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Copper nanoparticles have tendency to inhibit rosin modified surfactant induced aggregation of lysozyme

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ABSTRACT

Protein aggregation is associated with many serious diseases including Parkinson's and Alzheimer's. Protein aggregation is a primary problem related with the health of industrial workers who work with the surfactants, metal ions, and cosolvents. We have synthesized rosin-based surfactants, i.e., quaternary amines of rosin diethylaminoethyl esters (QRMAE), which is an ester of rosin acid with polyethylene glycol monomethyl ether. Here, we report the thermal aggregation of lysozyme induced by QRMAE at 65 °C and pH 7.4 for a given time period in which amorphous aggregates are formed and confirm that copper-nanoparticles have the ability to inhibit QRMAE-induced aggregation compared with zinc and silver-nanoparticles. Aggregation experiments was evaluated using several spectroscopic methods and dye binding assay, such as turbidity, Rayleigh light scattering, 1-anilino-8-naphthalene sulfonate (ANS), Thioflavin T (Th T), congo red (CR) and circular dichroism (CD), that was further supported by scanning electron microscopy (SEM) and SEM with EDX. The therapeutic use of nanoparticles and the fact that rosin possesses excellent film-forming properties, and that its derivatives have pharmaceuticals application such as micro encapsulation, coating and film forming, it's matrix materials are used for sustained and controlled release tablets, renders importance and application to the present study.

1. Introduction

The mechanism of protein aggregation process and how it results in cell damage is still unclear and a matter of intense debate [1]. When a protein is exposed to destabilizing conditions, it may unfold or undergo an aggregation process [2]. Proteins require a correctly folded conformation to exhibit their natural and biological activity [3], but under abnormal conditions, proteins enter an inaccurate folding pathway that is responsible for various conformational changes which ultimately cause many diseases, such as senile systematic amyloidosis, Alzheimer's disease, type II diabetes, or Parkinson's disease, formation of protein wine haze, expansion of eye lens in cataract [4–10]. Proteins aggregate in vitro under extreme conditions such as high temperature, extreme acidity or alkalinity, high pressure, and the presence of metal ions [11–14], surfactants, chemically synthesized surfactants and cosolvents [15–19]. These extreme conditions are responsible for a partially folded conformation of a protein that leads to protein aggregation within hours or days.

Quaternary amine of rosin diethylaminoethyl ester (QRMAE) was chemically synthesized from rosin/abietic acid by Atta et al., and it has the ability to induce protein aggregation within hours; however, rosin acid/abietic acid itself does not show this activity. Protein–surfactant interactions and aggregation have been widely studied recently because of their important applications in the cosmetics, pharmaceutical and chemical industries [20,21], but the rosin modified compound in relation to protein-interaction and aggregation has only been the subject of a few studies. Rosin, a plant product, is obtained from oleoresins of pine tree; the

Abbreviations: QRMAE, quaternary amine of rosin diethylaminoethyl ester; ANS, 1-anilino-8-naphthalene sulfonate; ThT, Thioflavin T; CR, congo red; CD, circular dichroism; Zn, zinc; Cu, copper; Ag, silver; NPs, nanoparticles; SEM-EDX, scanning electron microscopy-energy dispersive X-ray.

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major sources are *Pinus roxburghii*, *Pinus toeda* and *Pinus longifolia* [22]. Rosin primarily consists of abietic and pimeric-type rosin acids [23] that contain hydrophenthrine rings with cycloaliphatic and aromatic structures, which have pronounced hydrophobicity, accounting for its use in marine antifouling materials [24]. Rosin is a solid form of a non-volatile compound that is produced by heating, which vaporizes the volatile liquid portion of its terpene components. Rosin possesses excellent film-forming properties, and its derivatives are used in pharmaceutical applications such as microencapsulation, coating and film-forming; its matrix materials are used for sustained and controlled release tablets [25–27].

Surfactants play an important role in protein chemistry in unfolding or denaturing proteins by inducing conformational alterations in the secondary and tertiary structures of the protein [4,28]. Surfactants may either induce or inhibit the aggregation process which depends on the associated conditions [29–40]. Due to ever increasing therapeutic role of nanoparticles (NPs) we have chosen copper (Cu), silver (Ag) and zinc (Zn) NPs to investigate their effects on QRMAE-induced lysozyme aggregation. Lysozyme has approximately 60% homology with human lysozyme and is subject to hereditary non-neuropathic systemic aggregation [41–43]. Lysozyme contains 129 amino acid residues and consists of two domains (i.e., α and β); thus, lysozyme is the α + β class of protein [9,10,44], which is an excellent 'model protein' [45,46] for this study.

We report here the thermal aggregation of lysozyme induced by the modified rosin component QRMAE at 65 °C and pH 7.4 and the effect of NPs, which inhibit or promote the aggregation process. In the present investigation the lysozyme concentration employed was 5 μM and it was observed that a high temperature was required to complete the aggregation process within 2–3 h while at a temperature of 40 °C the same process would take days at such a low enzyme concentration. It is for this reason that the temperature of 65 °C was employed. Aggregate formation was monitored by means of turbidity, RLS, ANS, ThT, CD and SEM. It is well known that ThT binds to proteins that have amyloid aggregates and have a good fluorescence intensity at 485 nm, which directly depends on the amount of aggregate present in the sample [47]. ANS (1-anilino-8-naphthalene sulfonate) was used to monitor the hydrophobicity of the aggregates that were already present or eventually formed by QRMAE. The secondary and tertiary structure change in the protein was calculated by using CD, and the morphology of aggregated proteins was investigated by SEM and SEM-EDX. This study provides information regarding amorphous aggregates induced by QRMAE and aggregation prevention by NPs. Rosin-modified components are used by various pharmaceutical companies, industries, soft drink processing companies, gymnasts and athletic persons.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme (L6876), copper nanoparticles (774111), silver nanoparticles (576832), zinc nanoparticles (578002), Thioflavin T (ThT) and 1-anilino-8-naphthalene sulfonate (ANS) were procured from Sigma–Aldrich, whereas sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate buffer were from Qualigens, India. Double distilled water was used throughout the study. QRMAE (Mol. wt. 697) was synthesized as described in a previous work [48,49]. The schematic process of synthesis and chemical structures of rosin surfactant QRMAE are illustrated in Supplementary Fig. 1.

2.2. Protein concentration determination and sample preparation

A stock solution of hen egg white lysozyme was made in 20 mM sodium phosphate buffer at pH 7.4 and the protein concentration was determined spectrophotometrically using $\varepsilon_M = 37{,}970\ M^{-1}\ cm^{-1}$ at 280 nm [50] on a JASCO V-660 spectrophotometer. The buffer used in the experiments was filtered through a 0.45 μm Millipore Millex-HV PVDF filter, and the pH was measured using a Mettler-Toledo pH meter (model S20). Nanoparticles and QRMAE were dissolved in buffer. All reagents used in the study were of analytical grade.

2.3. Turbidity measurements

Turbidity measurements were performed on a JASCO V-660 double beam UV–vis spectrophotometer in a cuvette with a 1 cm path length. The turbidity of lysozyme and the presence of 0.7 mM surfactants and NPs were determined by monitoring the changes in absorbance at 350 nm. All of the samples were incubated at 65 °C before the time measurements.

2.4. Rayleigh scattering measurements

Rayleigh light scattering measurements were taken on a Shimadzu (RF-5301PC) fluorescence spectrophotometer at room temperature in a cuvette with a 1-cm path length. The samples were excited at 350 nm, and the spectra were recorded from 300 to 400 nm. Both the excitation and the emission slit width were set at 1.5 nm. The protein sample without surfactant and NPs served as the control. All of the samples were incubated at 65 °C prior to measurement.

2.5. Intrinsic fluorescence measurements

The intrinsic fluorescence measurements were carried out on Shimadzu (5301PC) fluorescence spectrophotometer equipped with a constant temperature holder attached to a Neslab RTE-110 water bath with an accuracy of ±0.1 °C. Intrinsic fluorescence was measured by exciting lysozyme at 280 and recorded from 290 to 500 nm because aromatic amino acid fluorescence is used as a probe of the local environment in a protein to determine the protein structure, dynamics and denaturation [51].

2.6. ThT binding assay

A stock solution of ThT was prepared in double distilled water and filtered with a 0.2 μm Millipore filter. The concentration of ThT was measured using a molar extinction coefficient $\varepsilon_M = 36{,}000\ M^{-1}\ cm^{-1}$ at 412 nm [50]. The protein samples, in the absence as well as in the presence of surfactants and NPs, were incubated at 65 °C. Post incubation, the samples were supplemented with a ThT solution at a ratio of 1:5 and were incubated for a further 30 min in the dark. The ThT was excited at 440 nm and spectra were recorded from 450 to 600 nm. The excitation and emission slit widths were set at 5 and 10 nm, respectively. Fluorescence measurements were made using a Shimadzu (RF-5301PC) fluorescence spectrophotometer.

2.7. ANS binding assay

A fresh stock solution of ANS was prepared in double distilled water and filtered with a 0.2 μm Millipore filter. The