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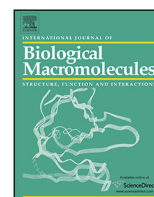

Initiating fibro-proliferation through interfacial interactions of myoglobin colloids with collagen in solution

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ABSTRACT

This work examines fibro-proliferation through interaction of myoglobin (Mb), a globular protein with collagen, an extracellular matrix fibrous protein. Designed colloids of Mb at pH 4.5 and 7.5 have been mixed with collagen solution at pH 7.5 and 4.5 in different concentrations altering their surface charges. For the Mb colloids, 100–200 nm sizes have been measured from Transmission electron micrographs and zeta sizer. CD spectra shows a shift to beta sheet like structure for the protein in the colloids. Interaction at Mb/Collagen interface studied using Dilational rheology, Quartz crystal microbalance with dissipation and Differential Scanning calorimetry show that the perturbation is not only by the charge compensation arising from the difference in pH of the colloids and collagen, but also by the organized assembly of collagen at that particular pH. Results demonstrate that positive Mb colloids at pH 4.5, having more% of entrained water stabilize the collagen fibrils (pH 7.5) around them. Ensuing dehydration leads to effective cross-linking and inherently anisotropic growth of fibrils/fibres of collagen. In the case of Mb colloids at pH 7.5, the fibril formation seems to supersede the clustering of Mb suggesting that the fibro-proliferation is both pH and hydrophilic-hydrophobic balance dependent at the interface.

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1. Introduction

Development of new and efficient biomaterials require an understanding of the molecular interactions that drive protein assemblies. In addition, modelling tissue disorders, fibrosis and breakdown of mechanical properties of the tissue is a recurring theme in the area of Biomaterials. Medically, renal interstitial fibrosis [1], scleroderma [2], sarcoidosis [3], idiopathic pulmonary fibrosis [4], and Dupuytren's disease (DD) [5] are some of the clinical examples that are being addressed in Systems Biology [6]. These disorders arise primarily from mis-folding of protein or peptide fragments leading to subsequent fibrillary aggregates that are implicated in the diseases themselves.

Among these different clinical disorders, DD is a fibro-proliferative disorder of the palm of the hands leading to digital contracture. Histology has shown increased levels of some types of collagen, myofibroblasts and myoglobin proteins in DD tissue and occasionally myoglobin is immobilized in the defective fibrils [6]. However, the exact mechanism by which the fibrillary aggregates of collagen and their interaction with myoglobin leading to alterations in mechanical properties of these combined systems is still not completely understood. Hence model studies need to be carried out that would give leads on possible biophysical and biochemical mechanistic pathways.

Normally, collagen, the most abundant protein is found in mammalian tissues and is a major component of the natural extracellular matrix (ECM). It has found use in the biomedical field due to its different unique hemostatic properties, low antigenicity and mechanical characteristics [7]. Appropriately reconstituted collagen is often used in tissue engineering applications [8]. Myoglobin is a monomeric water-soluble heme protein found in muscle tissue, working as an intracellular storage site for oxygen. During periods of oxygen deprivation it releases bound oxygen working which is used for metabolic purposes. It is water-soluble and contains 153 amino acids residues. Each myoglobin molecule contains one prosthetic group which is inserted into a hydrophobic cleft in protein [9]. The interfacial rheology of both Mb and collagen in dilute solutions and with small molecular additives have been studied by Sankaranarayanan et al. and Fathima and they have addressed the influence of the interfacial properties on the folding-misfolding of these proteins [10,11]. A number of research groups have addressed unusual aggregation of proteins and their molecular organized assemblies using different biophysical techniques. Bohidar et al. dealt with stability of protein colloids stabilized on

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polystyrene beads. They showed a time dependent coagulation of the proteins which had an anomalous dependence on NaCl concentration [12].

Kurinomaru and Shiraki in a recent review have highlighted the use of Aggregative protein–polyelectrolyte complex (PPC) biopharmaceutical application. In their review they have shown the usefulness of the protein formulation produced by aggregative PPC and their applicability for biomedical use [13].

Chandra et al. used relative intensities of SERS bands to analyse the orientation and the change in conformation of the Lysozyme molecules on the Ag surface with time. From the interaction kinetics of metal–protein complexes they concluded that Ag nanoparticles most likely interact with Trp123 which is in close proximity to Phe34 of the lysozyme molecule [14].

This work attempts to understand the anomalous interactions of colloidal myoglobin with monomeric and fibrillary aggregates of collagen in the context of changes in local as well as interfacial properties that could help understand fibro proliferative disorders.

In this work, we focus on the mixed films of proteins Myoglobin (Mb) assembled as colloids interacting with collagen, a fibrous protein at pH 4.5 and pH 7.5. Here the pH values are chosen above and below the pI of Myoglobin, thus making the colloidal Mb with different surface charges. The early steps in the assembly of the collagen molecules around the Mb colloids have been characterized using fluorescence spectroscopy and circular dichroic (CD) spectroscopy. Dilational rheological parameters have been analyzed to study the influence of collagen aggregation around Mb colloids at higher pH at the capsule/solution interface. Surface pressure-surface concentration isotherms of these colloids as pure spread films and with collagen introduced at the air/solution interface have been studied. Brewster angle microscopy (BAM) for different surface densities has been analyzed to study changes in morphology at the air/buffer interface. The combination of quartz crystal microbalance measurements with dissipation (QCM-D) and ζ-potential have been applied to provide information on layer structure, surface coverage and electrostatic interaction. Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) have been used to study the morphology of the aggregated structures.

The objective of using Mb in colloidal form has been to understand role of molecular congestion in Mb–Mb association and its role in driving the diffusion and aggregation/assembly of collagen around the colloid.

The elasticity and rheological properties of colloids as spread films on a drop are subjected to dilation through oscillation and it is assumed that for small deformations of the liquid drop, the mechanical response of the surface layer will be linear with the applied strain. The experiments presented here are in the linear response regime.

2. Experimental

2.1. Materials and methods

Collagen from tails of six month old albino rats (Wistar strain) were excised to extract the collagen. The teased collagen was then washed with 0.9% NaCl at 4 °C. Acetic acid extraction method [15]. was used and collagen precipitated with 5% sodium chloride followed by centrifugation. After centrifugation, the precipitate was collected and was dialyzed extensively against 50 mM phosphate buffer. The concentration of collagen in the solution was determined from the hydroxyproline content according to the Woessner method [16] with the average molecular weight of collagen as 300 kDa. The stock concentration of the extracted collagen was 9 μM and was used for further studies.

Horse heart myoglobin from Biozyme (Reinheitszahl 1/4 4.8) (San Diego, CA) was used without further purification. Before each experiment, the protein was dissolved in 20 mM 4-morpholinepropanesulfonic acid (Boehringer Mannheim, Ingelheim am Rhein, Germany) at pH 7.0 and was filtered with Millipore (Billerica, MA) Millex SV filters before use. Concentration of Mb solution was fixed at 10^{-6} M for all pH values. Few crystals of sodium dithionite were added to the Mb solution, for removal of oxygen after storing a gas tight sample container. The excess of dithionite was eliminated by gel filtration chromatography on a column (20 cm × 1.5 cm) filled with Sephadex G-25 (Pharmacia, Sweden). Acetate and phosphate buffers (10 mM) for pH 4.5 and pH 7.5 respectively have been used for all the experiments.

Chloroform and methanol have been purchased from Merck. Quartz surfaces used for the adsorption experiments have been immersed in freshly prepared chromic acid for half an hour, washed repeatedly with Milli-Q water, and then dipped into the mixture of sodium hydroxide and Milli-Q water for 15 min. The dipped slides are washed again using running Milli-Q water. Freshly cleaned slides stored in a desiccator for 3–5 h have been used for the measurements.

For the QCM measurements, gold coated quartz crystals have been first immersed in 2% sodium dodecyl sulfate (SDS) overnight. Before use, the quartz crystals are then rinsed thoroughly with water, dried under a stream of nitrogen, and cleaned with Harrick plasma cleaner for 20 min.

2.2. Colloid preparation

Myoglobin (Mb) colloids have been prepared by the method of Kuppevelt et al. with a combination of freezing, annealing and lyophilization technique [17]. For preparation of pure Mb colloids, the respective buffer solutions are introduced in liquid nitrogen for about two minutes and then protein is added dropwise and finally the frozen colloids are kept in the refrigerator at −35°C for 3 h, brought slowly to room temperature over 3 h and used for further experiments.

The colloids are stable for about a week. For the mixed Mb-Collagen system, the collagen solution (of pH = 4.5) kept in liquid N_2 mixed with Mb colloids (of pH = 7.5) and Mb (pH = 4.5) mixed with Collagen solution (pH = 7.5) at different proportions are used and the colloids analyzed using UV-Visible and fluorescence Spectra.

The stability of the colloids and the surface charge interaction of the Mb/collagen system have been monitored by particle size analyser and zeta potential measurements using Malvern Nano-ZS zeta sizer.

2.3. Interfacial properties of Mb colloids and in the presence of collagen

Freshly prepared solutions of the protein at different pH's were used for each measurement. Equilibrium surface tension values as a function of time were measured using an NIMA DST 9005 tensiometer fitted with a Wilhelmy plate (CHR1 chromatography paper) using a Teflon trough of capacity 30 mL. The surface was cleaned before each measurement ensuring that no aggregates of the protein were present at the surface at time t = 0. The dilational rheological parameters of the Mb colloids with the collagen solution at pH 4.5 and 7.5 were measured with the profile analysis tensiometer PAT-1 (SINTERFACE Technologies, Germany) with an accuracy of 0.1 mN/m and were thermostated at a temperature of 295 K. The solution drops were formed at the tip of a PTFE capillary immersed into a cuvette filled with a buffer saturated atmosphere. After the adsorption equilibrium, the solution drop was subjected to harmonic oscillations with frequencies (0.01–0.2 Hz) to measure the dilational elasticity. Accuracy in elasticity and viscosity

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