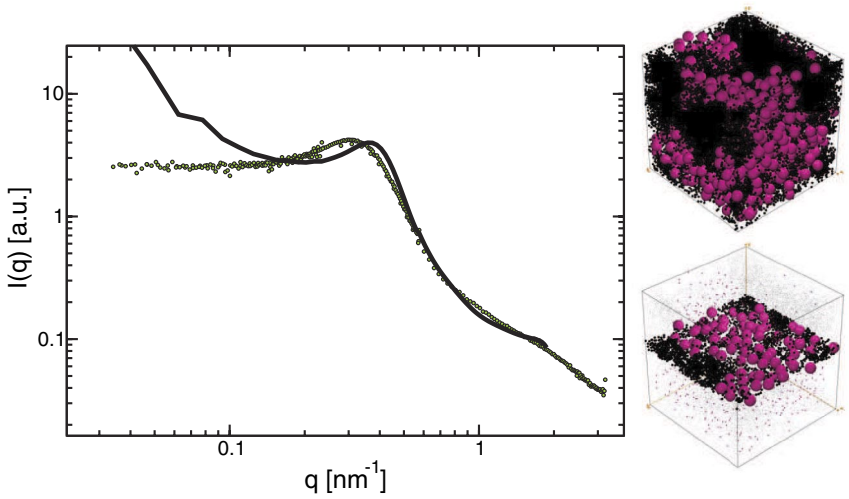

FIG. 2 (color online). Scattered intensity $I(q)$ of a mixture of α- and γB-crystallin ($C_\alpha = 50$) and MD computer simulation assuming mutual repulsion between unlike proteins. (Left) $I(q)$ of $C_\alpha = 50$ (open symbols) together with MD simulation of the system (full curve). (Right) Snapshot from MD simulation shown on the left. Assuming repulsive hard-sphere interactions only between α- (gray spheres) and γB-crystallins (black dots) leads to a strong segregation by protein type and the corresponding large density fluctuations would lead to a loss of transparency for visible light. Also shown is a slab of the box for this snapshot.

like proteins counterbalance and suppress segregation of the two proteins into large domains [25]. Thus transparency of the mixture is enhanced by a weak short-ranged interspecies attraction that considerably decreases T_c and the corresponding critical fluctuations at a given temperature.

We also performed simulations in which the attraction between α and γB was further increased, to above 100% of the γB-γB attraction. Such stronger attractions again resulted in enhanced instability (inset of Fig. 3). Thus the stability of these high concentration crystallin mixtures depends on α-γB attraction in a manner that is both extremely sensitive and nonmonotonic. Such nonmonotonic effects are a common feature of ternary liquid mixture phase separation [21,26].

There are studies suggesting that γ-crystallins inhibit the age-related aggregation of α-crystallins [27]. On the other hand, numerous investigations have suggested that inhibition of age- or heat-induced aggregation of γ- and β-crystallins is associated with the chaperone activity of α-crystallins [19,20,28]. In contrast to these stabilizing effects of α-γ interactions, it has recently been suggested that the observed increase in the noncovalent association of γ-crystallins to α-crystallins in aging bovine lenses might adversely affect the optical properties of the aged and/or cataractogenic lens [29], since strong attractions could lead to larger aggregates and increased scattering of light. While such a mechanism of increased protein-protein association is a possible route towards cataract [29], our experiments and simulations provide evidence that a weak and short-range attraction between α- and γB-crystallins can potentially enhance transparency in healthy lenses.

A variety of mechanisms could underlie this "stabilization due to mutual attraction", as a simple colloidal α-γB attraction can represent our observations. The molecular origins of the weak, short-range attraction remain a challenging, open question, as for many proteins including γB-crystallin and lysozyme [2,30].

Uniform, high concentration packing of crystallins, termed short-range order, suppresses refractive index fluctuations and reduces light scattering [7]. The chemically

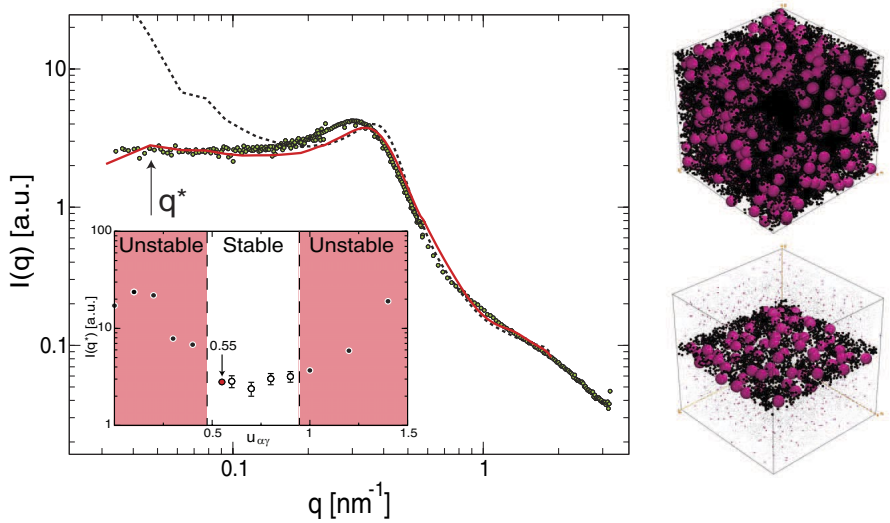

FIG. 3 (color online). $I(q)$ of an α- and γB-crystallin mixture ($C_\alpha = 50$) and simulations incorporating mutual attraction. (Left) SANS data from Fig. 2 (open symbols) and simulations without (dashed curve) and with added short-range attractions (full curve; $u_{\alpha\gamma} = 0.55 k_B T$) between unlike proteins. Inset: $I(q^* = 0.0467\ \mathrm{nm}^{-1})$ from simulations as a function of $u_{\alpha\gamma}$. The mixtures become unstable for $u_{\alpha\gamma} \lesssim 0.5 k_B T$ and $\gtrsim 1 k_B T$. (Right) Snapshot and slab representation of the box for this situation as in Fig. 2. The solution is homogeneous and does not give rise to increased light scattering.

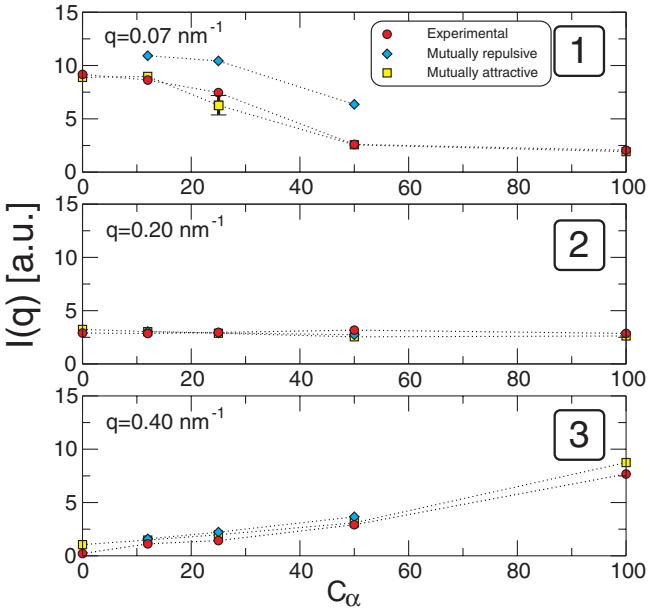

FIG. 4 (color online). Comparison of $I(q)$ obtained from simulations and SANS as a function of α-crystallin mixing ratio (C_α) in the α-γB mixtures. The predicted low-q portion of $I(q)$ is very sensitive to alpha-gamma mutual attraction, unlike the higher-q portions. $I(q)$ at different C_α are from SANS (●) and simulations without (◆) and with (■) mutual attractions. Shown are data at three scattering vectors, $q = 0.07$, 0.2, and 0.4 nm^{-1} [the numbered labels refer to the arrows in Fig. 1(c)]. Unless explicitly plotted, error bars are equal or smaller than the symbols.

specific origins of short-range order are key to understanding lens transparency. Not only do crystallins vary widely in short-range order properties, but sensitivity to intercrystallin interactions makes short-range order not a simple combination of individual crystallin properties, as shown here for α- and γB-crystallins. To understand transparency of crystallin mixtures, high concentration short-range order study, using liquid-state and colloid physics, remains a needed complement to low concentration investigations.

This investigation provides new insight into the stability and optical properties of lens protein mixtures relevant to cataract. The results also suggest mechanisms to tune colloid mixture stability, a topic of industrial importance. This demonstrates the importance of a colloid-oriented approach to proteins as a means of obtaining insight into questions of biological, medical, as well as fundamental soft matter physics relevance.

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