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Competitive interfacial adsorption of blood proteins

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Abstract

The competitive adsorption of blood proteins is of great importance for the treatment of thrombosis using a colloidal drug delivery system. The aim of this study is to investigate competitive adsorption of albumin (BSA) and human immunoglobulin G (HIgG) against fibrinogen (Fb). The competitive adsorption of blood proteins was investigated using interfacial rheology at physiological pH. The influence of bulk concentration, temperature and pH on the interfacial adsorption of protein molecules was determined at the air/aqueous interface. As expected, the results indicated that increase in bulk concentration enhanced the interfacial adsorption. Structure and molecular weight of the protein molecules under investigation had influence on interfacial adsorption leading to a competition at the interface. HIgG is more flexible and surface active molecule than BSA. Thus, HIgG replaced BSA and Fb at the air/aqueous interface. In the presence of Fb, BSA adsorbed rapidly initially and then, was replaced by Fb at the interface. The kinetics of displacement of albumin at the interface was rather slow. In conclusion, the investigation of competitive adsorption of blood proteins may be useful biotechnologically, as it will provide useful information for the production of an antithrombogenic material, which will adsorb albumin rather than Fb.

Keywords: Fibrinogen; Bovine serum albumin; Human immunoglobulin G; Competitive adsorption; Thrombosis

1. Introduction

There are significant evidences in epidemiological, clinical and experimental studies indicating a strong association between fibrinogen (Fb) level in plasma and pathogenesis of post-operative or post-traumatic thrombosis in human. Thus, competitive adsorption of blood proteins (bovine serum albumin (BSA), human immunoglobulin G (HIgG) and Fb) is of great importance regarding the treatment of such pathological complication (e.g. thrombogenesis) using a colloidal drug delivery system.

A major obstacle to the effective use of colloidal drug delivery devices developed for thrombogenesis may be opsonization as other colloidal drugs administered parenterally. After the administration of the drug, the first stage in the recognition process, which leads to opsonization, is the adsorption of blood proteins onto the interfaces of administered colloidal particles [1,2]. These processes depend on interfacial properties of the blood proteins. Therefore, this study was designed to investigate interfacial properties of blood proteins.

Interfacial adsorption of proteins can be investigated using microscopic interfacial analysis techniques such as FTIR spectroscopy and by physical techniques such as interfacial tension and pressure. The authors proposed a novel and better way of determining interfacial properties of protein layers in their previous studies [3]. In this study, interfacial properties of blood proteins were investigated using interfacial rheological measurements. Interfacial rheology measurements were conducted by means of a surface oscillatory technique. The data obtained with this technique provide information on molecular segmental kinetics of interfacial film formation, molecular conformational changes and intermolecular interactions [4–6].

Air/aqueous interface was used to simulate biological interface. The data obtained for interfacial adsorption of proteins follow the same trend of an initial rapid

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increase in interfacial elasticity followed by a slower increase phase as in the data of previous studies [7,8]. Also, Damodaran and Song [9] and Graham and Philips [10] have reported same trend in data for albumin adsorption. The interfacial rheological properties of adsorbed protein films were at a minimum at pH values close to their isoelectric pH (p*I*) [7,8]. At p*I* values, interfacial interactions are minimized due to the compact coiled conformation of the molecules. Damodaran and Song [9] showed that the decrease in surface pressure causes the extent of folding of albumin. Interfacial rheological data show the same affect on folding of the protein molecules. Increase in bulk concentration enhances interfacial adsorption.

BSA, HIgG and Fb have significantly different molecular weights, physicochemical properties and intramolecular folding. Molecular weight of BSA is 66,338 [11] and possesses a globular, flexible structure with 17 disulfide bonds which create 9 double loops and approximately 50% α-helix in the native state [12,13]. HIgG with the molecular weight of 150,000 [14] is a Y-shaped molecule. It consists of two heavy and two light chains which are cross-linked by disulfide bonds. Its structural motif is composed of two stacked β-pleated sheets surrounding an interior packed with hydrophobic residues. Fb has a double function yielding monomers that polymerize into fibrin and acting as a cofactor in platelet aggregation [15]. It is structurally a hexamer composed of two sets of three nonidentical chains (alpha, beta and gamma) linked to each other by disulfide bonds [15,16]. The molecular weight of Fb is 400,000 and carries zero net charge at pH 5.5 [17,18].

The interfacial rheology data indicated that adsorption of blood proteins takes place in a competitive process. Fb adsorption showed rapid or slow exchange or no exchange with other proteins at the interface. HIgG replaced Fb rapidly at the interface, whereas BSA was replaced by Fb rapidly initially and then slowly. These data may be useful for the design of an antithrombogenic drug.

2. Experimental

2.1. Materials

Bovine serum albumin (purified and essentially fatty acid-free), human immunoglobulin G (purified and lyophilized) and fibrinogen (from bovine plasma, purified and lyophilized) were purchased from Sigma Chemical Co. (St. Louis, MO) and ICN Biochemicals, Inc., USA, respectively. All other chemicals were analytical grades. All aqueous solutions were prepared with double distilled water.

2.2. Methods

Interfacial rheology of the adsorbed protein layers can be studied by different techniques: interfacial dilational measurements or interfacial shear experiments [8,19]. As no interfacial expansion or compression takes place causing disturbance to the interfacial layer and hence, escape of some of the molecules into the bulk phase during the shear experiments, the interface under investigation was sheared to a first-order approximation without altering the interfacial area. Thus, the interfacial pressure remained almost constant throughout the experiment preventing diffusion of protein molecules in or out of the interface. Interfacial shear measurements were performed by an MK2 Surface Oscillatory Rheometer (Surface Science Enterprises, UK) in this study. The rheometer consists of four connecting parts: a moving coil galvanometer; a platinum DuNouy ring, which sits parallel at the interface; a rheometer control unit which varies the driving frequency and monitors the amplitude and motion of the ring; and an IBM PC which processes the data from the interfacial rheometer.

Interfacial shear rheology measures both interfacial elasticity and viscosity. Sherriff and Warburton defined interfacial elasticity (G_s') (mN/m) and interfacial viscosity (η_s') (mNs/m)

$$G_s' = g_f 1.4\pi(f^2 - f_0^2) \tag{1}$$

and

$$\eta_s' = g_f I \times \mathrm{NC}\left(\frac{1}{X} - X_0\right) \tag{2}$$

where G_s' is interfacial elasticity; g_f is geometry factor; I is the moment of inertia; f^2 is the sample interfacial resonance frequency; f_0^2 is the reference interfacial resonance frequency; η_s' is the interfacial viscosity; NC is the number of cycles of integration; X is the mean amplitude at the sample interface; and X_0 is the mean amplitude at the reference interface. g_f is defined as a function of the radius of the ring:

$$g_f = 4\pi(R_1 - R_2) \tag{3}$$

where R_1 is the radius of the platinum ring and R_2 is the radius of the sample cell [7,19]. Calibration is carried out prior to each measurement. The rheometer is calibrated when the ring is set to oscillate in air using a series of standard inertia bars of known weight. Measurements are taken with respect to a reference interface, the air/double-distilled water interface. All measurements were repeated three times and the values plotted are the mean of these readings.

Sample solutions were freshly prepared in phosphate buffer solution of pH 7.4 and filled into a water-jacketed dish (internal diameter: 3.893 cm). Platinum ring was lowered to sit at the interface and the experiments were

performed at constant temperature maintained by a water bath (25 or 37±0.1 °C). Sample solutions used were freshly prepared. In all experiments, the interface was broken prior to each measurement by sweeping the interface with a clean glass rod. This allows the formation of a new film. The experiments were performed on a vibration-free platform. For each measurement, the instrument was run for 1 h. At the end of each measurement, the sample solutions were analyzed chromatographically for the presence of any degradation products. None were detected. Proteins are stable with respect to chemical degradation under the conditions studied.

3. Results and discussion

In order to obtain comparable data for competitive adsorption with Fb, the effects of bulk concentration and temperature on the interfacial properties of BSA and HIgG were investigated as conducted in our previous work. The effect of bulk concentration on interfacial rheology was measured for BSA at pH 7.4. The interfacial elasticity of BSA increases rapidly initially and then more gradually (Fig. 1). The initial rapid phase can be as a result of the migration of protein molecules to subsurface and then, adsorption into interface. The slower phase (rearrangement phase) may be due to conformational changes within individual molecules and to entanglement and interactions between adjacent molecules. The interfacial elasticity values increased with increasing bulk concentration (0.1–4%, w/v, pH 7.4). At pH 7.4, which is away from the isoelectric pH of BSA (p*I* 5.3) [7,8], the protein molecules are more hydrated and have an open, extended conformation due to charge repulsion. Intermolecular entanglement may be considerable and, therefore, interfacial elasticity is maximized. The effect of

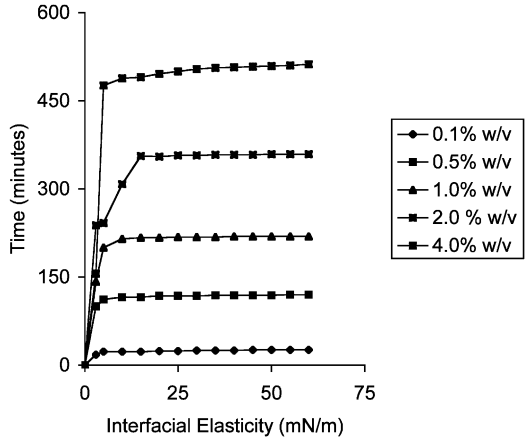

Fig. 1. The effect of bulk concentration on the interfacial elasticity of BSA at room temperature (pH 7.4; 100 mM).

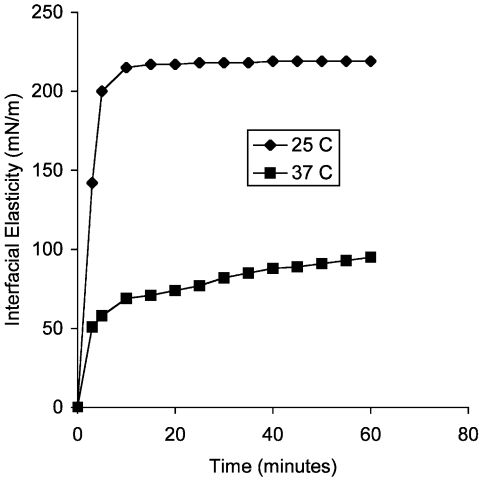

Fig. 2. The effect of temperature on the interfacial elasticity of BSA at air/aqueous interface (pH 7.4; 100 mM; 1.0%, w/v).

temperature on the interfacial elasticity of BSA was investigated at 25 and 37 °C. The protein molecules have much greater kinetic energy at 37 °C (Fig. 2). These data are in good agreement with those obtained in our previous studies [7,8].

The interfacial properties of HIgG adsorbed layers were investigated at the air/aqueous interface. Fig. 3 is a plot of interfacial elasticity of HIgG solutions with varying concentrations (0.5–1.5, w/v) at pH 7.4. Elasticity was not measurable at concentrations below 1.5%, w/v of HIgG at pH 7.4. As for BSA solutions, the interfacial elasticity of HIgG solutions was higher at 25 °C (Fig. 4). These data follow the same trend as in our previous studies for HIgG [7,8].

The investigation of interfacial rheology of adsorbed Fb was a bit problem as it formed a viscous solution in aqueous media. Therefore, the experiments were carried out using low concentration solutions without adding NaCl, which causes precipitation, at pH 7.4. Fb forms a

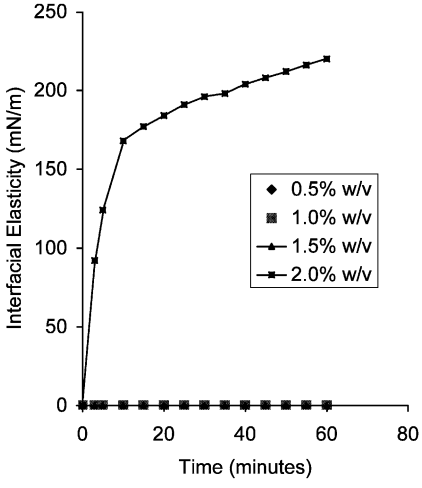

Fig. 3. The effect of bulk concentration on the interfacial elasticity of HIgG at the air/aqueous interface (25 °C; 100 mM; pH 7.4).

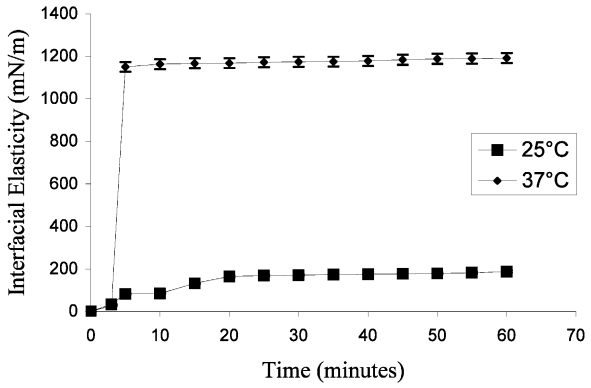

Fig. 4. The effect of temperature on the interfacial elasticity of HIgG (1.5%, w/v; 100 mM; pH 7.4).

viscoelastic film at the interface. Similarly, the elasticity values increased as concentration increased from 0.2 to 0.5%, w/v (Fig. 5). The effect of temperature on the interfacial elasticity values of Fb was studied at 25 and 37 °C. The interfacial elasticity values decreased as temperature increased (pH 7.4, 0.5%, w/v) (Fig. 6). This may be due to greater kinetic energy at 37 °C.

There is considerable amount of work done on investigation of adsorbed individual protein films. However, the use of a single purified proteins takes no account of likely competitive effects in real systems such as blood. Thus, interfacial adsorption of mixture of blood proteins was investigated. Fb (0.5%, w/v) was added to BSA solution (1%, w/v). In the presence of Fb, BSA adsorbs rapidly initially and then, replaced partially by Fb at the interface leading to a decrease in interfacial elasticity (Fig. 7). This may be due to faster migration of Fb to the interface than that of BSA. The kinetics of displacement of BSA at the interface is rather slow. In contrast, HIgG was not replaced by Fb or BSA when these proteins were added to HIgG solution and maintained its interfacial elasticity under the circumstances. This may be due to more flexible structure of HIgG and its greater interfacial activity.

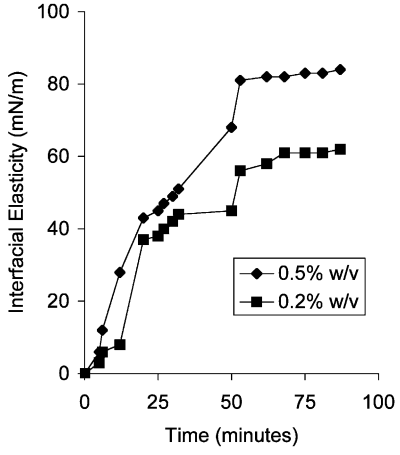

Fig. 5. The effect of bulk concentration on the interfacial elasticity of Fb at the air/aqueous interface (25 °C; 100 mM; pH 7.4).

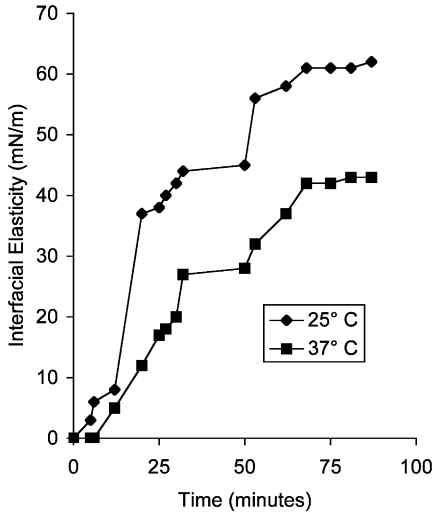

Fig. 6. The effect of temperature on the interfacial elasticity of Fb at the air/aqueous interface (100 mM; pH 7.4; 0.5%, w/v).

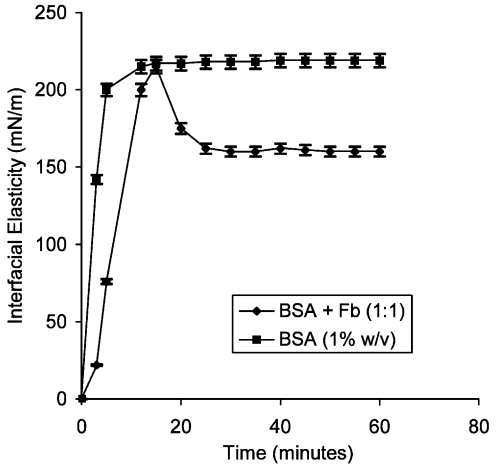

Fig. 7. The effect of Fb on the interfacial elasticity of BSA (1:1 mixture of BSA (1%) and Fb (1%, w/v) pH 7.4) at air/aqueous interface.

In short, the data obtained in this study indicate that interfacial properties of films adsorbed from mixed protein systems are significantly more different than those adsorbed from single-protein solutions. Competition between blood protein molecular space can be investigated using this methodology and hence, the prevention of interfacial adsorption of Fb molecules by the addition of BSA or HIgG can be determined. Bulk concentration and temperature had significant effects on the interfacial elasticity of blood proteins and therefore, on the rate of denaturation of these proteins. At high bulk concentrations, proteins form more elastic films. At physiological pH, HIgG replaces Fb and BSA at the interface. Albumin is replaced by Fb following an initial rapid adsorption phase.

In conclusion, rheological measurement can be useful in the study of conformational changes of blood proteins. Also, this technique is potentially useful in preformulation of an antithrombogenic drug [20–23].

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References

[1] D.J. Burgess, Colloids and colloidal drug delivery systems, in: J. Swarbrick, J.C. Boylan (Eds.), Encyclopedia of Pharmaceutical Technology, III, Marcel Dekker, New York, 1991, pp. 31–63.
[2] M.C. Manning, K. Patel, R.T. Borchandt, Stability of protein pharmaceuticals, Pharm. Res. 6 (1989) 903–905.
[3] D.J. Burgess, L. Longo, J.K. Yoon, A novel method of assessment of interfacial adsorption of blood proteins, J. Parent. Sci. (1991) 45–51.
[4] H.R. Kerr, B. Warburton, Surface rheological properties of hyaluronic acid solutions, Biorheology 22 (1985) 133–137.
[5] C.A. Moules, B. Warburton, The surface shear rheology of aqueous solutions of gum *Acacia senegal* in the presence of various second components, in: R.E. Carter (Ed.), Rheology of Food Pharmaceuticals and Biological Materials with General Rheology, Elsevier, Amsterdam, The Netherlands, 1990, p. 211.
[6] J.K. Yoon, D.J. Burgess, Investigation of interfacial stability of proteins using a surface oscillatory flow technique, in: Proceedings of the International Symposium of Controlled Release Bioactive Materials, 16, 1989, pp. 340–342.
[7] D.J. Burgess, J.K. Yoon, N.O. Sahin, A novel method of determination of protein stability, J. Parent. Sci. 46 (1992) 150–155.
[8] D.J. Burgess, N.O. Sahin, Interfacial rheological and tension properties of protein films, J. Colloid. Interf. Sci. 189 (1997) 74–82.
[9] S. Damodaran, K.B. Song, Kinetics of adsorption of proteins at interfaces: role of protein conformation in diffusional adsorption, Biochim. Biophys. Acta 954 (1988) 253–256.
[10] D.E. Graham, M.C. Philips, Proteins at liquid interfaces. I. Kinetics of adsorption and surface denaturation, J. Colloid. Interf. Sci. 70 (1979) 403–411.
[11] T. Peter, Jr., Serum albumin, in: F.W. Putman (Ed.), The Plasma Proteins, Academic Press, New York, 1975, p. 133.
[12] M.J. Hunter, F.C. McDuffie, Molecular weight structures on human serum albumin after reduction and alkylation of disulfide bonds, J. Am. Chem. Soc. 81 (1959) 1400–1403.
[13] P.Q. Behrens, A.M. Spiekerman, J.R. Brown, Structure of bovine serum albumin, Fed. Proc. 34 (1975) 591–594.
[14] S. Harrison, Principles of Internal Medicine, McGraw-Hill, New York, 1987.
[15] F. Doutremepuich, O. Aguejouf, E. Belougne-Malfatti, Fibrinogen as a factor of thrombosis: experimental study, Thromb. Res. 90 (1998) 57–64.
[16] R.A. Martinelli, A.S. Inglis, M.R. Rubira, Amino acid sequences of portions of the alpha and beta chains of bovine fibrinogen, Arch. Biochem. Biophys. 192 (1979) 27–32.
[17] D.W. Chung, M.W. Rixon, E.W. Davie, The biosynthesis of fibrinogen and the cloning of its cDNA, in: R.A. Bradshaw (Ed.), Proteins in Biology and Medicine, Academic Press, New York, 1982.
[18] S. Budowari, The Merck Index, 11th ed., Merck & Co, Rahway, 1989.
[19] M. Sherriff, B. Warburton, Measurement of dynamic rheological properties using the principle of externally shifted and restored resonance, Polymer 15 (1974) 253–258.
[20] K. Shiraishi, T. Ohnishi, K. Sugiyama, Preparation of poly(methyl methacrylate) microspheres modified with amino acid moieties, Macromol. Chem. Phys. 199 (1998) 2023–2028.
[21] S.M. Demos, S. Dagar, M. Klegerman, In vitro targeting of acoustically reflective immunoliposomes to fibrin under various flow conditions, J. Drug Target. 5 (1998) 507–518.
[22] M.T. Le, P. Dejardin, Simultaneous adsorption of fibrinogen and kininogen at a silica/solution interface, Langmuir 14 (1998) 3356–3364.
[23] A. Pothula, V.L. Serebruany, P.A. Gurbel, Pathophysiology and therapeutic modification of thrombin generation in patients with coronary artery disease, Eur. J. Pharmacol. 402 (2000) 1–10.