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The competitive adsorption of human proteins onto natural-based biomaterials

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This study aims to further the understanding of nanoscale structures relevant for cellular recognition on contact and interaction with natural-based materials. The correlation between surface characteristics and protein adsorption from unitary and complex protein systems was investigated with respect to altering the bulk chemistry of the substrate material. Polymeric blends of starch and cellulose acetate, polycaprolactone (SPCL) and ethylene vinyl alcohol (SEVA-C) were used. Different proteins, bovine serum albumin, human serum albumin (HSA) and human fibronectin (HFN), were selected for this study. The construction of adsorption isotherms is an important starting point towards characterizing the interactions between surfaces and proteins. In this study, albumin adsorption isotherms fit the Freundlich model and were correlated with the chemistry and morphology of surfaces. In addition, protein distribution, quantification and competition were measured using fluorimetry and visualized by confocal microscopy. The analysis of unitary systems demonstrated that the adsorption of HSA was generally lower than that of HFN. In the latter case, SPCL and SEVA-C blends reached adsorption values of 97 and 89 per cent, respectively. In studying the co-adsorption of proteins, an increase in both HSA and HFN on SEVA-C surfaces was observed. SPCL showed no substantial increase in the adsorption of the proteins in competitive conditions. The similarity of these materials with other polysaccharide-based materials increases the relevance of the presented results. This study provides valuable information for the development of strategies towards the control of protein orientation and functionality as the availability of cell signalling epitopes for a broader family of materials that continue to be a significant component of this field of research.

Keywords: adsorption; biomaterials; biodegradable materials; competition; proteins

1. INTRODUCTION

Analysing the interaction of proteins with the surfaces of materials intended for biomedical applications is fundamental for understanding cellular events and the overall host response. Furthermore, the importance of a variety of molecules in the biomedical field is known for several applications, including drug delivery, biomaterials, extracorporeal therapy and solid-phase diagnostics (Andrade & Vanwagenen 1983; Horbett 1994). To improve the understanding of the fundamentals of protein adsorption, many protein adsorption-modelling approaches have been successfully used (Morra 2000; Bajpai 2007). Most approaches treat the electrostatic and van der Waals interactions between the protein and the surface, and thus consider the effects of surface charge, protein size or solution ionic strength (Roth et al. 1996). Although the correlation between the adsorbed protein and the bulk concentration of the protein solution has been dealt with using many adsorption isotherm equations, the Langmuir equation, however, has been frequently applied owing to its lower complexity and broader applicability to various adsorption data (Bajpai 2007). The Langmuir isotherm performs a dynamic adsorption process with the reciprocation between adsorbed and unabsorbed bovine serum albumin (BSA) molecules, but disregards the interaction between these adsorbed molecules. On the contrary, the Freundlich isotherm can validate many adsorption processes facilitating the modelling of one or more interactions between adsorbed molecules or adsorbed molecules and the surfaces involved in the dynamic

process (Liu *et al.* 2006). The Freundlich model is an extension of the Langmuir model that is derived empirically in that it does not assume uniformity. However, it is still usually applied in a strictly empirical sense, which is of theoretical interest in terms of adsorption studies on energetically heterogeneous surfaces, such as natural origin biomaterials (Sharma & Agarwal 2001).

The adsorption of two factors from the human blood matrix (Jenney & Anderson 2000), fibronectin and albumin, onto the surface of polymeric blends was studied. These proteins were chosen on the basis of their biological effect and importance in a variety of biomedical applications. Furthermore, they represent two different types of proteins, small globular proteins (albumin) and large glycoproteins (fibronectin) (Carter & Ho 1994; Romberger 1997). Human fibronectin (HFN) is known to present RGD and PHSRN sequences with synergistic action responsible for substantial modulation of the biological activity subsequent to protein adsorption processes (Romberger 1997). Fibronectin has been considered for some time to facilitate and precede cell attachment to synthetic and natural surfaces when adsorbed in a favourable conformation (Potts & Campbell 1996), while human serum albumin (HSA) was selected considering its high concentration in the human blood plasma (Jenney & Anderson 2000). It is therefore likely that the results obtained for HSA and HFN will be representative of a large number of serum proteins. Moreover, authors have reported the ability of albumin to 'rescue' fibronectin molecules influencing its molecular conformation on hydrophobic surfaces (Grinnell & Feld 1981), which was also reported to facilitate cell adhesion-dependent processes (Lewandowska *et al.* 1989).

Starch-based biomaterials (SBBs) have been increasingly studied for applications in the field of tissue engineering, including their use as scaffolds for bone-related applications (Oliveira *et al.* 2002) and drug-delivery systems (Elvira *et al.* 2002). The SBBs selected are known to present different properties in terms of bulk chemistry, surface energy, wettability and phase interconnectivity (Alves *et al.* 2003, 2007; Pashkuleva *et al.* 2005, 2008). As part of previous research, their *in vitro* (Marques *et al.* 2002) and *in vivo* biological response (Marques *et al.* 2005) has been assessed. In these studies, the effect of the adsorption of different molecules onto the surface of SBBs was evaluated and the behaviour of immunological cells (Alves *et al.* 2003) and osteoblast-like cells (Alves *et al.* 2007) was investigated as a function of the pre-adsorbed proteins. Previous, *in vitro* studies have indicated that detectable amounts of proteins adsorb to SBB surfaces. Yet, the adsorption behaviour of biomolecules relevant for the cell–biomaterial interactions has yet to be understood (Alves *et al.* 2003). In the biomaterials field, it is important to know the protein concentration on the surfaces intended for different applications to engineer for functional performance and longevity. The similarity of these materials with other natural or natural-based material can motivate the development of strategies towards the control and availability of cell signalling epitopes for a family of materials that constitute a significant component of this field of research. To this

end, this quantitative approach aimed to determine further the relationship between substrate properties and protein adsorption kinetics and competition using fluorimetry and laser scanning confocal microscopy (LSCM) in both single and multiple protein adsorption studies. *In vitro* and *in vivo*, the cellular interaction was assessed for the adsorptive potential of HSA and HFN onto SBBs using single molecule solutions and then further for the competitive effectiveness of fibronectin against albumin using binary mixtures that were proportionally equivalent to the amounts of both proteins in human blood plasma. Analysis determined the composition of the proteinaceous layer on materials and the dynamics of deposition and competition of key modulators of the response to biomaterials and valuable therapeutic candidates.

2. MATERIAL AND METHODS

2.1. Materials and chemicals

BSA-Alexa Fluor 488 conjugates were obtained from Molecular Probes (The Netherlands) and purified HSA and HFN were purchased from Sigma-Aldrich (UK). Alexa Fluor 488 and Alexa Fluor 555 Protein Labelling Kits (Molecular Probes, The Netherlands) were used to fluorescently label proteins of human origin. The labelling procedure was conducted following the manufacturer's instructions, using Bio-Rad BioGel P-30 fine size exclusion purification resin to separate the uncoupled dye from labelled proteins. All aqueous solutions were prepared using purified deionized water and phosphate-buffered saline (PBS, Sigma-Aldrich, UK). Clear-bottomed black 24-well plates were used for fluorescence measurements (Biosera, UK) and tissue culture polystyrene (TCPS) coverslips were used as the reference surfaces (Sarstedt, UK).

2.2. Starch-based biomaterials

Biodegradable polymeric blends of corn starch with: (i) cellulose acetate (SCA), (ii) ethylene vinyl alcohol copolymer (SEVA-C) and (iii) polycaprolactone (SPCL) were investigated. The amount of starch was 50 per cent by weight (wt%) on SCA and SEVA-C and 30 wt% on SPCL. Ten millimetre disc samples were prepared by injection moulding and then sterilized by ethylene oxide in optimized conditions (Reis *et al.* 1997).

2.3. Protein adsorption studies

The physiological proportions of albumin and fibronectin in the human serum were matched for the preparation of the different solutions according to the published literature: $35 \, \text{mg ml}^{-1}$ of albumin to $0.4 \, \text{mg ml}^{-1}$ of fibronectin (Jenney & Anderson 2000). Adopting this approach allowed the data to be related to the analysis of the *in vivo* biological response to implanted surfaces.

A commercially available BSA-Alexa Fluor 488 conjugate was used as the model for adsorption studies and also to provide a reference control for the in-house conjugated human proteins. The fluorescent conjugation of HSA and HFN was conducted according to the

manufacturer's specifications. In brief, HSA and HFN protein solutions at a concentration of 2 mg ml^{-1} in standard PBS were mixed with 50 µl of sodium bicarbonate solution and allowed to react with Alexa Fluor dye for 1 h at room temperature. Subsequently, labelled proteins were separated from the unincorporated dye using fine size exclusion chromatography. The concentration and degree of labelling (moles of dye per mole of protein) were calculated following the supplier's instructions. UV-visible spectra of protein–dye conjugates (Bio-Tek Instruments, KC4 Data Analysis Software, USA) were used and the degree of labelling was calculated: 4.0 for HSA and 2.9 for HFN. Labelled proteins were stored at 4°C and used within one week of preparation. Probe-labelled proteins were used to study the surface concentration and distribution by fluorimetry and LSCM. HSA was labelled with Alexa Fluor 488 and HFN was labelled with Alexa Fluor 555, except for the confocal image analysis of HFN adsorption from unitary protein solutions, which was performed using Alexa Fluor 488 labelling.

2.3.1. Study of unitary protein systems. BSA was used for kinetic and isotherm studies. Adsorption studies were performed for the surface of SBBs using commercially available BSA–Alexa 488 conjugates, which were also utilized as the control for the experiments using proteins of human origin. The following solutions of BSA conjugates were prepared: 35, 70, 105, 140 and 175 µg ml^{-1} (pH 7.4), which corresponded to 0.1, 0.2, 0.3, 0.4 and 0.5 per cent (W/V) of albumin concentration in blood (Jenney & Anderson 2000).

Human protein adsorption onto the surface of SBBs was studied using purified HSA labelled with Alexa Fluor 488 and HFN labelled with Alexa Fluor 555. HSA–probe conjugates were studied at two different concentrations, 35 and 70 µg ml^{-1}, while HFN–Alexa Fluor 555 solutions were prepared at 0.4 and 0.8 µg ml^{-1} in PBS.

Saline solution was used as the control. Proteins and controls were incubated with samples for different time periods: 15, 30, 60, 120, 180, 240 and 420 min at 37°C. Samples were incubated in plates and made to adhere to the bottom of the wells. For each time point, protein solutions were removed, rinsed with ddH$_2$O and measurements/observations performed using the top surface of the samples. Experiments were performed at least three times under the same conditions and in triplicate ($n > 9$).

2.3.2. Protein competition: study of binary protein systems. Competition of proteins for the different starch-based surfaces was analysed using two-protein solutions: HSA and HFN fluorescently labelled with Alexa Fluor 488 and Alexa Fluor 555, respectively. Labelling and characterization of the conjugates were accomplished as described previously. A mixture of 70 µg ml^{-1} of HSA conjugate and 0.8 µg ml^{-1} of HFN conjugated with Alexa Fluor probes was used. The described solution corresponds to 0.2 per cent (W/V) of these molecules in human blood serum. Saline solution was used as the control. Proteins and controls

were incubated with samples for different time periods: 15, 30, 60, 120, 180, 240 and 420 min at 37°C. As for unitary protein systems, binary protein adsorption studies were conducted in triplicate and repeated at least three times ($n > 9$).

2.4. Fluorimetry

Samples were incubated as described for the analysis of protein adsorption. For each time point, fluorescence measurements were taken according to the conjugate spectroscopic properties using a Microplate Fluorescence Reader (Version FLx800T, Bio-Tek Instruments, USA). Measurements were automated for top probe detection, using one or two filter sets (488 and 555 nm) to assess labelled biomolecule kinetics and competitive adsorption. Acquired data were analysed using KCJunior Software (v. 1.31.5, Bio-Tek Instruments, USA).

Fluorimetry results were calibrated by assessing other contributions to the total fluorescence intensity. Background fluorescence was subtracted and the emission of SBB surfaces at the different wavelengths studied was analysed. Briefly, the different surfaces were incubated in protein-free PBS solution and the fluorescence intensities measured over time were corrected to rule out artefacts, such as absorption interferences from the polymer materials. The limit of detection, determined using an empirical approach, showed good sensitivity and low detection limits. Standard curves were prepared for the different tagged proteins by plotting increasing concentrations of tagged protein versus fluorescence. Protein adsorption data were presented as the arithmetic means/standard deviations of the mean (mean/SD). Finally, for each time point, samples were rinsed using ddH$_2$O and fluorescence was determined and represented against protein concentration.

2.5. Laser scanning confocal microscopy

A confocal laser scanning microscope (LSM 510 Zeiss, UK) was used to visualize the fluorescently labelled proteins adsorbed on the different starch-based materials. Alexa 488 probe was used in single-protein studies to label HSA and HFN molecules. For the study of binary protein systems, HSA and HFN were labelled with Alexa 488 and Alexa 555 probes, respectively. An argon laser ($\lambda = 488$ nm) and a HeNe laser ($\lambda = 543$ nm) provided the excitation of the protein–probe conjugates. Image analysis was performed using KS400 image analysis software (Imaging Associates, UK) to quantify protein distribution.

3. RESULTS AND DISCUSSION

3.1. BSA adsorption

BSA adsorption per unit area was higher for SCA and SPCL polymeric blends than for SEVA-C surfaces (figure 1). After 7 h, SCA and SPCL adsorbed 27 and 40 µg cm^{-2} of protein from the more concentrated solution of BSA–Alexa 455 (175 µg ml^{-1}), and SEVA-C adsorbed 13 µg cm^{-2}. Adsorption onto TCPS was substantially reduced when compared with the SBB
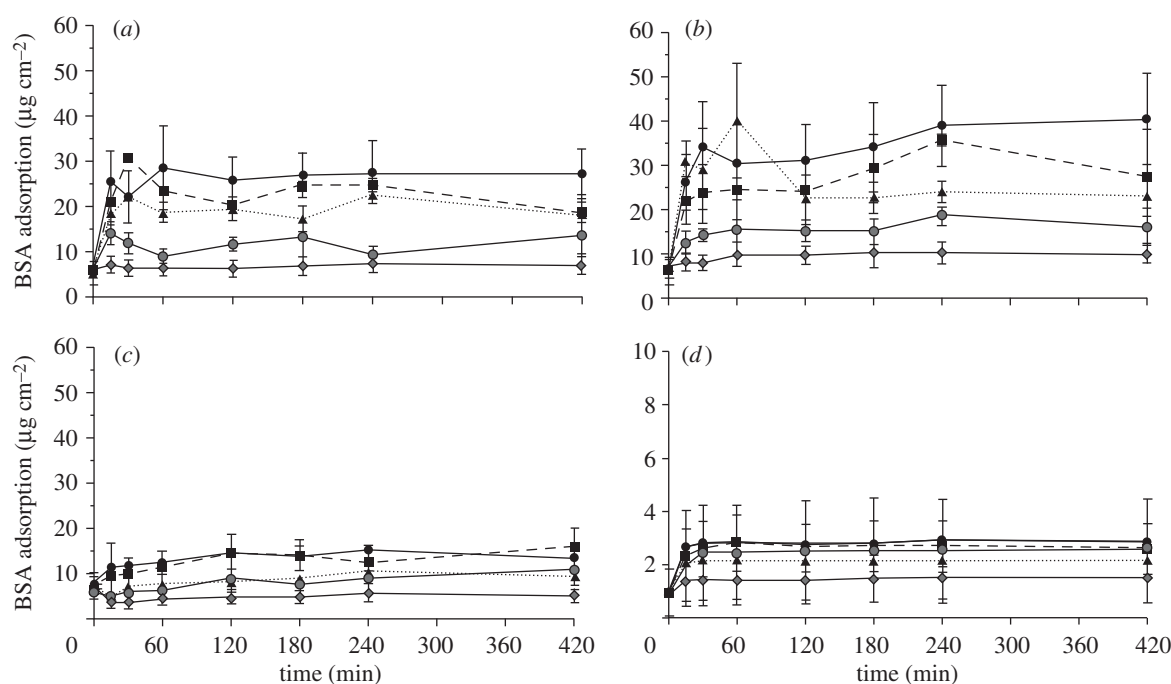
Figure 1. BSA adsorption onto (*a*) SCA, (*b*) SPCL, (*c*) SEVA-C and (*d*) TCPS surfaces. The different samples were incubated for 7 h in solutions of BSA ranging from 35 to 175 $\mu g\,ml^{-1}$. Values are means $\pm$ s.d. ($n = 5$). Grey squares, 30 $\mu g\,ml^{-1}$ BSA; grey circles, 70 $\mu g\,ml^{-1}$ BSA; black triangles, 105 $\mu g\,ml^{-1}$ BSA; black squares, 140 $\mu g\,ml^{-1}$ BSA; black circles, 175 $\mu g\,ml^{-1}$ BSA.

surfaces. In addition, SCA and SPCL profiles (figure 1*a,b*) indicated a fast burst of adsorption over the first 60 min of incubation, after which a pseudo-steady level was maintained. This indicated that adsorption could reach equilibrium within 60 min. In contrast, the shape of the kinetic curves indicated a slower adsorption of this molecule onto the SEVA-C surfaces (figure 1*c*).

The different amounts of adsorbed BSA and the shapes of the curves could be indicative of the heterogeneity characteristic of these different polymeric blends. The heterogeneity of the materials is known to vary in the order SCA > SPCL > SEVA-C as a consequence of increasing miscibility and interaction between the synthetic and natural polymeric phases (Alves *et al.* 2003). SCA is a non-miscible blend, while SPCL presents some interaction between the different phases. SEVA-C consists of the most homogeneous mixture presenting inter-penetrating networks (IPNs). Nevertheless, SEVA-C and SPCL present a similar surface roughness and, thus, similar surface area when compared with SCA polymeric blends (data not shown). In this sense, the fast increase in protein adsorption observed for SPCL (figure 1*b*) and the smoother slope obtained for SEVA-C samples (figure 1*c*) cannot be clarified based on roughness characteristics. Nevertheless, it is known that IPN mixtures can decrease the reorientation of the polymer chains at the liquid interface and slow down the response of the outermost region of the material to the environment, which could explain the distinct curve observed for SEVA-C from those obtained for SCA and SPCL.

The amounts of BSA adsorbed onto SCA, SPCL and TCPS at 60 min presented no obvious difference from that at 420 min. Figure 2 shows the adsorption isotherms calculated from the data obtained at 420 min. The results indicate that adsorption was strongly dependent on the bulk concentration of the protein solutions; the adsorbed amount gradually increased with the protein solution concentration. These observations may indicate that the HSA concentrations selected for this study did not fully cover or saturate the surface and that adsorption points were probably still available. The adsorption isotherms (figure 2) further indicated the affinity of BSA for the different surfaces; SPCL and SCA demonstrated the highest adsorption for increasing concentrations of BSA solutions. After 7 h of incubation with solutions of 35 and 175 $\mu g\,ml^{-1}$ of BSA, adsorption onto SCA and SPCL increased, respectively, to 20 and 30 $\mu g\,cm^{-2}$, while for SEVA-C this variation was around 8 $\mu g\,cm^{-2}$.

In previous studies performed to characterize the different polymeric blends, both wettability and surface energy were analysed. Contact angles were shown to progressively decline with increasing content of OH groups, indicating SEVA-C > SPCL > SCA ranking in terms of hydrophobicity, with contact angles of $80.0 \pm 2.6°$, $78.80 \pm 1.7°$ and $76.4 \pm 3.3°$, respectively ($p < 0.05$ for the comparison of SEVA-C and SCA contact angles) (Alves *et al.* 2007). The contact angle variation between the different materials was low for all surfaces and values were above 65°, which is considered to be the theoretical limit between hydrophobic and hydrophilic properties (Vogler 1998). Moreover, SEVA-C presented the lowest value for surface energy ($36.2 \pm 1.8\,Dyn\,cm^{-1}$) compared with SCA and SPCL (43.0 ± 2.7 and $46.9 \pm 1.0\,Dyn\,cm^{-1}$, respectively) (Alves *et al.* 2007). Previous research can be contradictory, concerning the effect of wettability on the adsorption of different molecules. Some authors have reported increased protein adsorption
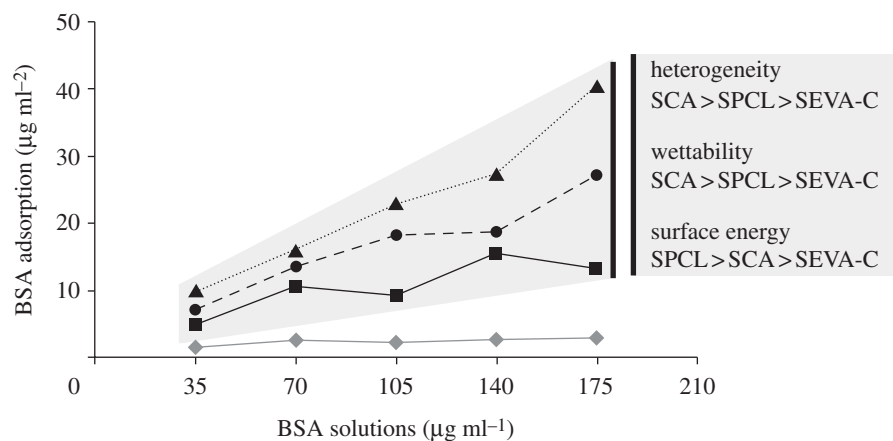
Figure 2. Isotherms of BSA adsorbed onto the different starch-based polymeric blends (equilibration time 7 h). Black circles, SCA; black squares, SEVA-C; black triangles, SPCL; grey triangles, TCPS.

onto hydrophilic substrates (Gessner *et al.* 2000), whereas the majority have found that proteins tended to adsorb more extensively onto hydrophobic surfaces (Nakanishi *et al.* 2001; Liu *et al.* 2006) such as SBBs. Surface energy has also been related to the affinity of proteins to surfaces, although there have been publications indicative of increasing surface energy leading to lower adsorption (Janocha *et al.* 2001). The results of surface energy previously obtained for SBB surfaces are in good agreement with published data. Michiardi *et al.* (2007) compared surface energy and protein adsorption behaviour for untreated and oxidized surfaces and showed that higher surface energy triggered albumin and fibronectin adsorption. Similarly, studies with PDLLA indicated an increase in albumin adsorption onto treated surfaces of higher surface energy (Alves *et al.* 2008). Considering previous results, it can be stated that these factors favoured higher levels of BSA adsorption: heterogeneity, wettability and surface energy, which characterized the different polymeric blends.

3.2. BSA adsorption isotherms: Langmuir and Freundlich models

The construction of adsorption isotherms is an important starting point towards characterizing the interactions between surfaces and proteins. To develop the fundamental understanding of protein adsorption, two typical adsorption models with the same degree of freedom (Andrade & Vanwagenen 1983), Langmuir and Freundlich isotherms (Docoslis *et al.* 1999), were used. The applicability of models having the same degree of freedom is known to provide theoretical insight rather than a mere comparison of model fitting (Maurya & Mittal 2006). The construction of the Langmuir isotherm provided two important pieces of information: the value of the maximum attainable concentration of bound protein in a monolayer formation and the equilibrium constant for the adsorption. The linear Langmuir equation can be expressed as

$$\frac{C}{Q} = \frac{1}{Q_m} C + \frac{1}{bQ_m}, \qquad (3.1)$$

where C is the BSA concentration at a certain time, Q and Q_m are the adsorption amounts for BSA at a certain time and the maximal adsorption amount, respectively, and b is Langmuir's equilibrium constant that describes the strength of interaction between the protein and the surface (Liu *et al.* 2006). The Langmuir isotherm was used to calculate b by plotting the adsorbed fraction versus the initial concentration. Finally, the slope of the isotherm curve, at zero concentration, represents the constant value (Docoslis *et al.* 1999).

The equilibrium adsorption constant can be determined by different methods. Other models such as the Freundlich model are more suitable for use with heterogeneous surfaces, but can only describe adsorption data over a restricted range (Ng *et al.* 2002)

$$\ln Q = \frac{1}{n}\ln C + \ln K. \qquad (3.2)$$

In this case, C, Q and Q_m are the same as in equation (3.1); n and K are constants at a specific condition. The constant K is a measure of the capacity of the adsorption and n is a measure of the intensity of adsorption (Liu *et al.* 2006; Bajpai 2007).

In the lower concentration range, changes in the bulk concentration produced large changes in the amount of protein adsorbed, resulting in a roughly linear increase in adsorption. However, as the bulk concentration is increased, adsorption is reduced and a plateau or a maximum adsorption level is reached. This type of adsorption behaviour is referred to as a Langmuir isotherm. In other cases, the increase in adsorption at high bulk concentration does not stop entirely, but presents a slow rise. This type of adsorption behaviour is referred to as a Freundlich isotherm.

The fit of the different models to the experimental data was analysed taking into account the coefficient of determination (r^2, table 1). All surfaces, except TCPS, showed a higher correlation fit for Freundlich (figure 3*b*) than for Langmuir isotherms (figure 3*a*). The fact that albumin adsorption onto TCPS surfaces was reduced could decrease protein–protein interactions and result in a better fit to the Langmuir model.
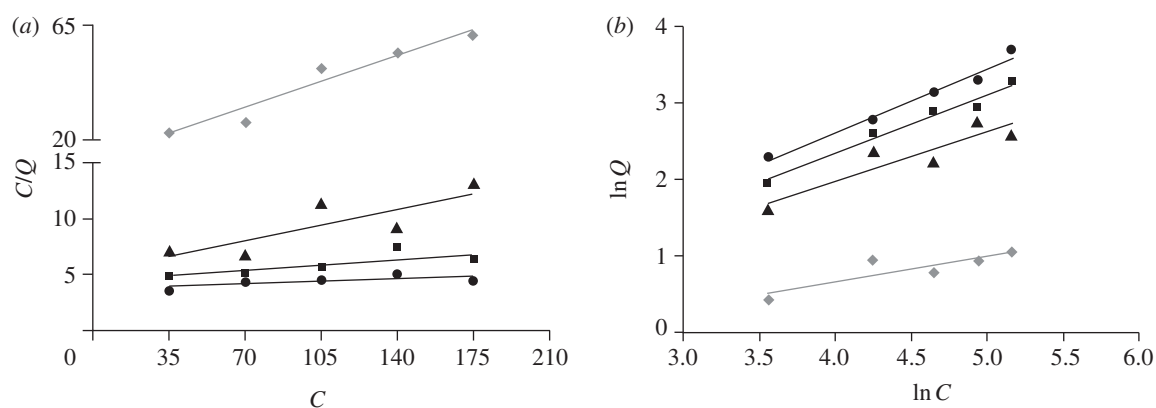
Figure 3. (*a*) Langmuir and (*b*) Freundlich fitting curves. Black squares, SCA; black triangles, SEVA-C; black circles, SPCL; grey squares, TCPS.

Table 1. Langmuir and Freundlich parameters for BSA adsorption onto starch-based materials.

materials	Langmuir parameters			Freundlich parameters		
	r^2	Q_m $(\mathrm{mg\,g^{-1}})$	b $(\mathrm{l\,mg^{-1}})$	r^2	n	K $(\mathrm{l\,g^{-1}})$
SCA	0.6538	66.14	0.01516	0.9651	1.27	0.46
SEVA-C	0.6678	24.19	0.00472	0.8263	1.58	0.57
SPCL	0.4276	144.90	0.03963	0.9757	1.21	0.50
TCPS	0.9446	3.36	0.00029	0.7595	2.94	0.49

The process of BSA adsorption onto SCA, SEVA-C and SPCL was more complicated than that of ideal adsorption. The interactions between adsorbed BSA molecules and the type of surface played an essential role in influencing these results. It is known that the Langmuir model disregards interactions between adsorbed proteins. Clearly, if there were no interactions among adsorbed BSA molecules, the Langmuir fit for SBB surfaces would be characterized by a higher r^2. Similarly, if the different surfaces did not affect the interaction, the correlation between both models should be the same. In previous studies, the deviation from the Langmuirean behaviour was explained on the basis of 'cooperativity' among the binding sites from evidence of multiple-dependent adsorption sites (Hutchens *et al.* 1988; Hutchens & Yip 1990).

The Freundlich isotherm, empirical in origin, is suitable for use with heterogeneous surfaces. This model can fit many adsorption processes, considering further the dynamics of protein adsorption, while taking into account interactions between adsorbed molecules or between adsorbed molecules and the surfaces studied (Liu *et al.* 2006). This could explain the improved correlations for the Freundlich fit for SBB surfaces. Freundlich's n parameter correlates with the averaged energies of adsorption; the lower the n the higher the affinity between solid and adsorbates (Robert *et al.* 2000). Using the model BSA protein, Freundlich's n (table 1) parameter obtained by isotherm studies (figure 2) evidenced higher adsorption affinity for SPCL and SCA.

3.3. Adsorption of human fibronectin and human serum albumin

The adsorption of Alexa Fluor 488-labelled HSA onto SBB surfaces was measured as a function of the incubation time (figure 4). The results obtained for HSA were similar to those presented for BSA. For SCA and SPCL, HSA curves presented similar shapes to that of BSA and showed an initial fast adsorption that was reduced to a semi-steady state after 60 min. SEVA-C showed a gradual increase in HSA adsorption and did not show a defined plateau over the time period and concentration studied, suggesting that maximum surface coverage was not reached. The increase in protein bulk concentration from 35 to 70 μg ml^{-1} induced an increase in adsorption. After 7 h, SCA and SPCL adsorbed around 16 and 21 μg cm^{-2} of protein when using the more concentrated solution of HSA (70 μg ml^{-1}), while SEVA-C adsorption was 8 and 3 μg cm^{-2} onto TCPS. In terms of percentage of adsorption, SPCL, SCA, SEVA-C and TCPS adsorbed 24, 18, 9 and 3 per cent (W/W), respectively, after incubation in 70 μg ml^{-1} of HSA.

In addition, protein coverage of the different surfaces was visualized (figure 5) and demonstrated heterogeneous HSA distribution on SCA, SPCL and SEVA-C surfaces.

HFN adsorption curves for all materials, except SCA (figure 6), were similar to HSA, demonstrating that the molecule species did not affect the kinetics adsorption onto SEVA-C, SPCL and TCPS.

Different from the trend observed for HSA adsorption, higher levels of HFN adsorption were obtained for SEVA-C and SPCL polymeric blends. After 7 h of incubation in 0.8 μg ml^{-1} solution, SPCL and SEVA-C adsorbed approximately 1.12 and 1.02 μg cm^{-2} of HFN. In addition, fibronectin adsorption onto SCA was lower and reached values of around 0.41 μg cm^{-2}, whereas TCPS adsorption was 0.08 μg cm^{-2}. In terms of percentage of adsorption, SPCL, SEVA-C, SCA and TCPS adsorbed 97, 89, 36 and 7 per cent (W/W), respectively, after incubation in 0.8 μg ml^{-1} of HFN.

Confocal microscopy was demonstrated to be applicable for protein observation (figure 7) as this supported the fluorescence measurements that demonstrated higher intensities for SPCL and SEVA-C polymeric blends.
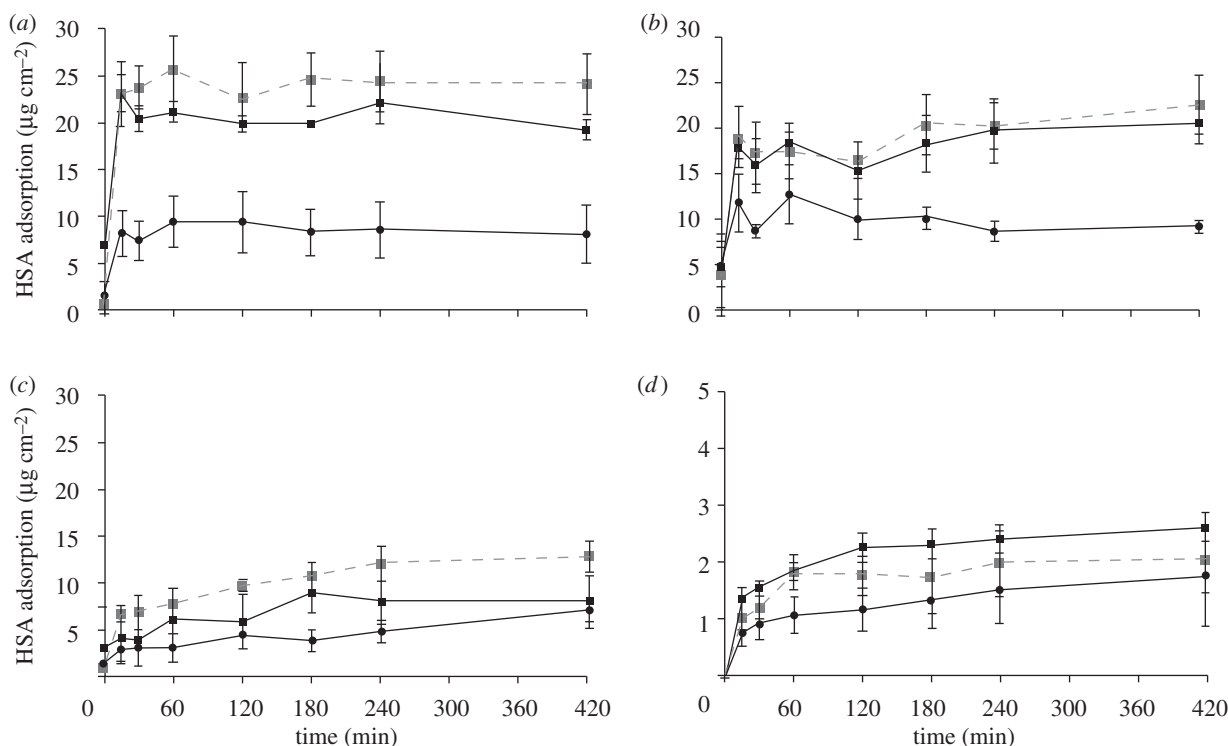
Figure 4. Adsorption of HSA under single and competitive conditions on the surface of (*a*) SCA, (*b*) SPCL, (*c*) SEVA-C and (*d*) TCPS. Continuous lines (black) correspond to unitary protein systems of 35 and 140 μg ml^{-1} of HSA and the dashed line (grey) represents the adsorption of HSA in the presence of 0.8 μg ml^{-1} of fibronectin. Values are means $\pm$ s.d. ($n = 3$). (*d*) Black circles, 35 μg ml^{-1} of HSA (unitary); black squares, 70 μg ml^{-1} of HSA (unitary); grey squares, 70 μg ml^{-1} of HSA (binary).
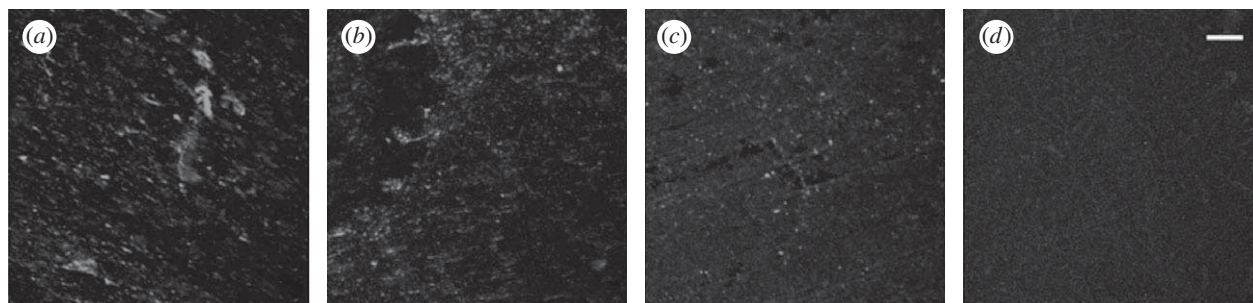

Figure 5. Confocal images of HSA−Alexa 488 adsorbed onto the surface of (*a*) SCA, (*b*) SPCL, (*c*) SEVA-C and (*d*) TCPS after 7 h incubation in 70 μg ml^{-1} of HSA−Alexa Fluor 488. Scale bar, 200 μm.

Moreover, the adsorption of HSA and HFN was in good agreement with a previously developed qualitative *in vitro* study that determined this by immunostaining and revealed higher HFN adsorption onto SEVA-C and SPCL surfaces than HSA, regardless of the higher bulk concentration of this last protein (Alves *et al.* 2003). An important result of the present study was the confirmation that SPCL surfaces presented the highest affinity for all the molecules studied. Moreover, the reduced adsorption of albumin onto SEVA-C indicated low levels of non-specific protein adsorption onto this blend when compared with SPCL and SCA surfaces.

Another relevant aspect of this study was the indication that the different proteins did not affect the adsorption curves. In fact, using BSA, HSA or HFN resulted in a similar curve shape for the same SBB surface. In contrast, adsorption onto SBBs was clearly surface regulated.

3.4. Protein behaviour in competitive conditions: binary systems

The single adsorption studies were investigated further in two additional experiments: (i) adsorption of HSA− 488 Alexa Fluor from a solution containing unlabelled HFN and (ii) adsorption of HFN−555 Alexa Fluor from a binary solution containing unlabelled HSA. The weight ratio of fibronectin to albumin was approximately 1 : 90, typical of that found in normal human blood serum (Jenney & Anderson 2000). The adsorption of albumin and fibronectin from binary mixtures
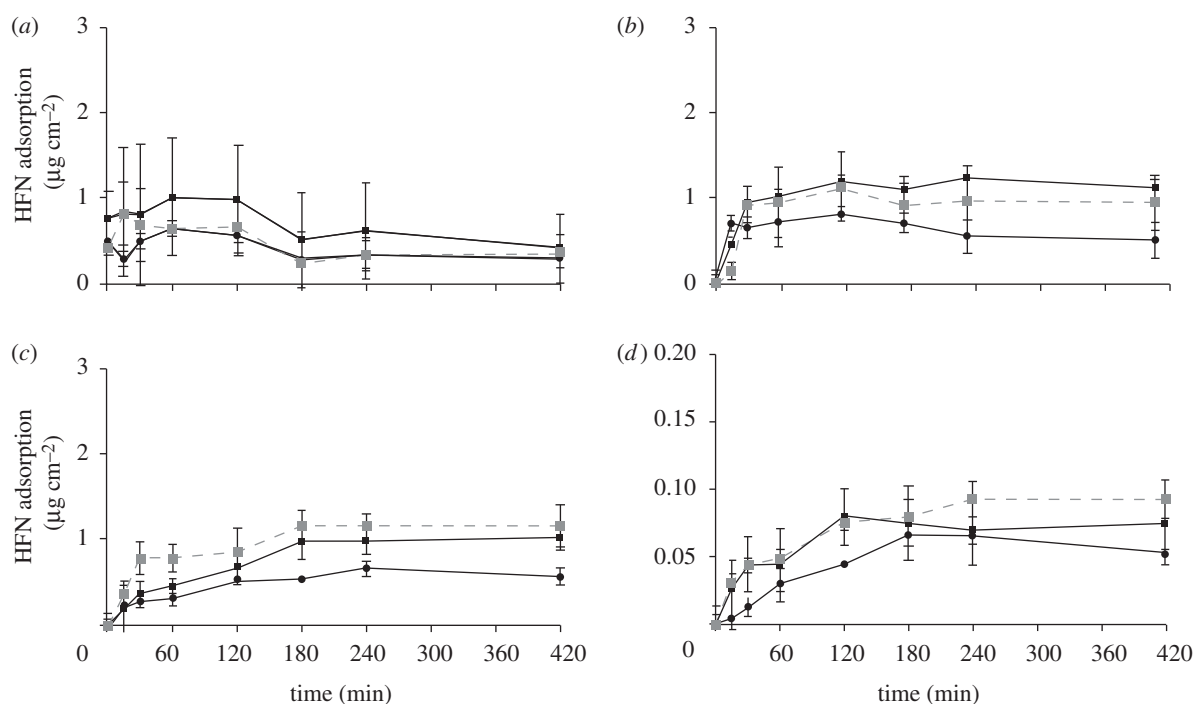
Figure 6. Adsorption of HFN under single and competition conditions on the surface of (*a*) SCA, (*b*) SPCL, (*c*) SEVA-C and (*d*) TCPS. Continuous lines (black) correspond to unitary protein systems of 0.4 and 0.8 $\mu g\,ml^{-1}$ of HFN and the dashed line (grey) represents the adsorption of HFN in the presence of 70 $\mu g\,ml^{-1}$ of HSA. Values are means $\pm$ s.d. ($n = 3$). (*d*) Black circles, 0.4 $\mu g\,ml^{-1}$ of HFN (unitary); black squares, 0.8 $\mu g\,ml^{-1}$ of HFN (unitary); grey squares, 0.8 $\mu g\,ml^{-1}$ of HFN (binary).
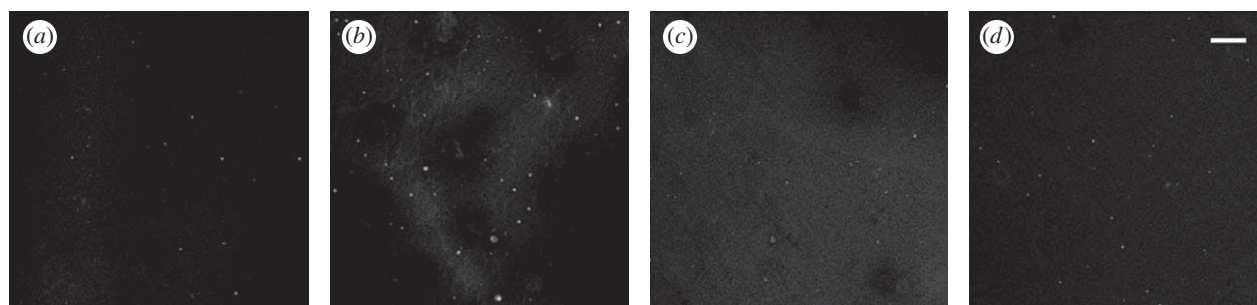

Figure 7. Confocal images of HFN–Alexa 488 adsorbed onto the surface of (*a*) SCA, (*b*) SPCL, (*c*) SEVA-C and (*d*) TCPS after 7 h incubation in 0.8 $\mu g\,ml^{-1}$ of HFN–Alexa Fluor 488. Scale bar, 200 μm.

was investigated as a function of time, indicated by the dashed lines in figures 4 and 6. In addition, the adsorption of molecules from HSA/HFN binary systems was assessed by LSCM (figure 8).

In competitive conditions for some of the SBB surfaces, a clear difference in HFN/HSA adsorption was determined for the single-protein solution versus the binary mixtures containing HFN and HSA. On the SCA surfaces, the adsorption of HSA was higher in competitive conditions (figure 6*a*) than in unitary systems. Furthermore, in HSA/HFN solutions, albumin substantially decreased fibronectin adsorption. The outcome of the competitive behaviour onto the SCA surface has been previously observed using other proteins. Brash's group (Cornelius & Brash 1997) showed a significant reduction in fibrinogen adsorption on different surfaces when high-molecular-weight

kininogen was co-adsorbed. In this study, results indicated that the presence of HSA had a negative effect on the level of adsorbed HFN. This effect could be brought about by the synergy of two mechanisms: first, by displacing HFN from the surface owing to the high affinity of HSA for this surface, and, second, by a molecular weight and concentration effect favourable for the adsorption of HSA. In contrast to SCA, the fluorescence intensities for single versus binary solutions indicated that adsorbed HFN was not displaced by albumin for the SPCL interface, even at much higher bulk concentrations of albumin. There was little increase or decrease in the fluorescence of both proteins, suggesting that, while there may be adsorption/desorption of the same species, there was no visible change in the total quantity of HFN at the interfaces (figures 4*b* and 6*b*). Finally, HSA
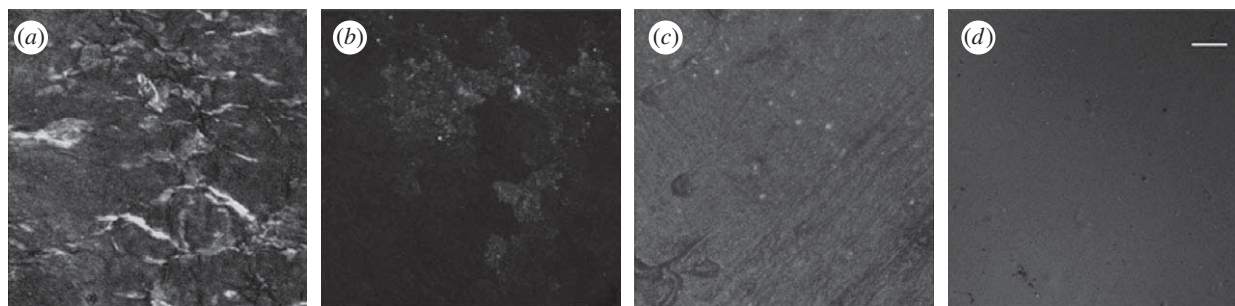
Figure 8. Confocal images of HSA/HFN adsorbed onto the surface of (*a*) SCA, (*b*) SPCL, (*c*) SEVA-C and (*d*) TCPS after 7 h incubation in binary solutions of 70 $\mu g\, ml^{-1}$ of HSA–Alexa Fluor 488 and 0.8 $\mu g\, ml^{-1}$ of HFN–Alexa Fluor 555. Scale bar, 200 μm.

adsorption on SEVA-C from single-protein solutions as well as from competitive systems was lower than that of the SCA and SPCL (figure 4*c*), while HFN reached the highest adsorption percentages (figure 6*c*). These surfaces were demonstrated to be highly resistant to non-specific protein adsorption from single albumin solutions. For binary systems (dashed lines), an increase in both HSA and HFN adsorption for SEVA-C surfaces was observed. Modulation of the biological activity of adsorbed fibronectin has been shown in several studies in which the ability of fibronectin adsorbed to various surfaces to support cell attachment or spreading was found to differ. Grinnell's group showed that fibronectin biological activity was strongly affected by the type of surface to which it was adsorbed as well as by whether albumin was co-adsorbed (Grinnell & Feld 1981, 1982). In these studies, fibronectin adsorbed to tissue culture grade polystyrene was able to support cell attachment and spreading, whereas fibronectin adsorbed to ordinary polystyrene did not support spreading unless some albumin was added to the fibronectin solution. Supplementing BSA to 2 $\mu g\, ml^{-1}$ fibronectin solutions was reported to enhance the fibronectin binding to both bacteriological and tissue culture grade polystyrene (Sukenik *et al.* 1990). In addition, Lewandowska *et al.* (1989) demonstrated that the conformation of fibronectin molecules can vary because of the chemical nature of the substrates. On hydrophobic surfaces pre-adsorbed with fibronectin, cell spreading was improved only when albumin was co-adsorbed, so-called 'albumin rescuing' (Lewandowska *et al.* 1989). The 'rescuing' phenomenon is similar to the effect Grinnell reported on polystyrene. This could explain the increase in HFN adsorption observed in binary conditions onto the SEVA-C and TCPS surfaces. In a later extension of that work, BSA mediated the modulation of the ability of the adsorbed fibronectin to induce neurite formation in neuroblastoma cells (Sukenik *et al.* 1990). The authors concluded that the cellular response was altered owing to a change in the conformation of fibronectin molecules as they interacted with the different chemical end groups. This rearrangement of fibronectin onto the surfaces indirectly detected by the analysis of the cellular response could also hinder the increased adsorbed amount of this protein determined in this study by fluorescence measurements.

The different natural-based surfaces were shown to provide different interactions with the proteins, which can control cellular function and ultimately explain the observed cell behaviour to starch-based materials.

4. CONCLUSIONS

Protein adsorption is an important question to address from a theoretical and biotechnological research viewpoint and is of great practical medical relevance. Our current analysis assessed for the first time the interaction between important interface molecules and the surfaces of starch-based materials. Several studies are ongoing applying natural-based materials in the biomedical field. The difficulty of studying polysaccharide-based surfaces creates a lack of detailed information relating to critical events such as the interfacial behaviour of biomolecules. There are many similarities between these materials and other polysaccharide-based materials to which these findings can be related, which enable these findings to be more broadly relevant beyond specifically the materials studied.

In this study, the adsorption isotherms and the competition of plasma proteins on SBBs have been elucidated. It has been determined that for BSA the adsorption onto SBB surfaces was characteristic of a Freundlich type; for SCA and SPCL, the equilibrium of adsorption was achieved within the first hour of incubation, while for SEVA-C the adsorption was slower. Albumin adsorption onto SBBs was affected by the material composition as well as by the concentration of the protein solution, preferentially adsorbing onto SCA and SPCL as evidenced by Freundlich's n parameter. Fibronectin adsorption reached higher values on SEVA-C and SPCL. There was no effect on the adsorption of HSA and HFN onto SPCL under competitive conditions. Fibronectin adsorption was reduced on SCA in the presence of albumin, for which adsorption simultaneously increased, while the opposite situation was observed for TCPS. Competitive conditions were favourable to the affinity of both molecules by improving the affinity of albumin and fibronectin onto SEVA-C surfaces. Fibronectin demonstrated a different adsorption activity for the different materials as assessed by single and competitive adsorption with albumin. This study enabled the assessment

of protein density and distribution that provides valuable information on the analysis of modulation of adsorption for the development of strategies towards the control of protein orientation and thus the availability of their cell signalling epitopes.

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