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Adsorption of serum α-1-microglobulin onto biomaterials

M. SANTIN*, M. CANNAS
Institute of Human Anatomy, Department of Medical Science, University of Turin, Via Solaroli 11, 28100 Novara, Italy

M. A. WASSALL, S. P. DENYER‡
Department of Pharmacy, University of Brighton, Cockcroft Building, Lewes Road, Brighton BN2 4GJ, UK

The adsorption of α-1-microglobulin (α-1-m) from serum to the surface of polymers with different physicochemical properties was investigated. Enzyme-linked immunosorbent assay showed binding of this protein to the surface of polystyrene (PS), polyvinyl chloride (PVC) and a polyurethane, Chronoflex, after water washing, but only trace levels could be detected on two polymethacrylate derivatives, polymethyl methacrylate and poly(2-hydroxyethyl methacrylate). α-1-m was selectively desorbed from the five materials by sequential washes of serum-conditioned surfaces with isopropanol solutions at increasing concentrations. The presence of α-1-m in the washing supernatants was detected by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The relative binding strength of α-1-m to each surface was evaluated as the isopropanol (IsoPOH) concentration required to desorb the protein from that surface. Analysis of bound proteins by SDS-PAGE conclusively demonstrated the binding of a range of serum proteins, including α-1-m, to all polymer systems, but with varying binding strengths. The majority of protein was removed by water washing for the polymethacrylate polymers, while varying concentrations of IsoPOH were required to desorb proteins from PS, PVC and Chronoflex. There was a correlation between the hydrophobic nature of the material, determined by water contact angle measurements, and adsorption of α-1-m. Immunoblotting of isopropanol-eluted proteins by α-1-m antibodies showed the positive staining of a 29 kDa protein as well as selected bands within a molecular weight range of 40–200 kDa, suggesting the adsorption of this protein as both free and complexed forms. The ability of α-1-m to adsorb on to material surfaces and to participate in events relevant to the biocompatibility of a polymer, such as bacterial infection or inflammation control, suggests the need for further characterization of the properties of this protein.

1. Introduction

The implantation of artificial materials in the body triggers an inflammatory response which is the first of a series of events leading to the wound healing process [1]. Implant-related inflammation is induced by the adsorption of proteins such as C3a and C5a fragments of the complement cascade which act as both chemotactic agents and receptors for phagocytes such as neutrophils and macrophages able to secrete hydroxyl radicals and lysosomal enzymes [2]. A massive induction of these products may lead to damage of the material surfaces and of the surrounding tissues with consequential failure in performance of the prosthesis [3]. The vast majority of the studies designed to elucidate the mechanisms of biomaterial-induced inflammation have followed the adsorption of these proteins with a view to understanding their effects on phagocyte activation [4, 5]; little information is available concerning the adsorption of serum proteins able to inhibit inflammatory events.

α-1-m is a 29 kDa glycoprotein produced in the liver [6, 7] which has been found to inhibit *in vitro* granulocyte migration and lymphocyte activation [8]. This protein has been identified in several biological fluids such as serum, urine and cerebrospinal fluid [9, 10] and immunological studies have also demonstrated the presence of the protein in lymphocyte-rich organs and in connective tissue [11, 12]. α-1-m belongs to the lipocalin superfamily, a group of similarly folded proteins carrying hydrophobic prosthetic

*Also: Department of Pharmacy, University of Brighton, Cockcroft Building, Lewes Road, Brighton BN2 4GJ, UK.
‡Author to whom all correspondence should be addressed.

groups [13], and is associated with a yellow–brown chromophore [14]. This protein is also called protein HC (human complex) because half of the plasma-borne material is complexed with IgA [15, 16]; furthermore, α-1-m complexes with human serum albumin, rat plasma fibronectin [17] and α-1-inhibitor [7].

Recently, we reported the adsorption of urine α-1-m to the surfaces of biomaterials for urological applications [18] and related its adsorption to the adhesion of an uropathogen, *Pseudomonas aeruginosa* B4, to urine-treated polystyrene [19].

The purpose of this work was to study the adsorption of serum α-1-m to several biomaterials and to relate amounts bound and binding strength to the physicochemical properties of the polymers used. Enzyme-linked immunosorbent assay, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were employed to detect and characterize α-1-m adsorption.

2. Materials and methods

2.1. Preparation of polymer films

Polystyrene (PS), polymethyl methacrylate (PMMA) and polyvinyl chloride (PVC) (Fluka, Gillingham, Dorset, UK) and a polyurethane, Chronoflex AL-50 (Polymedica, Woburn, MA, USA), were dissolved in chloroform to 5% (wt/vol) solutions. Poly(2-hydroxyethyl methacrylate) (PHEMA; Sigma, Poole, Dorset, UK) was dissolved in 1:1 tetrahydrofuran/65% (vol/vol) ethanol solution to produce a 5% (wt/vol) solution. Polymer films were cast and dried overnight at 37 °C. Discs (7 mm diameter) were cut from the prepared films.

2.2. Preparation of specimens for dynamic contact angle (DCA) analysis

Acid-washed 24 mm square glass coverslips were silanized by immersion in dimethyl dichlorosilane (Fluka) for 5 min, washed three times with acetone and deionized water, dried in air at 65 °C and dip-coated in 0.1% (wt/vol) polymer solution.

2.3. DCA analysis of polymers

Two-cycle DCA analysis was carried out by immersion of the specimens in HPLC-grade water at 10 $\mu m\,s^{-1}$ by a computer-driven DCA Analyser and software (Cahn Instruments, Manchester, UK). Data were expressed as mean ± standard deviation of the advancing, θ_a, and receding, θ_r, contact angle values from six analyses. Statistical analysis was carried out by Student's *t*-test ($P \leq 0.05$).

2.4. Evaluation of adsorbed serum α-1-microglobulin levels by enzyme-linked immunosorbent assay (ELISA)

Serum, pooled from 25 healthy blood donors, was kindly donated by Dr M. H. Wilcox, Clinical Microbiology and Public Health Laboratory, Cambridge, UK. Protein adsorption was carried out by incubation of phosphate-buffered saline (PBS)-equilibrated polymer discs in 200 µl serum for 1 h at 37 °C in 96-well microplates (Dynatech, West Sussex, UK). The specimens were washed three times with 200 µl 0.5% (wt/vol) gelatin solution in phosphate buffered saline; pH 7.3 (PBS) and incubated with 200 µl 1% (wt/vol) gelatin in PBS for 1 h at 37 °C. After three washes, the specimens were incubated with 100 µl 1:800 diluted rabbit anti-human α-1-microglobulin antibodies (Dako, Golstrup, Denmark) in 0.1% (wt/vol) gelatin solution in PBS for 2 h at 37 °C, washed again with 0.5% (wt/vol) gelatin in PBS and finally incubated with goat anti-rabbit IgG antibodies conjugated with horseradish peroxidase (Dako). The specimens were washed five times with 200 µl 0.5% (wt/vol) gelatin in PBS and incubated with 100 µl 0.1 M citric acid, 0.2 M Na_2HPO_4 solution, pH 5.0, containing 0.03% (wt/vol) H_2O_2 and 0.03% (wt/vol) *o*-phenylene diamine for 10 min at room temperature. Finally, the reaction was stopped with 50 µl 20% (wt/vol) sulphuric acid; 100 µl of each sample was transferred to a microplate where the first row of wells were used as blanks. The samples were read at 490 nm by a Biorad Model 520 microplate reader (Biorad, Hemel Hempstead, UK). A standard curve with a linear coefficient of 0.996 was obtained by evaluation of purified urine α-1-microglobulin (Calibiochem, Nottingham, UK) in the range of concentration 0.5–10.0 $\mu g\,ml^{-1}$). Data were calculated as mean ± standard deviation of bound α-1-m ($ng\,mm^{-2}$) from ten analyses, after subtraction of the non-specific antibody adsorption values obtained from control polymer discs not treated with serum. Statistical analysis was carried out by Student's *t*-test ($P \leq 0.05$).

2.5. Evaluation of adsorbed serum α-1-microglobulin binding strength

The binding strength of α-1-m adsorption on polymer surfaces was evaluated by selective elution of the adsorbed protein from the tested materials. After incubation with serum, the discs were washed three times with distilled water and with isopropanol/water solutions at increasing concentration (10%, 30%, 50%, 70% by volume). Each wash was carried out with 200 µl washing medium for 20 min at static conditions. The supernatants were freeze-dried and resuspended in 15 µl SDS-PAGE sample buffer [2% (wt/vol) SDS, 5% (vol/vol) 2-mercaptoethanol, 10% (wt/vol) glycerol and 0.02% (wt/vol) bromophenol blue in 62.5 mM *tris*-HCl; pH 6.8], except for the supernatant from the first water wash, which was dissolved in 50 µl. 10 µl of each sample was loaded on a 10% acrylamide gel and the electrophoresis was carried out at 100 V constant voltage by the Laemmli method [20] using a Miniprotean II system (Biorad, Hemel Hempstead, UK). SDS-PAGE was also carried out on 10 µl 1:100 diluted serum in water. Protein bands were detected by the silver stain procedure [21] using a Biorad Silver Stain Plus kit.

Immunoblots of 1:100 diluted serum and of serum proteins desorbed from water and isopropanol

(IsoPOH)-washed PS were performed by the method of Brunette [22] using rabbit anti-human α-1-m antibodies. Briefly, proteins were transferred on to nitrocellulose membranes using the mini-transblot module (Biorad), the membranes were washed with PBS, incubated for 2 h with 1% (wt/vol) gelatin in PBS, extensively washed with PBS and incubated with rabbit anti-human α-1-m antibodies (50 $\mu g\,ml^{-1}$ in 0.1% (wt/vol) gelatin PBS solution) overnight at room temperature under shaking. The membranes were washed in PBS, incubated with horseradish peroxidase-conjugate goat anti-rabbit IgG antibodies for 1 h at room temperature, washed again in PBS and finally developed in 50 mM *tris* pH 7.6 containing 0.03% (wt/vol) 4-chloro-1-naphthol and 0.03% (vol/vol) H_2O_2 as substrates.

3. Results

3.1. DCA analysis of the polymers

Table I shows the contact angle analysis, expressed as θ_a and θ_r mean ± standard deviation, of the tested polymers. Although θ_a values for PS, PMMA and PVC were not significantly different, θ_r suggested a hydrophobic scale in the order PVC > PS > PMMA > Chronoflex > PHEMA.

TABLE I Dynamic contact analysis of polymers (mean ± standard deviation; $n = 6$)

Polymer	Dynamic contact angle values (deg)	
	Advancing contact angle, θ_a	Receding contact angle, θ_r
PHEMA	78.0 ± 5.0	33.8 ± 0.4
PMMA	91.8 ± 2.0	62.9 ± 3.5
Chronoflex	83.4 ± 1.7	56.1 ± 1.9
PS	94.3 ± 4.3	67.2 ± 2.8
PVC	91.4 ± 1.3	71.3 ± 2.3

3.2. Evaluation of adsorbed serum α-1-microglobulin levels by ELISA

Fig. 1 shows the amounts of serum α-1-microglobulin adsorbed on the tested polymers. Polystyrene encouraged the highest amount of serum α-1-microglobulin sorption (0.75 ± 0.26 $ng\,mm^{-2}$). Polyvinyl chloride and Chronoflex showed lower amounts (0.31 ± 0.13 $ng\,mm^{-2}$ and 0.24 ± 0.07 $ng\,mm^{-2}$ respectively), while only trace amounts of α-1-m adsorption was detected on PHEMA and PMMA.

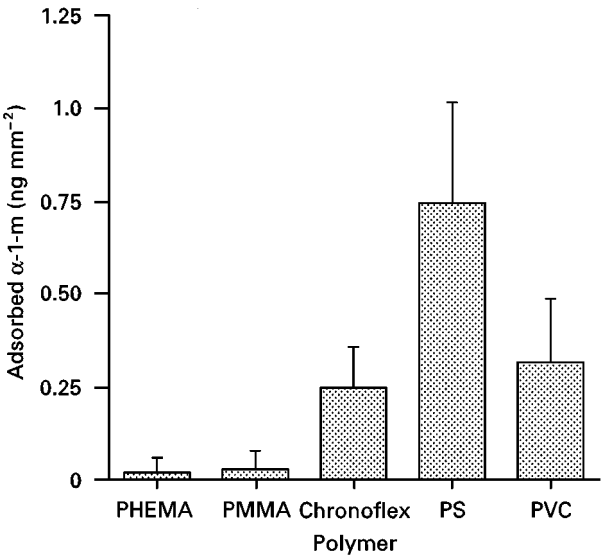

Figure 1 ELISA of serum α-1-microglobulin bound to polymer-coated surfaces. Data expressed as mean ± standard deviation; $n = 6$.

3.3. Evaluation of adsorbed serum α-1-microglobulin binding strength

Fig. 2 shows the electrophoretic pattern of 1:100 diluted serum (lane 1) and the corresponding immunoblotting for α-1-m (lane 2) against the molecular weight standards (lane 3). Positive reaction of α-1-m antibodies was obtained for the band at 29 kDa (lane 2, arrow) and for other bands at higher molecular weight range 40–200 kDa (lane 2, bracket).

The SDS-PAGE protein profile of the supernatants, obtained from washes of serum-conditioned surfaces with water and IsoPOH are shown in Figs 3 and 4. Desorption of 29 kDa α-1-m was detected in the first water wash of all the tested serum-conditioned polymers (Figs 3 and 4, lane 1, arrows). Complete α-1-m desorption took place after two water washes in the case of PHEMA (Fig. 3a, lanes 1 and 2) and with the first water wash in case of PMMA (Fig. 3b, lane 1). Chronoflex (Fig. 4a) released α-1-m in the supernatants of the water washes and with 30% IsoPOH (lanes 1, 2, 3 and 5 arrows). Elution peaks of α-1-m in 30% and 50% IsoPOH wash supernatants were

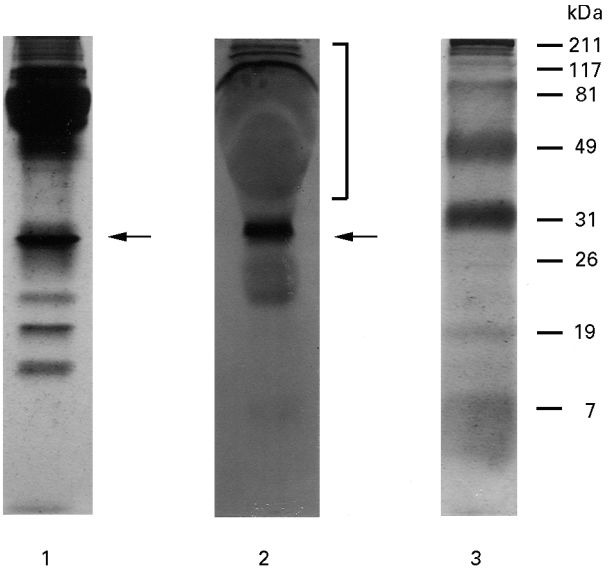

Figure 2 SDS-PAGE (lane 1) and immunoblot (lane 2) of serum α-1-m. Lane 3 shows molecular weight standards. Arrow indicates 29 kDa α-1-m; bracket indicates high molecular weight bands positive to rabbit anti-human α-1-m antibodies.

detected for PS (Fig. 4b, lanes 5 and 6), with a large amount of the protein released in the first and second water washes (lanes 1 and 2) and traces were present after washing of the surface with the third water wash,

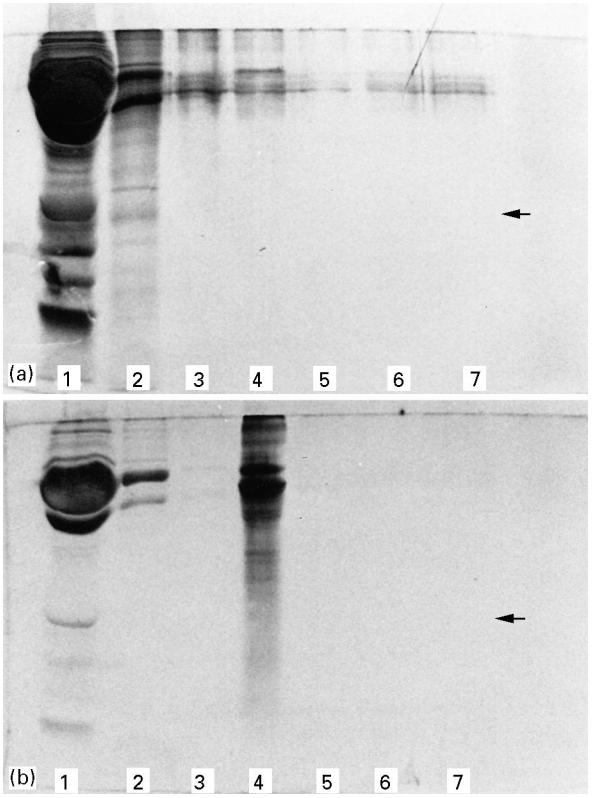

Figure 3 SDS-PAGE of serum α-1-m desorbed from (a) PHEMA and (b) PMMA. Lanes 1–3, water washes; lanes 4–7, 10%, 30%, 50% and 70% IsoPOH washes. Arrow indicates 29 kDa α-1-m.

10% and 70% IsoPOH (lanes 3, 4 and 7). No obvious desorption of 29 kDa α-1-microglobulin was achieved for PVC in the chosen range of IsoPOH concentrations except for a faint band at 70% IsoPOH (Fig. 4c, lane 7).

Fig. 5, lanes 1–3 show α-1-m identification by immunoblotting in 30%, 50% and 70% IsoPOH eluate from serum-conditioned PS. In 30% and 50% IsoPOH supernatants a band at 29 kDa as well as several bands at high molecular weight were found (lanes 1 and 2). It is evident that in 70% IsoPOH washes, no 29 kDa form of α-1-m was present, but a positive reaction to the antibody was still detectable in the 40–200 kDa range (lane 3).

4. Discussion

This study investigated the adsorption of α-1-m to a panel of polymers with different physicochemical properties. Three parameters were studied: the DCA of the materials; the amount of α-1-m adsorbed; and the strength of interaction between protein and surface. Dynamic contact angle force profiles for all the test materials showed an hysterisis loop, indicating chemical surface heterogeneity [23]. The advancing contact angle reflected the interaction of the water with the non-polar regions of the dry surface, while θ_r values were indicative of the polar domains of the wetted surface after immersion in water. The substantially lower θ_r values obtained by DCA over those of the corresponding θ_a, indicate that the surface of the polymer reorientated as it was moved between the air and water environment [24]. While DCA

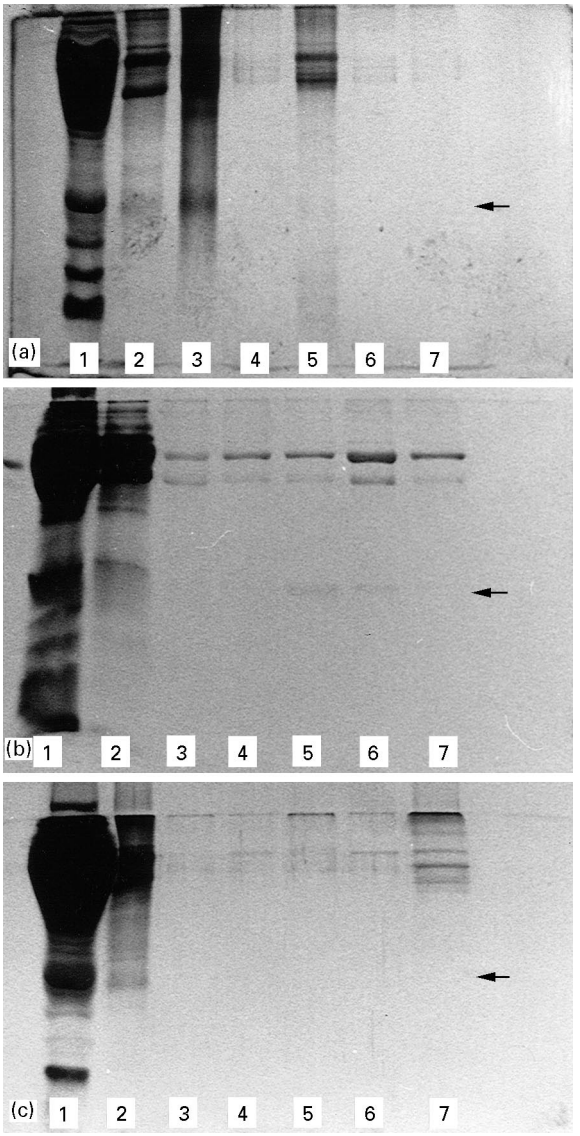

Figure 4 SDS-PAGE of serum α-1-m desorbed from (a) Chronoflex, (b) PS and (c) PVC. Lanes 1–3, water washes; lanes 4–7, 10%, 30%, 50% and 70% IsoPOH washes. Arrow indicates 29 kDa α-1-m.

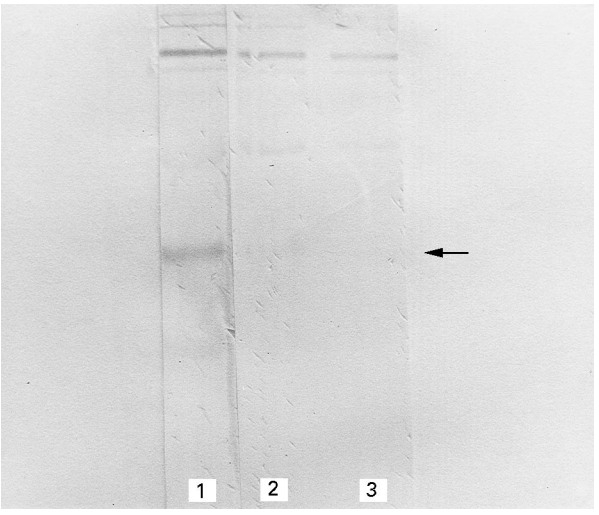

Figure 5 Immunoblot of serum α-1-m desorbed from PS with IsoPOH washes. Lane 1, 30% IsoPOH; lane 2, 50% IsoPOH; lane 3, 70% IsoPOH. Arrow indicates 29 kDa α-1-m.

measurements cannot provide information about the surface structure at a molecular level, they do make it possible to draw comparisons between the hydrophobicity of the material and protein adsorption. For the evaluation of protein adsorption to a biomaterial, the receding contact angle, indicating the properties of the surface when it is exposed to an aqueous environment, may be more appropriate.

α-1-m possesses hydrophobic prosthetic groups [13] and binds to hydrophobic molecules [25]. Comparison of the protein adsorption and DCA data, showed that the two most hydrophobic materials (PVC and PS) adsorbed the greatest amount of α-1-m, while PHEMA, the most hydrophilic material, adsorbed only trace amounts of the protein. The θ_r for the remaining two polymers, PMMA and Chronoflex, were not significantly different; α-1-m adsorbed to Chronoflex in greater amounts than to PMMA. Dynamic contact angle values for PMMA have been reported in the literature to be in the range $\theta_a = 71–74^\circ$ and $\theta_r = 52–59^\circ$ [26] offering a rather more hydrophilic surface than that described here. This method of analysis is sensitive to slight changes in the composition of the material [23] and the variation from the reported values may be attributed to variations in the molecular weight or purity of the PMMA used.

Studies of the binding strength between the protein and polymeric materials highlighted a further correlation between surface hydrophobicity and α-1-m adsorption. It was evident that for PHEMA and PMMA, α-1-m was readily desorbed by water washes, suggesting a weak attachment of the protein. Because the ELISA procedure included several washing steps after protein conditioning of the surfaces, it is not surprising that α-1-m remained in minor amounts. Stronger α-1-m binding was observed for Chronoflex and PS where complete desorption occurred only when the serum-conditioned surfaces were washed, respectively, with 30% and 50% IsoPOH.

An apparent contradiction between the two methods, used to investigate protein adsorption, emerged when α-1-m adsorption to PVC was evaluated. Although ELISA indicated a significant adsorption of α-1-microglobulin on this material, SDS-PAGE showed an extensive desorption of the protein within two water washes with only trace amounts appearing at 70% IsoPOH. This would imply a substantial remnant of strongly bound α-1-m which was resistant to IsoPOH removal but responsive to ELISA detection.

These apparently contradictory results prompted us to investigate α-1-m adsorption not only as a free 29 kDa form, but also as high molecular weight complexes. Serum proteins desorbed from PS by IsoPOH washes were blotted on to nitrocellulose membrane and immunodetected with the α-1-m antibody. The results obtained clearly showed the presence of high molecular weight bands positive for α-1-m antibody. The staining of high molecular weight bands may be ascribed to artefacts due to a low specificity of the antibodies used, but it is more likely to be due to the ability of α-1-m to form macromolecular complexes. Previous reports, as well as preliminary studies carried out in our laboratory, show the ability of α-1-m to form macromolecular complexes with other proteins [15–17]; the adsorption of this protein may occur as both free and complexed form.

5. Conclusion

In this paper we highlight the adsorption of serum α-1-m to the surface of several polymers. From the results it may be concluded that (i) the binding of α-1-m depends on the hydrophobic character of the material and (ii) the adsorption may occur as both free 29 kDa form and high molecular weight complexes with other proteins. The ability of the 29 kDa α-1-m to adsorb on to material surfaces and to participate in events such as inflammation [8] and bacterial infection [19] suggests a possible role in the modulation of implant-related inflammatory behaviour warranting study of its role in other aspects of biocompatibility.

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