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ALBUMIN AFFINITY BIOMATERIAL SURFACES

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Abstract

Recently, considerable progress has been made in designing biomaterial surfaces which possess enhanced albumin affinity. Two derivatization methods for producing albumin binding biomaterial surfaces, based on an albumin affinity dye, cibacron blue, have been developed. Both surface derivatization methods were found to enhance the binding of albumin to an implant grade polyetherurethane. Evaluations of the enhanced albumin affinity demonstrated the binding to be both selective and reversible. Surfaces having such enhanced albumin affinity were found to be minimally thrombogenic and to discourage the adhesion of bacteria which might otherwise cause device-centered infections. We conclude that albumin affinity surfaces, such as these, may be useful in the design of non-thrombogenic and infection resistant biomaterials.

Key Words: Albumin, affinity, biomaterial, bioactive, biocompatibility, surface.

Introduction

Thrombosis is a major complication affecting polymeric blood-contacting medical devices (Lindsay, 1980; Leininger et al., 1987). These devices tend to be thrombogenic due, in part, to the surface adsorption of a variety of plasma proteins which can initiate coagulation and activate platelets (Zucker and Vroman, 1969; Kim et al., 1974; Abolom et al., 1987; Collins et al., 1987; Fukumura et al., 1987; Salzman et al., 1987; Sevastianov, 1988; Ito and Imanishi, 1989). This procoagulant activity of many polymeric devices prevents their use in vivo or greatly diminishes their useful lifetime. Besides being procoagulant, these same devices can also serve as foci for infection, promoted by the tendency of certain microorganisms to adhere to, and colonize, their surfaces (Christensen et al., 1982; Lowy and Hammer, 1983; Gower et al., 1986; Hogt et al., 1986, 1987; Schmitt et al., 1986; Jansen et al., 1989). For these reasons, there is a real medical need to create polymeric materials which are less thrombogenic and less liable to harbor pathogenic bacteria.

One potential solution to the problem of surface adsorption of procoagulant proteins and cells on polymeric materials is based on the selective adsorption of albumin. Indeed, in some experimental systems, surfaces coated directly with albumin have demonstrated diminished adherence of cells and, especially, platelets (Zucker and Vroman, 1969; Kim et al., 1974; Abolom et al., 1987). Unfortunately, thus far, there has been no fully effective way of creating polymeric materials with surfaces which selectively bind albumin in vivo.

One prior promising attempt was based on the well-known ability of albumin to bind free fatty acids; surfaces displaying straight-chain 16- or 18-carbon alkyl groups were made in hopes that this might enhance surface albumin affinity (Munro et al., 1981). Indeed, the addition of alkyl chains did increase the surface affinity for albumin during brief incubations (Frautschi et al., 1983; Eberhart et al., 1987; Pitt and Cooper, 1988) and improved thromboresistance in an acute canine ex vivo experiment (Grasel et al., 1987). However, these
materials are not entirely selective for albumin binding and may, due to the highly hydrophobic nature of the surface, encourage the subsequent denaturation of adsorbed protein (Andrade and Hlady, 1986).

Our attempt to engineer polymeric surfaces with enhanced albumin affinity is based on the ability of albumin to bind a variety of triazine dyes such as cibacron blue. In fact, a commonly used technique for purifying albumin relies on this affinity. The purification technique involves the passage of solutions containing albumin, e.g., plasma, over an affinity support matrix to which cibacron blue dye is covalently bound (Travis et al., 1976). A variety of affinity support matrices including agarose (Sigma Chem. Co., St. Louis, MO), Sephacryl® (Sigma), Sephadex (Sigma), Sepharose (Sigma), etc. have been used (Kopperschlager et al., 1982). In our work, we have used a 2 million molecular weight dextran. We felt that dextran would act as an appropriate hydrophilic "spacer," thus preventing the close surface approach and consequent denaturation of bound albumin. We also anticipated that the dextran spacer would be highly stable in vivo due to the fact that mammals lack dextranase enzymes. Furthermore, cibacron blue dye is quite stable at extreme pHs. Therefore, although the catabolism of this dye in vivo has not been characterized, we felt that it would likely resist degradation. We also predicted that polymeric materials displaying "blue dextran" (dextran coupled with cibacron blue dye) would spontaneously, selectively and reversibly bind albumin upon contact with blood and certain other body fluids. With this in mind, two methods were developed to incorporate blue dextran on the surface of polymeric materials. (1) The first employs a co-solvation technique for incorporating blue dextran throughout a polyurethane. (2) The second method covalently attaches blue dextran to the surface of the polyurethane. The resultant polymeric materials from both derivatization methods demonstrate an increase in albumin affinity. This increase in albumin affinity confers a number of desirable properties, including increased thrombo-resistance and decreased bacterial adherence.

**Materials and Methods**

**Incorporation of blue dextran**

The co-solvation technique for incorporating blue dextran throughout a polyurethane has previously been described (Keogh et al., 1992). Briefly, 0.3 g of blue dextran (BD) (Sigma Chemical Co., St. Louis, MO) was dissolved in 1.5 ml of deionized (DI) water. This mixture was then added to 20 g of N,N-dimethylacetamide (DMAC) (Fisher Scientific Co., Pittsburgh, PA). To this blue dextran/solvent mixture, 1.0 g of raw 2363-55D pellets, an implant grade polyetherurethane, was added. The resultant mixture was then shaken overnight to allow complete solvation of the polyurethane. The solvated blue dextran/polyurethane mixture was then cast into film (0.3 mm thick) on Mylar® (Custom Coating and Laminating Corporation, Worcester, MA) release sheets or tubing (2 mm internal diameter, ID) on glass mandrels and dried overnight in a vacuum oven at 50°C to remove the solvent.

The covalent attachment of blue dextran to the surface of the polyurethane also has been previously described (Keogh and Eaton, 1994). Briefly, raw pelletized 55D was extruded into film (0.3 mm thick) using a RC 0625 Randcastle (Little Falls, NJ) vertical screw extruder and into tubing (2 mm ID) using a Harrel (Norwalk, CT) horizontal screw extruder. No processing aids were added prior to extrusion. The film was then cut into 1 cm² sample disks. Both tubing and sample disks were cleansed in reagent grade ethanol (Sigma) for at least two hours to remove most surface contamination, thoroughly rinsed in DI water and allowed to dry. Extruded 55D samples were then placed into a surface grafting solution consisting of 11.2 M acrylamide (AAm) (Eastman Kodak Co., Rochester, NY), 1.1 M N-(3-aminopropyl)methacrylamide (APMA) (Eastman Kodak), 400 mM nitric acid (HNO₃) (Aldrich Chemical Co., Milwaukee, WI) and 40 mM ammonium cerium IV nitrate [(NH₄)₂Ce(NO₃)₆] (Aldrich) in DI water. Grafting was allowed to proceed for 30 minutes before the samples were removed and thoroughly rinsed in DI water to remove any residual monomer.

Sodium metaperiodate (NaIO₄) (Sigma) oxidized dextran (Sigma), 2 million molecular weight, was then coupled through Schiff's base formation to the AAm/APMA grafted polyurethane surface. Dextran was oxidized by placing 5.0 g of dextran in 100 ml DI water containing 5.0 mg NaIO₄, incubating in the dark for 3 hours and then dialyzing against DI water for 24 hours to remove any residual periodate. The AAm/APMA-grafted polyurethane was placed in the oxidized dextran solution at 25°C and stirred. After 1 hour, 3 mg/ml sodium cyanoborohydride (NaCNBH₃) (Sigma) was added, and the samples were incubated with stirring for 2 hours at 25°C, thus stabilizing the Schiff's bases which had formed between the aldehydes of oxidized dextran and the amino groups of APMA. The samples were then removed and rinsed thoroughly in DI water.

Cibacron blue 3GA dye (Sigma) was then covalently coupled to the AAm/APMA-dextran derivatized polyurethane by incubating the derivatized polyurethane for 6 hours in a dye solution containing 6.5 mM cibacron blue dye, 0.3 M NaCl (Sigma) and 0.7 M NaHCO₃ (Sigma) in DI water. The blue dextran surface-derivatized polyurethane was then rinsed thoroughly in DI water.
Surface characterization

The extent of blue dextran derivatization on polyurethane surfaces was assessed with several techniques. Fourier transform infrared spectroscopy with attenuated total reflectance optics (FTIR/ATR) was performed with a SPC 3200 Bio-Rad (Cambridge, MA) spectrometer and a 45° germanium ATR crystal. Electron spectroscopy for chemical analysis (ESCA) was performed on material surfaces using a 5400 Perkin-Elmer Physical Electronics (Eden Prairie, MN) ESCA spectrometer. Scanning electron microscopy (SEM) was performed on material surfaces following mounting and sputter-coating with gold. The samples were viewed using a JSM-35CF JEOL (Peabody, MA) SEM.

Albumin affinity determinations

The extent of surface binding of albumin to blue dextran bulk-derivatized 55D sample disks (1 cm²) (hereafter, "bulk-derivatized 55D") and blue dextran surface-derivatized 55D sample disks (1 cm²) (hereafter, "surface-derivatized 55D") was estimated by incubation of derivatized samples in solutions containing radiolabeled human albumin. Sample disks of bulk-derivatized, surface-derivatized and non-derivatized 55D were first rinsed in isotonic phosphate buffered saline (PBS), pH 7.4, at 25°C for 15 minutes prior to incubation for 1 hour at 25°C in 125I-labeled human albumin diluted in PBS (final concentration = 15.7 μg/ml; specific activity = 8.5 μCi/mg protein) (Mallinkrodt, St. Louis, MO). Following incubation, the samples were thoroughly rinsed with PBS, placed in scintillant and then counted in a scintillation counter.

The selectivity of the binding of albumin by bulk-derivatized, surface-derivatized and non-derivatized 55D in the presence of 125I-labeled fibrinogen (15.7 μg/ml; specific activity = 115 μCi/mg protein) (Amersham, Arlington Heights, IL) was determined using 1:1 mixtures of albumin and fibrinogen. Fibrinogen was used as a competitive protein since it is well known that fibrinogen readily adsorbs to most medical device surfaces and because surfaces with adsorbed fibrinogen tend to be thrombogenic (Zucker and Vroman, 1969; Kim and Lee, 1991). Disks of bulk-derivatized, surface-derivatized and non-derivatized 55D were exposed to a 1:1 by weight 125I-labeled albumin/unlabeled fibrinogen or 125I-labeled fibrinogen/unlabeled albumin mixture (final protein concentration, 15.7 μg/ml) for 1 hour at 25°C. In making these mixtures, the albumin added to 125I-labeled fibrinogen by the supplier was taken into account. The samples were then thoroughly rinsed with PBS, placed in scintillant and counted in a scintillation counter.

Proteins that remain adherent to the surface of polymeric materials despite thorough washing in sodium deoxycholate sulfate (SDS) are considered denatured and irreversibly bound (Bohnert and Horbett, 1986; Rapoza and Horbett, 1989; Chinn et al., 1991, 1992). Therefore, the reversibility of albumin binding to blue dextran-derivatized surfaces was determined by measuring the elutability of previously adsorbed protein using a 1% SDS (Sigma) wash. Disks of bulk-derivatized, surface-derivatized and non-derivatized 55D were incubated for 5 hours in 125I-labeled albumin (15.7 μg/ml) at 25°C. The samples were then rinsed with PBS and incubated in PBS alone or in a 1% SDS solution for 1 hour at 25°C. After incubation, the samples were rinsed in PBS, placed in scintillant and counted in a scintillation counter.

Reversibility of albumin binding was also estimated by the release of 125I-labeled albumin by subsequent incubation in solutions of unlabeled albumin. Again, bulk-derivatized, surface-derivatized and non-derivatized 55D disks were incubated for 5 hours in 125I-labeled albumin (15.7 μg/ml) at 25°C. The samples were then rinsed in PBS followed by immersion in PBS containing 1 mg/ml unlabeled human serum albumin for 1 hour at 25°C. The extent of displacement of bound 125I-labeled albumin by free unlabeled albumin was then determined by scintillation counting of both disks and supernatants.

Finally, the extent to which surface binding of albumin by blue dextran-derivatized polyurethane is mediated specifically by an association between the immobilized cibacron blue dye and albumin was determined by using solution-phase blue dextran to competitively elute bound albumin. Bulk-derivatized, surface-derivatized and non-derivatized 55D disks were incubated in 125I-labeled albumin (15.7 μg/ml) for 5 hours at 25°C. The samples were then rinsed in PBS and incubated in PBS alone or in PBS containing soluble blue dextran (5 mg/ml) for 1 hour at 25°C. Following incubation, the samples were rinsed in PBS, placed in scintillant and counted in a scintillation counter.

Thromboresistance measurements

As one indication of the tendency of blue dextran-derivatized polyurethane to affect clotting of whole blood, limited studies using a Baumgartner (1973) apparatus and a Chandler (1958) blood loop were performed. For investigations using the Baumgartner apparatus, venous blood was drawn from a human subject (who had not taken aspirin within the last two weeks) into a plastic vessel containing citrate. These studies were approved by the Committee on Research Involving Human Subjects, University of Minnesota. The citrate-anticoagulated blood, maintained at 37°C, was then pumped through the Baumgartner (1973) perfusion chamber containing bulk-derivatized and non-derivatized 55D tubing samples (2 mm ID and 5 cm in length). Blood flow was
maintained at a rate of 140 ml/min (shear rate of 800 s\(^{-1}\)) for five minutes. The samples were then removed, fixed and stained with Diff-Quik (Baxter Healthcare Corp., McGraw Park, IL) stain set. Adherent platelets were quantified using a light microscope (field size of 3.7 \(\times\) 10\(^{-3}\) mm\(^2\)).

For investigations using a Chandler (1958) blood loop, venous blood was again drawn from a human subject into a plastic syringe containing no anticoagulant. One-milliliter aliquots of whole human blood were then delivered immediately into (1) a 12 mm x 75 mm glass test tube, (2) an ethanol-sterilized non-derivatized 55D tubing sample (2 mm ID and 30 cm in length) and (3) an ethanol-sterilized surface-derivatized 55D tubing sample (also 2 mm ID and 30 cm in length). One end of each of the polyurethane tubes was looped around and connected to the other end via an external silicone sleeve. The 1 ml aliquot of whole blood was purposely not enough blood to fill the entire tube, thus forming an air-blood interface which tends to accelerate the clotting of whole blood. The polyurethane tubes were then attached to a vertical circular plate and rotated at 9 rpm at 37°C until the blood clotted. The glass test tube was gently tilted every 30 seconds at 37°C until the blood clotted.

After 16 hours (the long incubation due to the fact that the blood within the surface-derivatized 55D tubing samples never clotted), the blood was expelled from the sample loops. The tubing samples were then vigorously rinsed in PBS and placed in 2.5% glutaraldehyde (Sigma) solution overnight. Following mounting and sputter-coating with gold, sample surfaces were examined with a JEOL JSM-35CF SEM for adherent thrombus.

**Bacterial adherence assay**

Bacterial adherence to implant surfaces is thought to be a major mechanism underlying the high frequency of device-associated infections. We, therefore, carried out limited bacterial adherence studies with bulk-derivatized, surface-derivatized and non-derivatized 55D samples using *Staphylococcus (S.) epidermidis*. *S. epidermidis* (an uncatalogued "slime"-producing clinical isolate obtained from an infected catheter by the Microbiology Laboratory at the University of Minnesota) were grown overnight in Brain Heart Infusion broth (Difco Co., Detroit, MI) in a shaking water bath at 37°C. The bacteria were then concentrated centrifugally and washed three times in isotonic saline solution and resuspended in sterile Hanks' balanced salt solution to 1.05 \(\times\) 10\(^9\) cfu/ml. Bulk-derivatized, surface-derivatized and non-derivatized 55D sample disks (1.0 cm\(^2\)), both with and without preadsorption of albumin (15.7 \(\mu\)g/ml) for 4 hours at 25°C, were immersed in this bacterial suspension with gentle mixing for 1 hour at 25°C. After incubation, the samples were thoroughly rinsed in sterile isotonic saline solution and placed in 5 ml of sterile saline solution. The samples were then gently sonicated at 20 W for 1 minute to release adherent bacteria. This power setting was experimentally determined not to decrease the viability of solution-phase *S. epidermidis*. After sonication, the numbers of displaced bacteria were enumerated by pour-plating appropriate dilutions as previously described (Ma and Eaton, 1992).

**Results**

**Surface characterization**

As reported earlier (Keogh, 1992; Keogh and Eaton, 1994), the blue dextran bulk derivatization technique partially obscured the polyurethane surface as estimated with FTIR/ATR and ESCA. In contrast, the blue dextran surface derivatization technique completely obscured the polyurethane surface as estimated with FTIR/ATR and ESCA, and the resultant surface coating had a thickness of approximately 2 \(\mu\)m, as estimated with SEM.

**Albumin affinity**

Blue dextran surface-derivatized 55D was found to bind approximately twice as much \(^{125}\)I-labeled albumin as did non-derivatized 55D and roughly 50% more than bulk-derivatized 55D (Fig. 1). This enhanced albumin
Figure 2. Relative adsorption of albumin and fibrinogen to non-derivatized 55D, bulk-derivatized 55D and surface-derivatized 55D disks. Triplicate samples were incubated in either a 1:1 by weight $^{125}$I-labeled albumin/fibrinogen or $^{125}$I-labeled fibrinogen/albumin mixture (final protein concentration, 15.7 $\mu$g/ml) for 1 hour at 25°C and thoroughly rinsed in PBS. The surface associated $^{125}$I-labeled albumin or $^{125}$I-labeled fibrinogen was determined by scintillation counting. Results are expressed as the mean percentage of total protein adsorbed ± 1 SD.

Table 1. The number of platelets spontaneously adherent to non-derivatized 55D tubing and bulk-derivatized 55D tubing. Triplicate samples were placed in a Baumgartner apparatus and exposed to flowing citrate-anticoagulated whole human blood for 5 minutes at 37°C. Adherent platelets were quantified using light microscopy. Results are expressed as the mean number of platelets/mm$^2$ ± 1 SD (20 fields of size 3.7 x 10$^3$ mm$^2$; non-derivatized differs from bulk-derivatized 55D at p < 0.01, Student's t-test, two-tailed).

<table>
<thead>
<tr>
<th>Sample</th>
<th># Platelets/mm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-derivatized 55D</td>
<td>23,000 ± 8,700</td>
</tr>
<tr>
<td>bulk-derivatized 55D</td>
<td>81 ± 154</td>
</tr>
</tbody>
</table>

binding appears to be selective. As shown in Figure 2, when non-derivatized 55D is incubated with equal proportions (weight/weight) of fibrinogen and albumin, bound fibrinogen predominates. However, similar experiments with blue dextran-derivatized surfaces

Figure 3. Percent elution of albumin previously bound to non-derivatized 55D, bulk-derivatized 55D and surface-derivatized 55D disks by SDS, albumin or solution-phase blue dextran. Triplicate samples were incubated in $^{125}$I-labeled albumin (15.7 $\mu$g/ml) for 5 hours at 25°C and thoroughly rinsed in PBS. The samples were then incubated for 1 hour at 25°C in a 1% SDS solution, a solution of 1 mg/ml soluble unlabeled albumin or a solution of 5 mg/ml soluble blue dextran. The surface associated $^{125}$I-labeled albumin was determined by scintillation counting. Results are expressed as the mean percentage of elutable albumin ± 1 SD (p < 0.01 non-derivatized or both types of derivatized samples, Student's t-test, two-tailed).

Table 2. Times at which 1 ml of freshly shed whole human blood clotted in non-derivatized 55D tubing, surface-derivatized 55D tubing and glass test tubes. Results shown are from a single experiment involving duplicate samples. These results are representative of three additional and independent experiments (all four blood samples from a single donor). Overall, for glass and non-derivatized 55D, the time at which clotting occurred varied by no more than 10%.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Clotting Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>glass test tube</td>
<td>5.75 minutes</td>
</tr>
<tr>
<td>non-derivatized 55D</td>
<td>9.5 minutes</td>
</tr>
<tr>
<td>surface-derivatized 55D</td>
<td>&gt; 16 hours</td>
</tr>
</tbody>
</table>
revealed an albumin preference of almost 70% in the case of bulk-derivatized 55D, and over 95% in the case of surface-derivatized 55D. Furthermore, less than 30% of the albumin bound to non-derivatized 55D was elutable by SDS, solution-phase blue dextran or albumin (Fig. 3). However, albumin binding to blue dextran-derivatized surfaces was found to be much more reversible and ligand specific. As shown in Figure 3, at least 70% of the albumin which binds to bulk-derivatized 55D is released by SDS and appears to be ligand specific (i.e.,

is released by solution phase albumin and blue dextran). In the case of surface-derivatized 55D, more than 95% of the bound albumin appears to be bound reversibly to the surface incorporated blue dextran.
Albumin affinity biomaterial surfaces

Thromboresistance

When tubing samples were studied under flow in a Baumgartner (1973) device, large numbers of platelets were found to adhere to non-derivatized 55D, whereas almost no detectable platelets were adherent to bulk-derivatized 55D (Table 1). Importantly, the large numbers of platelets adherent to non-derivatized 55D were activated, as indicated by the spread pseudopodia of adherent platelets (Fig. 4A). In contrast, the few platelets found on bulk-derivatized 55D were not activated by morphological criteria (Fig. 4B).

Bacterial adherence

Finally, as shown in Figure 7, the binding of S. epidermidis to bulk-derivatized 55D surfaces is more than 70% less than that to non-derivatized 55D surfaces when the surfaces were pre-incubated with albumin.
More strikingly, however, bacteria are almost completely incapable of adhering to the surfaces of surface-derivatized 55D which has been pre-incubated with albumin.

Discussion

Thrombosis and infection are among the most frequent iatrogenic problems associated with implantable and blood-contact medical devices. Especially in the case of devices made of polymeric elastomers, these adverse effects may be initiated by rapid formation of a layer of adsorbed, apparently denatured, proteins on the surface. This chaotic array of adsorbed proteins may be thrombogenic. Furthermore, the adsorption of fibrogen in particular has recently been shown responsible for the acute inflammatory effects of at least one type of implanted biomaterial (Tang and Eaton, 1993; Tang et al., 1996). Finally, this layer of denatured host proteins may form a substratum, permitting the adherence and colonization of device surfaces by microorganisms.

One technique for preventing the chaotic adsorption of proteins and cells on medical device surfaces is by way of an albumin coating. Numerous investigators have found that coating of a biomaterial surface with albumin "passivates" the surface by diminishing thrombosis, the adherence of pathogenic bacteria and, perhaps, the inflammatory response. Unfortunately, despite the fact that albumin is the predominant plasma protein, many other proteins typically have at least an equal affinity to the medical device surface. Therefore, selective coating of a medical device surface with albumin typically does not occur in vivo. Furthermore, direct attachment of albumin to the device surface can contribute to the aforementioned problems since irreversibly bound albumin eventually degrades. For these reasons, there have been some attempts to engineer medical device surfaces with enhanced albumin affinity. As described earlier, one strategy was based on the well-known ability of albumin to bind free fatty acids with high affinity. Unfortunately, these materials are not entirely selective for albumin binding and may, due to the highly hydrophobic nature of the surface, even encourage the denaturation of adsorbed protein.

Our work has been based on the premise that the development of effective and compatible blood-contact devices depends on creating surfaces which can regulate, without denaturing, the types and amounts of protein that adsorb on the surface. Therefore, we employed the concept of affinity chromatography to produce biomaterial surfaces which selectively and reversibly bind albumin in vivo. One very effective affinity chromatography technique widely used for separating albumin from plasma involves the use of cibacron blue dye (Travis et al., 1976). In fact, albumin from most mammals will bind tenaciously (Kd ~ 10^-7, dissociation constant derived from Scatchard plot) to immobilized cibacron blue dye (perhaps through a specific interaction with the bilirubin binding site on albumin) (Leatherbarrow and Dean, 1980). While other proteins and, especially, adenine nucleotide-dependent enzymes also bind to immobilized cibacron blue dye, albumin is by far the predominant plasma protein which binds this dye (Travis et al., 1976).

Based on cibacron blue dye, two derivatization methods for producing albumin affinity biomaterial surfaces were developed. Evaluations of albumin affinity of polymeric materials incorporated with cibacron blue dye demonstrate that both methods produce surfaces which preferentially bind albumin even from mixtures containing equal amounts of both fibrinogen and albumin. Furthermore, albumin is bound to these surfaces by a ligand-affinity mechanism in as much as previously bound albumin is readily released by solution-phase albumin or blue dextran. In addition, these cibacron blue derivatized materials have an unexpectedly potent anti-thrombogenic character, retarding non-cellular clotting as well as platelet adherence. Finally, these hydrophilic albumin affinity surfaces discourage the adherence of potentially pathogenic bacteria. We conclude that surfaces with similar characteristics may hold some promise for the development of less thrombogenic biomaterials which also are less liable to foster device-associated infections by adherent microorganisms.

Acknowledgements

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References


Chinn JA, Posso SE, Horbett TA, Ratner BD


Discussion with Reviewers

C. Tsai: How long will the blue dextran last on the surface?

Authors: As stated in the text, we felt the dextran spacer would be highly stable in vivo due to the fact that mammals lack dextranase enzymes. Also, since cibacron blue dye is quite stable at extreme pHs and, although the catabolism of this dye in vivo has not been characterized, we felt it would likely resist degradation. However, we have not carried out any long term implantation studies to directly measure the time.

C. Tsai: Do other plasma proteins, such as IgG, high molecular weight Kininogen (HMWK), Factor XII and complement proteins, which all can have a negative impact on blood compatibility of foreign materials, bind to your surfaces?

Authors: Unfortunately, the binding of purified plasma proteins such as IgG, HMWK, Factor XII and complement proteins has not been characterized. However, we earlier demonstrated that when blue dextran surface-derivatized material was directly immersed in whole human plasma, very few other proteins aside from albumin could be detected electrophoretically following elution from the material surface (Keogh and Eaton, 1994). We also previously demonstrated the ability of blue dextran bulk-derivatized material to selectively bind 125I-labeled albumin in the presence of increasing amounts of albumin-depleted human serum (Keogh et al., 1992).

C. Tsai: Since dextran contains many hydroxyl (-OH) groups, have you performed any complement activation tests for blue dextran-derivatized surfaces?

Authors: No formal tests have been performed for assessing complement activation.

C. Tsai: Have you looked into any other index for measuring platelet activation such as thromboxane B2 or PF4 since the lack of platelet adhesion does not necessarily mean the lack of platelet activation?

Authors: It is absolutely correct that platelet adherence alone does not prove that platelets were not being activated. We have not yet carried out studies which measure platelet activation directly.

C. Tsai: In your radiolabeled protein adsorption studies, what were the percentages of free iodide (i.e., labeling efficiency) in the 125I-labeled albumin and 125I-labeled fibrinogen solutions and will free iodide adsorb to blue dextran-derivatized surfaces?

Authors: We are confident that our radiolabeled protein solutions contained little or no free iodide since inclusion of large amounts of unlabeled NaI failed to change the amounts of surface associated 125I-labeled albumin or 125I-labeled fibrinogen found adherent to derivatized or non-derivatized surfaces.

C. Tsai: In your Chandler (1958) loop study, blue dextran surface-derivatized tubing showed no clotting phenomena (Table 2). Could you elaborate on this?

Authors: At present, we have no firm explanation for the anti-thrombogenic activities of these surfaces. However, as shown previously (Keogh and Eaton, 1994), the blue dextran surface may activate antithrombin III in a heparin-like fashion. If so, this may be sufficient to explain the long-term anticoagulant effects which we have found.

S.L. Goodman: Do you have any evidence on how well the blue dextran modified surfaces maintain their albumin binding affinity when in actual use? Does the blue dextran detach, degrade, or otherwise lose activity over time? If so, is there any toxicity or other problems associated with circulating or soluble blue dextran?

Authors: Our best response to the durability question is that we do not know. As indicated above, we have not done the requisite long-term implantation or blood contact studies. However, we do suspect that the blue dextran will be relatively stable, even compared to heparin-derivatized surfaces which can potentially be attacked by heparinases known to be present in mammals. We believe the toxicity of blue dextran is unknown.

S.L. Goodman: Do you have any direct measurements of the concentration of blue dextran which is on the derivatized surfaces, that is, other than the albumin adsorption measurements? If so, how does the surface concentration of blue dextran compare to the concentration of adsorbed albumin? Might the surface concentration of blue dextran be further optimized for reducing thrombogenicity and bacterial adherence?

Authors: We have not yet attempted to measure the actual concentration of blue dextran which is on the derivatized surfaces. However, we do believe that the surface concentration of blue dextran or cibacron blue dye might be further optimized for reducing thrombogenicity and/or bacterial adherence.

J.A. Chinn: Does blue dextran modification of polyurethanes affect their mechanical properties?

Authors: As we reported (Keogh and Eaton, 1994), we saw no affect on the mechanical properties of blue dextran modified polyurethane.
J.A. Chinn: Is blue dextran modified polyurethane chemically stable? Do samples contain leachable materials?

Authors: The modified samples we tested did not appear to leach any material and, as mentioned above, we currently do not know the chemical in vivo stability of blue dextran modified polyurethane materials.

J.A. Chinn: What affect did incubation of test samples with ethanol have on mechanical as well as surface properties of this material? Was material extracted from the polymer during incubation with ethanol, and, if so, how did this affect your results?

Authors: We have not observed any significant effect of ethanol rinsing on the mechanical properties of the polyurethane materials. Depending on the length of extraction, we sometimes observe some extracted material which we currently have not characterized. However, we believe the extracted material to be either processing aids Dow Chemical Co. may have added, low molecular weight urethane or possibly a combination of both.

J.A. Chinn: It appears from Figure 6B that modified substrate is very rough. How did surface roughness affect your results? Did you account for the increase in surface area relative to unmodified polyurethane in your adsorption/elution calculations?

Authors: We believe the observed roughness on surfaces of derivatized material is a drying artifact, one which may reflect the sites of primary "nucleation" of the acrylamide. We currently do not know of a way to calculate precisely the surface area of the modified surfaces.

J.A. Chinn: Protein adsorption is highly temperature and medium composition dependent. Can you extrapolate your results to physiological conditions, i.e., adsorption from whole blood at 37°C?

Authors: We cannot extrapolate our room temperature protein binding studies to whole blood at 37°C, however, our blood loop results which were performed at 37°C did provide favorable results.

J.A. Chinn: Does human serum albumin (HSA) absorb into the dextran matrix?

Authors: It is possible that some albumin may absorb into the dextran matrix.

J.A. Chinn: You conclude that the increase in HSA binding observed with modified polyurethane is due to ligand-receptor interaction between the immobilized dye and the protein. How does your data indicate ligand-receptor interaction rather than non-specific absorption and adsorption, followed by non-specific elution?

Authors: Once again, elution of pre-bound ¹²⁵I-labeled albumin by solution phase unlabeled albumin and by solution phase blue dextran clearly indicates ligand binding. Furthermore, adsorbed albumin is not displaced by dextran alone or proteins other than albumin.

J.A. Chinn: Was elutability of fibrinogen measured? If fibrinogen and HSA were equally elutable, would that result effect your interpretation of the HSA binding data?

Authors: Fibrinogen is elutable. However, this does not affect interpretation of our results but reflects the fact that hydrophilic surfaces, regardless of the functionalities exposed, do not encourage the irreversible hydrophobic denaturation of bond proteins.

J.A. Chinn: How do you explain the low retention of albumin by modified polyurethane given a $K_d = 10^{7}$?

Authors: We are not sure what you mean by low retention. It seems to us that retention of $-1$ monolayer after vigorous washing is pretty good. Incidentally, the $K_d$ probably refers to non-cooperative binding of cibacron blue by albumin, whereas it is quite possible that in the case of polymeric cibacron blue on dextran, the binding is cooperative.

J.A. Chinn: Frautschi et al. (1987, personal communication) report enhanced albumin adsorption from buffer to alkylated polyurethanes, and subsequent low elutability after incubation with SDS. They attribute high retention to strong ligand (alkyl residue)-receptor (albumin) interaction with native (not denatured) albumin. Further, Munro et al. (1983) propose that adsorbed protein denatures, then desorbs. Subsequently, a native albumin molecule adsors to the freed alkyl residue. In contrast, Bohnert and Horbett (1986) interpret low elutability of adsorbed protein as denaturation. Which adsorption/elution mechanism is most consistent with your data?

Authors: We would agree with Bohnert and Horbett (1986) that low elutability suggests surface denaturation. Such avid surface association does not indicate strong ligand-receptor interaction, particularly where low elutability extends to a denaturing agent such as SDS.

J.A. Chinn: Sefton (1993) reports that hydrogel surfaces consume platelets, though few platelets are observed adherent to the surface. Does dextran act as a hydrogel in your system? What does your data in Table I and Figure 4A indicate about non-adherent platelets?

Authors: We agree that some hydrogel surfaces can consume platelets, but we disagree with the statement that all hydrogel surfaces consume platelets. In fact, we recently have reported on a sulphonated hydrogel surface which appeared not to consume platelets during a blood
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loop experiment (Keogh et al., 1996).

**J.A. Chinn:** Does low bacterial adherence indicated in Figure 7 represent a kinetic or an equilibrium effect?

**Authors:** We are not sure if the observed decrease in bacterial adherence is attributable to a kinetic or an equilibrium effect, but suspect the latter.

**R.C. Eberhart:** When surfaces are exposed to blood, plasma, or protein solution, non-specific binding takes place, i.e., binding not associated with the specific interaction between the ligand and the protein. Albumin and other proteins may have some affinity for dextran (Birkenmeier, 1984, personal communication). Therefore, there may have been some degree of the albumin binding and retention due to non-specific association with the dextran and not the cibacron blue. Although the results of this paper are impressive, perhaps an instructive control surface would have been unmodified dextran.

**Authors:** We have measured albumin binding to dextran-derivatized surfaces (i.e., lacking the final cibacron blue modification) and found some binding. This binding does not show ligand specificity (i.e., selectivity for albumin) but is reversible.

**R.C. Eberhart:** Immersing the sample into a protein solution is not a very good technique for protein adsorption studies as the air/water interface is known to denature protein. Therefore, as you lower your sample into the solution, you are passing it through a layer of denatured protein which may or may not still have the cibacron blue binding site intact. Therefore, as in Langmuir-Blodgett films, a film of denatured protein may be physically attached to your surface by this process independent of any cibacron blue specific binding. Therefore, in the future, I would suggest modifying your method of protein binding assays so that the adsorbed proteins do not have opportunity to denature and your surfaces have a chance to become fully hydrated prior to protein exposure. Based on this, do you believe that the protein binding results presented here are indicative of what might be seen clinically if your surface were to be used in cardiopulmonary bypass tubing, for example?

**Authors:** Yes, we believe our protein binding results would be indicative of what might be seen clinically in cardiopulmonary bypass tubing. The main thrust of our binding studies was to investigate the reversible binding of the proteins under study. The results with albumin would, if anything, have been made less impressive if we had been accidentally adsorbing a layer of pre-denatured albumin. But we agree that there are better ways to do these sorts of protein binding studies.

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**Additional References**

