A New Method for Membrane Construction on ePTFE Vascular Grafts: Effect on Surface Morphology and Platelet Adhesion

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ABSTRACT: Vascular grafts made of expanded polytetrafluoroethylene (ePTFE) are widely employed in reconstructive surgery. Despite their successful use for replacement of large diameter blood vessels, ePTFE vascular grafts with internal diameters of less than 6 mm uniformly fail as a result of blood clot formation. To reduce the ePTFE reactivity to platelets, a new method for membrane construction on the inner graft surface was developed. The membrane, made of a chitosan–poly(vinyl alcohol) blend and non-ionic detergent, is constructed by adsorption of a thin liquid film and air drying. The chemically modified ePTFE surface exhibits both morphological changes and a markedly reduced reactivity to platelets in *in vitro* studies. Chemical activation of the chitosan–poly(vinyl alcohol) blend, to trigger desired protein binding, may yet serve as a complementary surface modification approach. © 1997 John Wiley & Sons, Inc. J Appl Polym Sci **63:** 1393–1400, 1997

Key words: polytetrafluoroethylene (ePTFE) vascular graft; platelet; chitosan; poly(vinyl alcohol); membrane coat

INTRODUCTION

Vascular grafts made of expanded polytetrafluoroethylene (ePTFE) are widely employed in reconstructive surgery.[1] Despite their successful use for replacement of large diameter blood vessels, ePTFE vascular grafts with internal diameters of less than 6 mm uniformly fail as a result of blood clot formation.[2,3] Several approaches to minimize the ePTFE surface effect on blood clotting have been attempted. These include surface modification by construction of a molecular outer layer accomplished by adsorption graft polymerization or activation and chemical binding of antithrombogenic mediators,[4–7] as well as removal of air trapped within the ePTFE surface interstices.[8] However, these methods have had limited success. An alternative useful method, which has not been yet attempted, is the construction of a polymer-based membrane coating on the vascular graft surface, to simultaneously affect both its chemical and morphological characteristics. Such a membrane should be (1) biocompatible, (2) sta-

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ble under physiological conditions, (3) nonthrombogenic, and (4) able to trigger and support the healing process by providing a good surface for attachment and growth of endothelial cells. Two hydrophilic polymers, chitosan and poly(vinyl alcohol), seem to comply with several of these requirements. Chitosan, a commercially available polysaccharide made of 2-aminoglucose obtained by deacetilation of chitin, is both biocompatible and able to enhance healing of ePTFE prosthesis.[9] Chitosan films made by air drying of its solution in diluted acetic acid have also been found to support Madin Darby Canine Kidney (MDCK) cells growth.[10] Both chitosan films and an absorbed chitosan monolayer are, however, highly thrombogenic, as evidenced by their use in the sealing of Dacron-made grafts and their ability to trigger formation of an intense thick blood coagulum.[11–13] Chemical modification of chitosan effects clotting time; furthermore, a correlation between clotting time and specific modification/s was observed,[14] suggesting that the chitosan thrombogenic properties may be systematically altered.

Poly(vinyl alcohol) (PVA) is another biocompatible, commercially available, and potentially useful polymer for the treatment of the ePTFE surface. Small caliber vascular graft tubes made of PVA stayed patent *in vivo* for 1 month.[15] Despite the desired nonthrombogenic property of these tubes, they were mechanically weak.[16] Attempts to use chitosan–PVA blends for cardiovascular graft coatings have not been reported, even though strong molecular interactions between chitosan and PVA in polymer blends were observed.[17] Films made of a chitosan–PVA blend similarly exhibited high tensile strength.[18] Based on these observations, this study investigated the possibility of constructing a membrane coating comprising chitosan, as the "backbone" component, and PVA, as a polymeric nonthrombogenic component on the ePTFE surface, with the purpose of affecting both the morphology and the chemical nature of the ePTFE surface.

EXPERIMENTAL

Materials

Chitosan (Cat. No. 22741) was purchased from Fluka, Buchs, Switzerland. Poly(vinyl alcohol) (PVA, MW 125,000, Cat. No. 29791) and Triton X-100 (Cat. No. 30632) were purchased from BDH, Poole, England. Glutaraldehyde (Cat. No. 4239) was from Merck. An expanded polytetrafluoroethylene (ePTFE) vascular graft, with a regular wall and internal diameter of 8 mm, was a generous gift from Impra, Inc., Tempe, Arizona.

Preparation of Polymer-coating Solutions

(a) Chitosan solution: A 3% (w/v) solution in 1% (v/v) aqueous acetic acid was prepared by suspending 3 g chitosan in 99 mL water for 15 min, followed by the addition of 1 mL glacial acetic acid. The mixture was stirred overnight at room temperature, autoclaved (17 min at 121°C), and filtered through a gauze pad. The solution was stored at room temperature. Dilutions of this stock solution were made with 1% (v/v) aqueous acetic acid. (b) PVA solution: A 5% (w/v) solution was prepared by suspending, while mixing, 5 g PVA in 100 mL of water warming to gentle boiling and simmering for 30 min. The PVA solution was stored at room temperature. (c) Chitosan–PVA blend solutions: Blends were prepared by mixing 4 vol of 3% chitosan stock solution with 3.8 vol of 1% (v/v) aqueous acetic acid, 1.2 vol of 5% PVA stock solution, and 1 vol of 1% (v/v) aqueous Triton-X-100, resulting in 1.2% (w/v) chitosan–0.6% (w/v) PVA–0.1% Triton X-100 combination. Similarly, other combinations were prepared as detailed in Table I.

In some experiments, surfaces coated with 0.6% chitosan were air-dried or not and then treated for 15 min at room temperature with a 1% glutaraldehyde solution made in standard phosphate-buffered saline (PBS), pH 7.4. After activation, the discs were rinsed in PBS to remove excess of nonbound aldehyde. The activated coating was then treated with 0.5% (w/v) human serum albumin solution in PBS for 1 h at room temperature. Excess nonbound albumin was removed by washing in PBS. Alternatively, as a control, the activated coating was treated for 1 h at room temperature with 0.1*M* Tris [Tris(hydroxymethyl)aminoethane] buffer solution, pH 7.4, to block the aldehyde groups.

Coating of ePTFE Discs

Discs, 6 mm in diameter, were prepared by cutting vascular graft material made of ePTFE as previously described.[19] To coat the discs, each disc was held on the tip of a needle (26G1/2) and gradually dipped in the coating solution. Special care was taken to avoid entrapment of air bubbles. Excess liquid was carefully drained and the discs

Table I Preparation of Chitosan–PVA Solutions

Final Blend Composition (%, w/v) (Chitosan/PVA/Triton X-100)	3% Chitosan	5% PVA	1% Acetic Acid	1% Triton	Water
1.2/0.6/0.1	4.0	1.2	3.8	1.0	—
0.6/0.3/0.1	2.0	0.6	6.4	1.0	—
1.2/0.0/0.1	4.0	—	5.0	1.0	—
0.0/0.6/0.0	—	1.2	—	—	8.8

were allowed to air dry overnight at room temperature.

Platelet Isolation and Binding Assay

Venous blood was collected from healthy human volunteers using protocols approved by the Robert Wood Johnson Medical School Ethics Committee. Freshly drawn blood was anticoagulated with acid–citrate dextrose (56 m*M* sodium citrate, 65 m*M* citric acid, 104 m*M* glucose) supplemented with prostaglandin E1 (1 μM Sigma). Platelets were isolated as previously described.[20] Platelet-rich plasma was prepared by centrifugation of the blood at 180*g* for 20 min at room temperature. Platelets were pelleted from the platelet-rich plasma by centrifugation at 1000*g* for 16 min at room temperature. The pellet was gently resuspended in platelet-poor plasma. Prostaglandin E1 (1 μM, Sigma) and apyrase (1 unit/mL, Sigma) were added to each isolation step to minimize platelet activation. To label platelets, 500 μCi ^{51}Cr (5 mCi/ml in normal saline, ICN Biomedicals) was added to each mL of resuspended platelet solution. The resulting solution was incubated at 37°C for 60 min. Platelets were isolated from the plasma by filtration through a Sepharose 2B column equilibrated with Walsh buffer (137 m*M* NaCl, 2.7 m*M* KCl, 1 m*M* $MgCl_2$, 3.3 m*M* NaH_2PO_4, 20 m*M* HEPES, 0.1% albumin [Sigma], pH 7.4). The plasma-free platelet count was adjusted to $2–3 \times 10^8$ platelets/mL in the Walsh solution.

Platelet adhesion was studied by using untreated 96-well microtiter plates (Corning). Untreated and polymer-coated ePTFE discs were placed at the bottom of the wells and were then plasma-coated with 100 μL/well of platelet-poor plasma for 1 h at room temperature. Nonadherent proteins were removed by aspiration and the wells were washed three times with PBS (200 μL/well), incubated with an albumin solution in PBS (500 μg/mL; 200 μL/well), to prevent potential nonspecific adhesion to the discs and/or well surfaces, and washed again with PBS (200 μL/well). In later experiments, identical results were achieved without the albumin block.

^{51}Cr-labeled platelets were added to each well (100 μL/well) and incubated for 60 min at room temperature. Wells were washed three times with PBS (200 μL/well) to remove nonadherent platelets. Residual buffer was removed by aspiration. Adherent platelets were lysed into lysis buffer (2% SDS, 66 m*M* Tris, pH 7.4; 100 μL/well). The lysates were collected and ^{51}Cr-labeled platelet adherence was quantitated using a gamma counter (LKB Wallac, Modelk 1282). Platelet adhesion to polymer-coated discs was expressed as counts per minute. Each condition was run in triplicate. Error bars represent the standard error of the mean.

Scanning Electron Microscopy

Uncoated and polymer-coated ePTFE discs were fixed in 2% glutaraldehyde in phosphate buffer (0.05 mol/L) at 5°C for 2 h. The discs were rinsed in the phosphate buffer and postfixed in 1% aqueous osmium tetraoxide at 5°C for 1 h. After additional rinsing, the discs were dehydrated through a graded ethanol series and critical point-dried in a Blazer QFD 020 critical point drier (Baltec Products Inc., Middlebury, Conn.). The samples were then sputter-coated with gold palladium in a Baltec SCD 004 sputter coater (Baltec Products Inc.). The specimens were observed with a Hitachi S450 scanning electron microscope (Hitachi Inc., Danbury, CN).

X-ray Photoelectron Spectroscopy (XPS) Analysis

The atomic composition of uncoated and coated ePTFE surfaces was analyzed by X-ray photoelectron spectroscopy (also known as ESCA; electron

spectroscopy for chemical analysis) on a Kratos XSAM-800 instrument with AlKα radiation. For all samples, a survey spectrum was obtained for a 1000 eV window at a take-off angle normal to the surface or a grazing take-off angle of 75°. High-resolution spectra were obtained using 20 eV windows for individual XPS peaks corresponding to C1*s* with two components at 287 and 294 eV (the latter corresponding to C bonded to F [CF_2 or CF_3]), F1*s* (691 eV), O1*s* (533 eV), and N1*s* (398 eV). The average atomic composition was determined based on the measured peak intensities using the software provided by Kratos.

RESULTS

Microscopic Evaluation of the Modified ePTFE Surface

Examination of the inner surface of an untreated ePTFE cardiovascular graft by scanning electron microscopy (SEM) reveals an uneven surface topography rich in microcavities that are formed by large polymeric backbone strands interconnected by a network of much finer polymeric fibrils [Fig. 1(A)]. Treatment of the ePTFE surface with a chitosan solution, prepared as described by Malette et al.[11] (2 mg/mL chitosan), did not affect the surface microstructure [Fig. 1(B) vs. (A)]. A unimolecular absorption of chitosan onto the ePTFE surface may thus account for the enhanced reactivity of the chitosan-treated surface to blood.[11–13] Attempts to treat the ePTFE surface with more concentrated chitosan solutions (10–30 mg/mL; i.e., 1–3% [w/v]) were equally unsuccessful as these solutions did not coat the ePTFE surface (data not shown). In contrast, addition of a low concentration (0.1%) of the nonionic detergent Triton X-100 into the chitosan solution readily allowed for the adsorption of a fine liquid film onto the ePTFE surface [Fig. 1(C)]. Furthermore, following air drying, the fine ePTFE fibrils appeared to be, in part, sealed by a polymeric film that significantly reduced the fine microcavities number. Treatment of the ePTFE surface with a PVA solution (6 mg/mL; i.e., 0.6% [w/v]) resulted in an even more pronounced surface alteration; the adsorbed PVA film, once dry, formed a continuously sealing membrane [Fig. 1(D)]. Significantly, application of a chitosan–PVA blend (1.2%/0.6% combination, containing 0.1% Triton X-100) to the ePTFE surface also effectively sealed the membrane, thus eliminating the fine microcavities [Fig. 1(E)].

The surface composition of the coated and uncoated ePTFE surfaces was next examined by XPS. Analysis of the uncoated ePTFE surface revealed mainly two elements, C and F, at an atomic concentration of C1*s* = 24% (of which C bonded to F represented 82%) and F1*s* = 75%. An attenuation in the peak intensities corresponding to F, and C with F, was expected for the coated surfaces. As expected, coating of the ePTFE surface with 1.2% chitosan, 0.6% PVA, or a blend of the two resulted in a decrease in the atomic concentration of F and C with F, with a parallel increase in the concentration of C1*s* and O1*s* due to surface coating. The atomic concentration of nitrogen detected on either the chitosan-coated or chitosan–PVA blend-coated ePTFE surface was 1.2% or less and was therefore disregarded. The atomic composition of the chitosan–PVA blend-coated ePTFE surface was C1*s* = 48% (of which C bonded to F represented 12%), F1*s* = 27%, O1*s* = 24%, and N1*s* = 1%. The atomic composition of the chitosan-coated surface was C1*s* = 33% (of which C bonded to F represented 34%), F1*s* = 51%, O1*s* = 15%, and N1*s* = 1%, whereas that of the PVA-coated surface was C1*s* = 30% (of which C bonded to F represented 52%), F1*s* = 60%, and O1*s* = 10.0%. These data indicated that coating of the ePTFE surface with PVA, chitosan, or the chitosan–PVA blend was achieved.

Effect of Membrane Coating on Platelet Adhesion

To assess the impact of the membrane coat on the graft reactivity to platelets, platelet adhesion to untreated and modified ePTFE discs was examined. To allow for plasma protein adsorption onto the surface, all surfaces were first incubated with platelet-free-plasma for 1 h. In some experiments, the surfaces were next treated with an albumin-containing solution to block potential nonspecific binding sites. ^{51}Cr-labeled platelets were then exposed to the plasma-coated surfaces for 1 h, nonadherent platelets were removed by washes, and adhesion was quantitated by gamma emission (see Experimental and Ref. 20 for details).

Compared to platelet adhesion to the untreated ePTFE discs, a 5–20% *increase* in platelet adhesion to the chitosan-coated ePTFE discs was observed in three independent experiments [representative data from one experiment is shown in Fig. 2(A)]. The correlation between

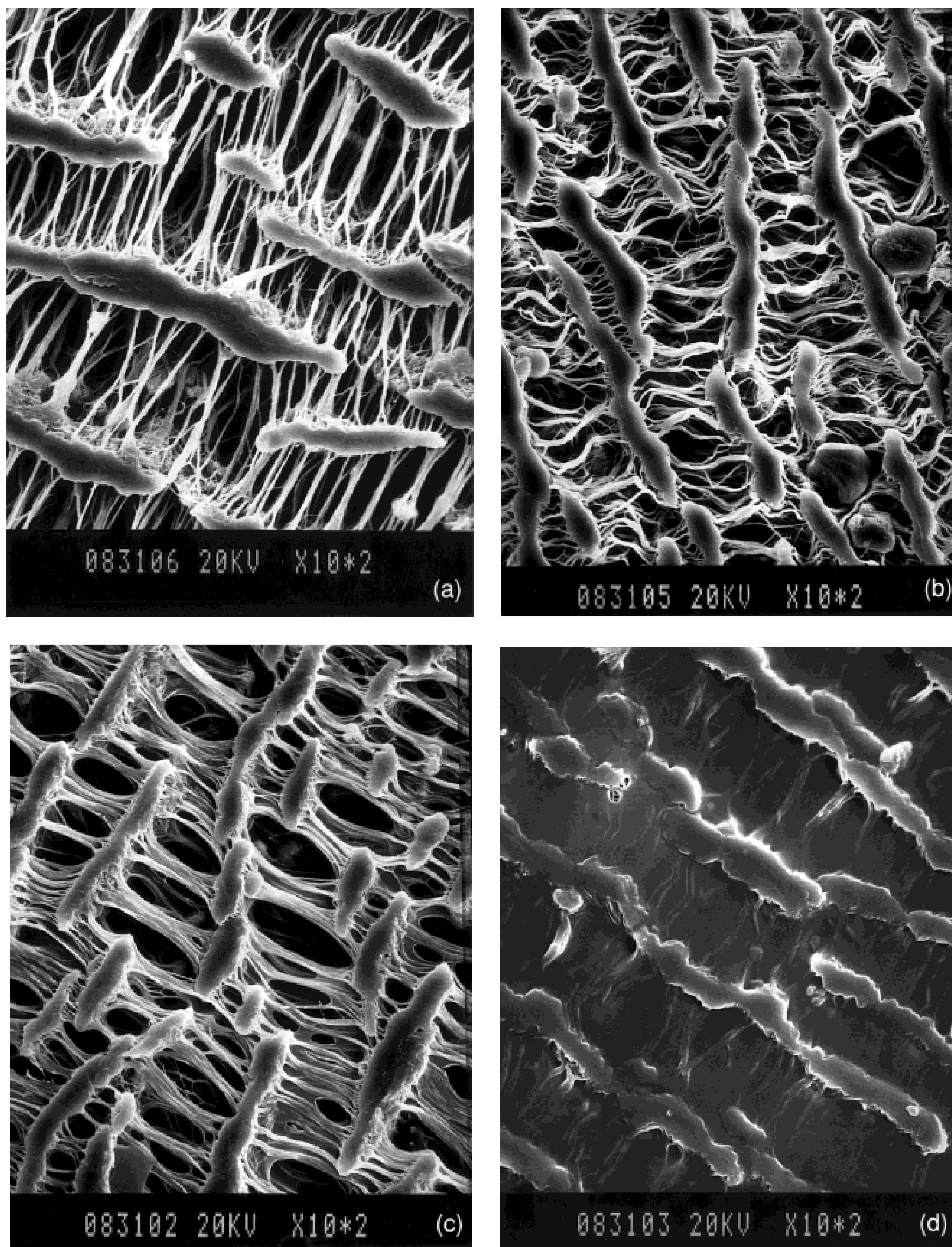

Figure 1 Coating effect on ePTFE surface morphology. The ePTFE surface was (A) untreated, or treated with (B) a diluted chitosan solution (after Ref. 11), (C) a chitosan-Triton X-100 solution (1.2% [w/v] and 0.1% [v/v], respectively), (D) a PVA solution (0.6% [w/v], or (E) a chitosan–PVA–Triton X-100 blend (1.2% [w/v], 0.6% [w/v], and 0.1% [v/v], respectively). Original magnification 1000×.

the chitosan concentration and the increase in platelet binding to the ePTFE surface seen in Figure 2(A) was not reproduced in other experiments. The increase in platelet binding to the chitosan-coated ePTFE surface is consistent with the observed effect of chitosan alone on blood coagulation.[11] In contrast to the stimulatory effect of the chitosan coat on platelet adhe-

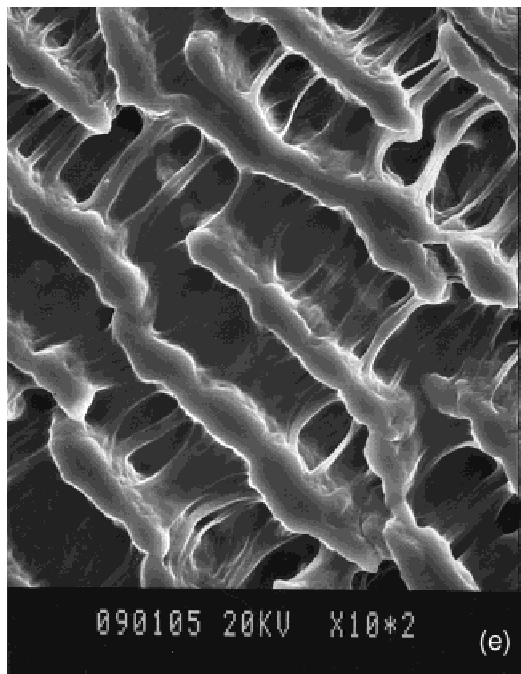

Figure 1 (*Continued from the previous page*)

sion, coating of the ePTFE surface with PVA solutions (0.5–5%) reduced binding by 67–85% [representative data from one experiment is shown in Fig. 2(B)]. These results are consistent with the noted low reactivity of PVA tubings to blood *in vivo*.[15]

The effect of several chitosan–PVA blend coatings on platelet adhesion was next examined. In one set of experiments, a representative of which is shown in Figure 2(C), platelet adhesion to chitosan–PVA blend coatings at weight ratios of 2 : 1, 3 : 2, 1 : 1, and 1 : 2, was examined. Platelet adhesion to the chitosan–PVA blend-coated surfaces was reduced by 53–67% as compared to the control untreated surface. In another set of experiments, two chitosan–PVA blend coatings at weight ratios of 2 : 1, 1.2%/0.6%, and 0.6%/0.3% were found to decrease platelet binding to the surface by 80% [Fig. 2(D)]. Identical results were observed with discs treated with the chitosan/PVA blends without Triton X-100 (data not shown). The close similarity between the results obtained with the chitosan–PVA blend and those obtained with PVA alone suggest that when coated with the chitosan–PVA blend the PVA is the predominant surface-active polymer.

One of the potential benefits of chitosan as a coating ingredient is that its amino groups can be readily activated. To examine this potential application, ePTFE discs coated with 0.6% chitosan were air-dried or not and then activated with glutaraldehyde. The activated coating was next treated with human serum albumin (HSA) or with Tris buffer solution to block the activated sites. Albumin was used in these experiments because of its low reactivity to platelets.[20] Data shown in Figure 3 suggest that binding of albumin to the chitosan-coated discs effectively reduced platelet binding to these surfaces by 55–60%. This effect was not observed if HSA was omitted and replaced by a Tris wash. Furthermore, treatment of the ePTFE discs with an HSA containing solution did not significantly affect platelet binding to the surface. Taken together, these results suggest that chitosan can provide the linking element for protein binding to ePTFE.

DISCUSSION

The hydrophobic nature of the ePTFE surface, as well as its morphology, are considered prime factors in the thrombogenicity of vascular grafts. The ePTFE membrane coat described in this study was designed to significantly alter the graft surface morphology, leading to the possible elimination of the graft microcavities. Our data suggest that treatment of the ePTFE surface with PVA indeed generates a smoother surface. The PVA-coated surface is most probably also more hydrophilic than is the uncoated ePTFE surface. The change in surface properties correlated with a reduced reactivity of the modified surface to platelets. Significantly, similar results were obtained when chitosan and PVA were used in combination. These results are extremely encouraging in respect to the potential of constructing a bicomponent membrane combining the high mechanical stability expected of chitosan (see Refs. 17 and 18) with the highly desirable nonthrombogenic surface properties observed for PVA. Furthermore, as data presented in Figure 3 suggest, the chitosan component may provide yet an additional advantage: Its amino groups can be readily activated (e.g., by glutaraldehyde treatment), thus providing the necessary linking side chain for complementary protein binding to the already modified surface. In conclusion, our results demonstrate the feasibility of using chitosan–PVA blends for the construction of a membrane coat-

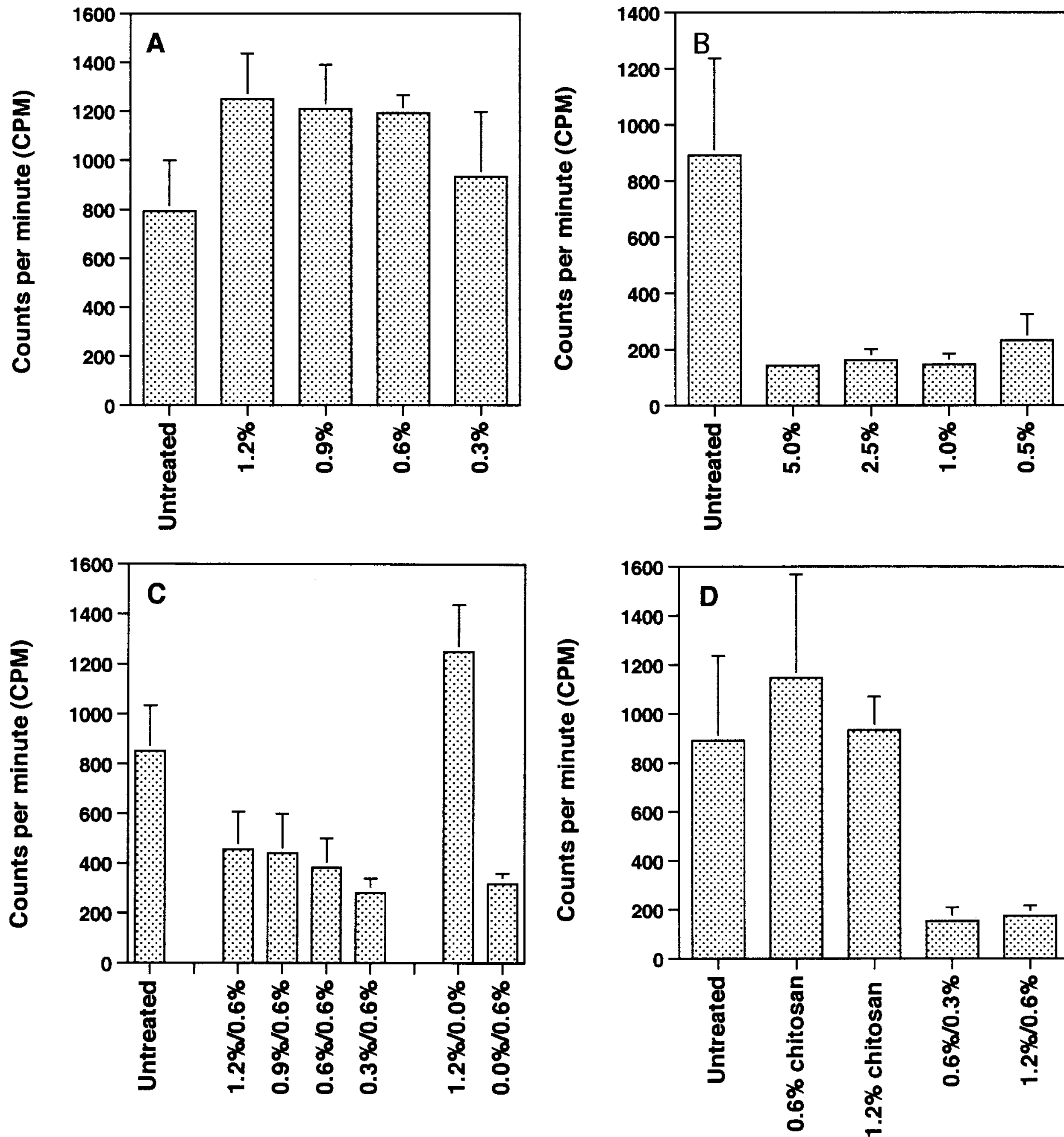

Figure 2 Coating effect on platelet binding. The ePTFE discs were (A) untreated or treated with 0.3–1.2% chitosan solutions (containing 0.1% [v/v] Triton X-100), (B) untreated or treated with 0.5–5.0% PVA solutions, or (C) untreated or treated with chitosan/PVA blends (containing 0.1% [v/v] Triton X-100). The blends tested contained chitosan and PVA concentrations of, respectively, 1.2%/0.6%, 0.9%/0.6%, 0.6%/0.6%, and 0.3%/0.6%. Platelet binding to discs coated with 1.2% chitosan (containing 0.1% [v/v] Triton X-100) or 0.6% PVA was examined for comparison; (D) untreated or treated with two chitosan/PVA blends (containing 0.1% [v/v] Triton X-100) at weight ratios of 2 : 1, 1.2%/0.6%, and 0.6%/0.3% or chitosan at two concentrations, 1.2% and 0.6%. Platelet binding is expressed as counts per minute. Error bars indicate the standard error of the mean.

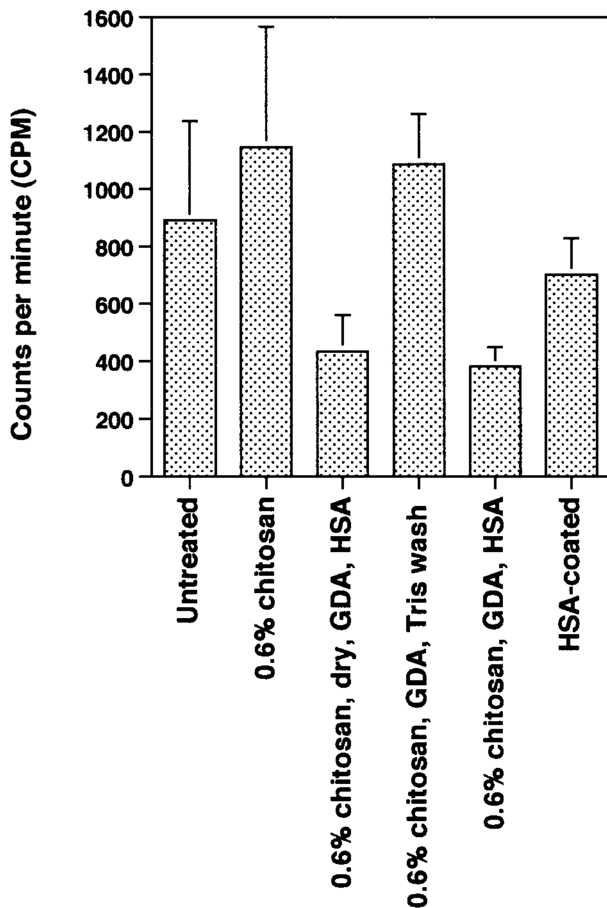

Figure 3 Platelet binding to chitosan-activated discs. The ePTFE discs were untreated or coated with a 0.6% chitosan solution (containing 0.1% [v/v] Triton X-100). The chitosan-coated surfaces were air-dried or not and then activated for 15 min with 1% glutaraldehyde. The surfaces were then rinsed and treated for 1 h with 0.5% human serum albumin (HSA) or 0.1*M* Tris buffer. ePTFE discs treated for 1 h with 0.5% HSA were also examined. Platelet binding is expressed as counts per minute. Error bars indicate the standard error of the mean.

ing on biomaterial surfaces that exhibits low reactivity to platelets.

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