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Favourable Interfacial Characteristics of A2 Milk Protein Monolayer

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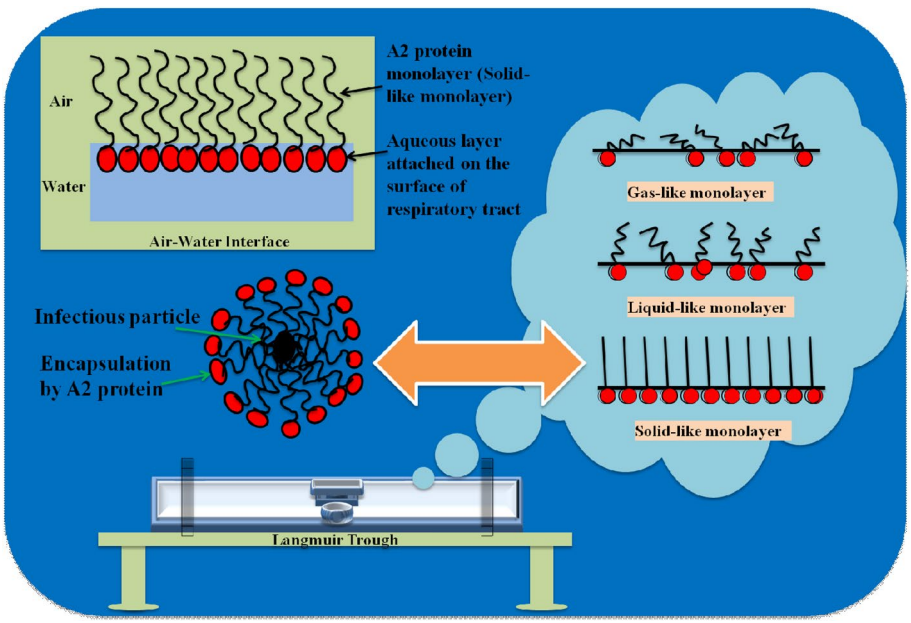
Abstract

Shielding of the specific body organ using the biocompatible material helps preventing direct exposure of that part to the foreign entities responsible for infections. Here we show the potential of the A2 milk protein recovered from the milk of cow from Indian origin for possible prevention of the direct exposure to other foreign molecules. We measured the surface pressure of the monolayers of different types of protein samples using Langmuir isotherm experiments. The surface pressure measurements for the monolayer of four types of protein macromolecules have been carried out using the Wilhelmy plate micro pressure sensor. We studied the self-organization of different protein macromolecules and their monolayer compression characteristics. The electrochemical behaviour is studied using electrochemical impedance spectroscopy. We found the highest surface pressure for the monolayer of A2 protein. Further, it is also found that A2 protein exhibited the highest surface activity amongst the other proteins. This property can be effectively used for making the envelope of the A2 protein surrounding the targeted entity.

Graphical Abstract



Keywords A2 protein · Casein · Competitive interfacial activity · Coronavirus · Gir cow milk · Langmuir monolayer isotherm

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Introduction

The novel coronavirus has now spread worldwide and caused many deaths reported worldwide. The World Health Organization defined the disease caused by this virus—COVID-19—as a pandemic (Naspro and Pozzo 2020; Sohrabi et al. 2019). COVID-19—relevant to severe acute respiratory syndrome (SARS) is caused by a betta-coronavirus named SARS-CoV-2, which affects the lower respiratory tract (Sohrabi et al. 2019). The chloroquine—an approved malaria drug—has been found to be a potential candidate for therapeutic efficacy against COVID-19 based on clinical trials and cell culture studies (Wang et al. 2019; Gao et al. 2020; Hu et al. 2020). A Recent report also mentions that Remdesivir (GS-5734)—a drug developed by Gilead Sciences, is also under trial for the treatment of COVID-19 (Bras 2020). Possible clinical-phase vaccine candidates for COVID-19 are also reported in the literature (Thanh et al. 2020). It has been reported that this novel coronavirus has typical structure with the 'spike protein' (Li 2016) in the membrane envelope, and expressed other polyproteins, nucleoproteins, and membrane proteins such as RNA polymerase, 3- chymotrypsin-like protease, papain-like protease, helicase, glycoprotein, and accessory proteins (Lu et al. 2015; Zhou et al. 2020; Fan et al. 2020). Besides the routine diet, a specific dosage of immunity boosters is very beneficial for maintaining healthy functioning of respiratory system which is of prime concern in the pandemic COVID-19. In addition to the development of new drugs and vaccines for this fast spreading pandemic, it is also essential to have the nutritional diet habit to sustain the health against such infectious pathogens. Milk protein is one of the significant nutrients known for good health.

The concept of the interfacial science can be effectively utilized for protecting the possible targeted area of the body organs prone to get infected by the virus or any other foreign entity. Biocompatible coating of the molecular-size thickness with suitable mechanical strength to offer reasonable vulnerability to the foreign entity can be a promising solution to avoid direct exposure of the body organs to foreign entity. The surface energy of the substrates and the surface activity of the molecules decide the preferential adsorption of the molecules at the interface. The interfacial characteristics of the biocompatible molecules can be effectively used to cover the surface of the substrates or even that of the tiny particles. The ability of the molecules to preferentially accumulate at the interface is useful for this type of applications, and can be best studied by their surface activity and interaction forces. Phenomena occurring on surfaces are very complicated to study experimentally and theoretically (Cragnell et al. 2017). Langmuir developed an experimental technique to study the behaviour of amphiphilic molecules (Sun 1994). The studies on monolayers also led to the development of thin films for variety of applications. Many cells of the human body are made up of biological macromolecules containing proteins, lipids, carbohydrates, etc. for proper functionality (Schöne et al. 2016). These biological macromolecules are essential to life and all living things which contain10,000 or more atoms having macromolecular size in the range of 100 to 1000 A˚(Trurnit 1960). Biological macromolecules do exist in nature as well as they can also be synthesized in the laboratory. The rigidity of macromolecules depends on the nature of the chains and their environment (Lee et al. 2007).

Milk contains various types of the minerals, vitamins, and proteins (Wal 1998). Casein protein and whey protein are well explained in the literature (Phadungath 2005; Fox 2008; Nelson and Cox 2009). Casein protein fractions are composed of four types, i.e. alpha S1, alpha S2, beta-β, and k-casein, and can be subdivided into A1 protein and A2 protein (Spuergin et al. 1996; Chatchatee et al. 2001). A2 milk protein is one of the best natural anti-oxidants and prevents the formation of serum cholesterol. It is also found to be effective for the treatment of cancers with remarkable boost up of the immune system (Tronin et al. 1996). The physiochemical property and biological activity of proteins depend on the intermolecular interactions. Several key factors such as multiple electrostatic, hydrogen bonding, hydrophobic interactions, ionic strength, pH, and temperature play essential roles in protein adsorption (Gupta et al. 2005; Bogomolova et al. 2009; Sindhuja et al. 2016).

Macromolecules in the form of a thin protein film need to be explored for the interactions with the surrounding media. In this paper, four different types of milk proteins have been examined with the help of the Langmuir monolayer technique and electrochemical impedance spectroscopy. Four different types of protein samples used in the study were—A2 protein, recovered casein protein, commercially available casein protein, and whey protein. Milk of the cow from Indian origin (*Gir* cow) is known to contain A2 protein, which is considered very beneficial, and full of nutrient value for human health. The protein recovered from this milk need to be studied for the interaction with that of the lipids and other proteins. The behaviour of the A2 milk protein can be predicted from the information of its monolayer characteristics. The present work explores the useful interaction of this protein which can be useful to eliminate the harmful effects of foreign entities and other infectious diseases.

Materials and Methods

Materials

Milk of the cow of Indian origin (Gir cow) was procured, and used to recover protein. Commercially available casein protein was purchased from Hi-Media chemicals Ltd., Mumbai, India. Gold standard whey protein was purchased from the Optimum Nutrition, USA. Protein was recovered from the milk of Gir cow through the acid precipitation method. To compare the behaviour of A2 milk protein with other recovered protein, milk was purchased from the local supermarket, and casein was recovered from it using acid precipitation method. Deionized ultrapure water (18.2 MΩcm, MerckMillipore ELIX10, Bengaluru, India) was used for all the experiments.

Experimental Method

We used an indirect method to predict the interfacial interaction of the A2 protein by exploring its surface pressure characteristics. Surface pressure–area isotherms and surface pressure relaxation were studied for the samples. The A2 protein behaviour was compared with the recovered casein protein from the milk from local supermarket, commercially available whey protein and casein. The experiments were performed for the surface pressure measurements and for the electrochemical impedance spectroscopy (EIS) analysis. The principle and working mechanism of the EIS is explained in more detail in our previous work (Dhopte and Lad 2020). The schematic diagram for the Langmuir monolayer isotherm is shown in Fig. 1.

Recovery of Proteins by Acid Precipitation Method

The well-known procedure developed by Holler, as explained in (Fox et al. 2004; Xiao et al. 2019) was used to recover protein from the milk of Indian Gir cow, and from that available in the local supermarket. Fresh, undiluted milk, the fat of which was removed by centrifugation at 4000 rpm, was cooled to 10 °C, and was used for further processing. Protein recovered from the local supermarket milk has been mentioned hereafter as recovered casein protein, and that from the milk of Indian Gir cow is mentioned as A2 protein. We prepared 10% acetic acid solution in deionized water, and used it to adjust the pH of the milk sample 4.5. Then we filtered the solution with the help of Whatman filter paper. The protein samples were washed with water, dried, and kept in a freezer at 4 °C till further use in the experimentations.

Surface Pressure Measurement Technique

The Langmuir trough (Apex Instruments Pvt. Ltd., Kolkata, India) with teflon double barriers was used to measure the surface pressure of the monolayer of various samples. The surface pressure measurements were recorded by surface pressure (π) v/s area per molecule (a), and surface pressure (π) v/s time (t). Here we studied the samples of A2

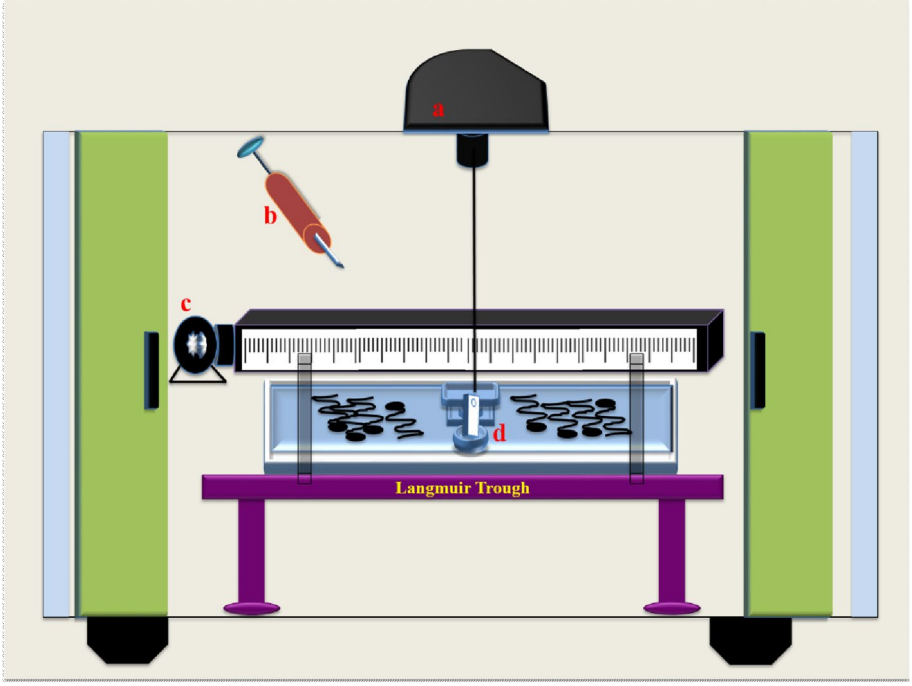

Fig. 1 The schematic diagram for the experimental setup: **a** surface pressure sensor, **b** microsyringe, **c** barrier motor, **d** Whilhelmy plate

protein, casein protein, commercially available casein protein and commercially available whey protein for surface pressure isotherm characteristics at the air–water interface. We repeated the experiments twice to confirm the reproducibility. The aspirator pump was used to remove the contaminants if any, prior to spread the sample on the subphase. The double barriers of the trough were moving at a speed of 1 mm/min to generate compression isotherms. A Hamilton Gastight® microsyringe procured from (Sigma-Aldrich, India) was used to spread the solution carefully on the surface of the subphase. 50 µL of spreading solution with pH 8, containing 2 mg/mL of the sample, was spread on the aqueous subphase in the Langmuir trough maintained at 28 °C. As explained earlier, Wilhelmy plate microbalance assembly was used as a surface pressure sensor (Dhopte and Lad 2020). The height of Wilhelmy plate was adjusted so that approximately one third of the plate height was dipped in to the liquid.

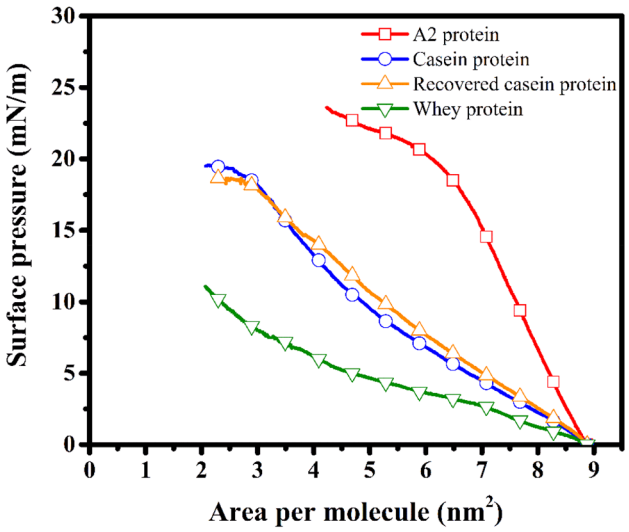

Fig. 2 Surface pressure-area isotherms for A2 protein, casein protein, commercially available casein protein and whey protein

Impedance Analysis

The three-electrode system containing a platinum counter electrode, a platinum working electrode, and Ag/AgCl reference electrode has been used for electrochemical impedance analysis. Further details of the system and the protocol are mentioned in Dhopte and Lad (2020). Electrodes were purchased from Bioanalytical Systems Inc. (West Lafayette, IN). The impedance measurements were performed at 1 V, 1 A over the frequency range from 100 Hz to 1 mHz using the potentiostate (Vertex 1A, IVIUM Technologies, The Netherlands).

Results and Discussion

Surface Area Isotherms

Surface pressure (π) v/s area per molecule behaviour is shown in Fig. 2. Proteins are amphoteric polymers containing hydrophobic and hydrophilic residues, and therefore exhibit affinity with a wide range of surfaces. It is well known that intermolecular interactions and intra-molecular interactions cause the protein adsorption process. These interactions are incorporated by hydrogen bonding, hydrophobic effects, van der Waals interactions, and electrostatic forces, which determine the conformation of the proteins at the interface (Carrera Sánchez et al. 1998a; Elderdfi and Sikorski 2018; Chen et al. 2019). During the compression, the phase transition occurred sequentially, that of gas-like monolayer, expanded liquid-like monolayer, condensed liquid-like monolayer, and solid-like monolayer phases with consequently increasing surface pressure. Further compression was resulted in the collapse of the monolayer. The maximum surface pressure was noticed for monolayers of all the samples at which the monolayers were remained stable prior to their collapse. Compression of the monolayer results in comparatively closer orientation of the molecules at the air–water interface, hence give rise to the surface pressure. The surface tension of the subphase was 71.6 mN/m which was considered as a base line for calculation of surface pressure of monolayer. Interestingly, the maximum surface pressure of the monolayer of the A2 protein sample was the highest amongst other samples, as revealed from Fig. 2. This shows that the mechanical strength of the monolayer of A2 protein is the highest for the same mean surface area per molecule. We obtained the maximum surface pressure values of 23.75 mN/m, 19.85 mN/m, 18.65 mN/m, and 12.65 mN/m for A2 protein, casein protein, purchased casein protein, and whey protein, respectively.

Electrochemical Impedance Analysis

Electrochemical impedance measurements were performed for four different types of protein samples using EIS technique. The EIS response presented an electrical fingerprint of the samples exhibiting an insight of their stability behaviour. Open circuit potential was observed for the potential difference between working and reference electrodes at equilibrium. The reaction process as a whole can be represented by Nyquist plot with impedance real part Z′ and imaginary part Z″, for an electric circuit composed of various resistances, shown in Fig. 3. An ideal Nyquist plot reveals the initial region as solution resistance. The semicircle diameter corresponds to the charge transfer resistance produced by redox reactions at the interface with the electrode. The

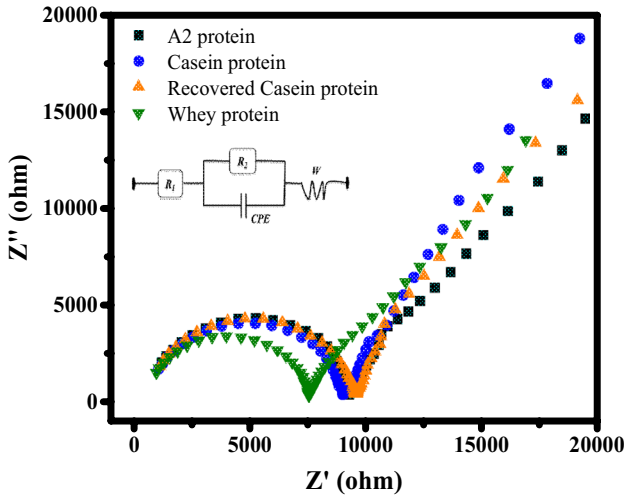

Fig. 3 Electrochemical impedance analysis for protein samples. Equivalent circuit parameters: R_1-solution resistance, R_2-charge resistance, *CPE* constant phase element, and *W* Warburg constant

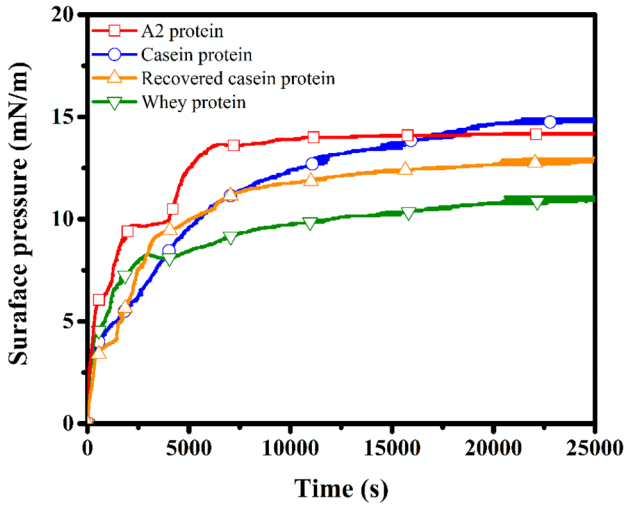

Fig. 4 Surface pressure measurements with respect to time of relaxation for protein samples

diagonal straight line corresponds to the current impedance due to diffusion from the solution to the interface. This measurement can be helpful for single frequency scan, to distinguish between two or more electrochemical reactions taking place, and also provides information about diffusion through a passive film, capacitive behaviour of the system as well as the electron transfer rate of reaction of protein molecules (Gomes et al. 2019). Double repetitions of the impedance analysis confirmed the reproducibility of the experimental results. Figure 3 reveals that as there was no appearance of additional semicircles it is clear that the electrode surface was free from impurities. The charge transfer resistance and impedance were higher for the A2 protein sample than other samples.

Surface Pressure-Relaxation Time Behaviour

The surface pressure-relaxation time relation is shown in Fig. 4, which shows the relaxation effects of various protein molecules. This technique provides information on the comparative surface activity of different protein samples. As evident from Fig. 4, the surface became saturated with the molecules at the fastest rate for A2 protein molecules. The competitive surface activity as revealed from Fig. 4 suggests that A2 protein molecules have higher affinity to adsorb at the interface hence this makes them a more promising candidate to bind the surface of the virus. Further, it also shows that A2 protein molecules offered the highest equilibrium surface pressure than that for the others. The behaviour of whey protein and casein protein samples is in agreement with that reported in the literature (Carrera Sánchez et al. 1998b; Rodriguez Patino et al. 2006; Dhopte and Lad 2020). Hence, it is confirm that amongst these four types of the samples, A2 protein molecules have the highest affinity to orient themselves at the interface. This characteristic is helpful for generation of the thin film of the molecules at the interfaces or at the surfaces of the substrates. Furthermore, it is clear that the A2 protein monolayer offers the highest maximum surface pressure at all the stages of compressions, ultimately resulting in comparatively more vulnerability of the monolayer towards the outer phase or foreign entity. Hence, such stable, and with comparatively higher mechanical strength, the A2 protein monolayer can be effectively used for protecting the substrate from exposing it to the harmful environment.

Conclusion

Different experiments have been performed to evaluate the surface activity of various protein samples. The protein recovered from the milk of indigenous cow of Indian origin (Gir Cow) has exhibited excellent surface activity and stability. The high surface pressure of the monolayer of the A2 protein reveals its potential to shield the internal body tissues to prevent their exposure to harmful foreign entities. The present work provides a new research direction to study the competency of the A2 protein to interact with the SARS CoV-2 to suppress the detrimental effects of the novel coronavirus and their successive variants. The surface pressure isotherms provide the information of the A2 protein macromolecules for their efficacy to orient at the interfaces. Further, the type of the monolayer or the compactness of the surface-oriented macromolecules ultimately governs the strength of the monolayer. Additionally, the isotherms reveal that the strength of the monolayer of A2 milk protein

is higher than the rest of the candidates, and hence, A2 protein macromolecules can be preferentially used to cover the surface of the substrates, which may prevent the intimate contact of the substrates with the infection causing entities. The competitive surface activity and the strength of the A2 milk protein monolayer can be effectively used for arresting the coronavirus using cage-like structures for which further research to evaluate the interaction of the A2 milk protein with infectious microscopic organisms may be helpful.

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Data Availability Data of the work presented here may be available on request till 6 months from the date of first online publication of this paper.

Declarations

Conflict of interest Authors declare that there is no conflict of interest.

Consent Statement/Ethical Approval Not required.

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