Interaction of antithrombin III with surface-immobilized albumin–heparin conjugates

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The interaction between antithrombin III (ATIII) and albumin–heparin conjugates covalently coupled onto carboxylated polystyrene beads either in buffer containing albumin or in plasma was studied using 14C-labeled ATIII. Binding isotherms of ATIII were modeled using a summation of two Langmuir equations. These equations describe the binding of ATIII to two different sets of binding sites, one with a high, the other with a low affinity for ATIII. The average binding constants for the binding of ATIII to these sites are $9 \times 10^5$ L/mol and $0.3 \times 10^6$ L/mol, respectively. The binding of ATIII to surface binding sites with a high affinity for ATIII was correlated with the presence of specific ATIII binding sites in the immobilized heparin. Binding of ATIII from albumin solutions to binding sites with a low affinity for ATIII was dominated by nonspecific binding of ATIII to the immobilized heparin. A third small fraction of the surface bound ATIII is probably adsorbed to sites on the surface not covered with heparin. In the case of the binding of ATIII to the heparinized surface from plasma solutions, a fraction of initially adsorbed ATIII was desorbed by other plasma proteins. This desorption in combination with direct competition between ATIII and other plasma proteins resulted in lower ATIII surface concentrations using plasma as compared to the ATIII surface concentrations obtained using albumin solutions. The binding of ATIII to nonspecific binding sites was almost completely inhibited in the presence of plasma proteins. The amount of ATIII bound to immobilized heparin via specific ATIII binding sites was 30% lower in plasma solutions as compared to the specific binding of ATIII using albumin solutions. It is concluded that the accessibility of immobilized heparin for ATIII in plasma decreases by binding of heparin–binding proteins onto the immobilized heparin and/or by adsorption of other plasma proteins on the heparinized surface.

INTRODUCTION

Heparin frequently has been applied for the modification of polymeric surfaces. The covalent immobilization of heparin onto a biomaterial surface usually results in a stable heparin coating that exerts anticoagulant activity if the immobilized heparin molecules are sufficiently mobile and accessible for antithrombin III (ATIII) and clotting proteins. One-third of heparin molecules in commercially available unfractionated heparin contains a pentasaccharide sequence with a high affinity for ATIII. For the potentiating effect of heparin on ATIII in the inhibition of clotting proteases, it is essential that ATIII be bound to heparin via this specific binding site. Other plasma proteins, such as histidine-rich glycoprotein (HRGP), platelet factor four, and vitronectin, also bind to heparin and thereby reversibly neutralize its action, probably by competition with ATIII and clotting proteases, for binding to the negatively charged mucopolysaccharide.

It is well known that when a biomaterial is brought into contact with blood or plasma, usually protein adsorption onto the surface will take place. When a surface contains covalently bound heparin, the question arises as to how the interaction of immobilized heparin with ATIII is influenced by the presence of other plasma proteins. In this study we have focused on the interaction of ATIII with a well-defined and characterized heparin-containing surface. This surface was obtained by the modification of carboxylated polystyrene beads with covalently bound albumin–heparin (alb-hep) conjugates. A coating consisting of albumin and heparin combines the beneficial effects of surface-bound heparin with passivation of the surface with an albumin layer. When Hennink et al. studied the interaction of ATIII with a polystyrene substrate preadsorbed with albumin–heparin conjugates, the highest amounts of antithrombin III were bound by surfaces modified with alb–hep conjugates with a high affinity for

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ATIII. It was concluded that the interaction of ATIII with the alb-hep-conjugates-coated surfaces takes place via the heparin moiety of the conjugate.

Hypothetically, three types of interactions between ATIII and a heparinized surface can be distinguished. The first one is a specific interaction between ATIII and ATIII binding sites on the surface-immobilized heparin. ATIII molecules also can bind to the immobilized heparin via nonspecific binding sites. The third type of interaction involves the adsorption of ATIII on surface areas not covered with heparin. Parameters that influence these interactions are the nature and immobilization method of heparin, including the use of spacers, the nature of the material surface, and the medium used for the ATIII adsorption, especially the presence or absence of other (heparin-binding) proteins.

This study is to elucidate the interactions between ATIII and polystyrene surfaces coated with covalently immobilized alb-hep conjugates with different affinities for ATIII. The nature and the contribution of the different interactions of ATIII with the heparinized surfaces in both a buffer system containing albumin and in plasma were investigated.

MATERIALS AND METHODS

Labeling and purification of ATIII

Human ATIII was supplied by the Dutch Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. The lyophilized material (5.2 U/mg) contained ±5% immunoglobulin (IgG) against hepatitis. The ATIII was labeled by reductive methylation using 14C-formaldehyde (27.2 mCi/m mole, Amersham, UK) in the presence of NaCNBH3 (Merck, Darmstadt, Germany). In a typical labeling experiment, 100 mg ATIII was reconstituted with 5 mL distilled water. 14C-formaldehyde (125 µCi) was added under light stirring. Aliquots of 100 µL NaCNBH3 (1 g/L) were added every 15 min for 3 h. The labeled ATIII was purified on a heparin–Sepharose column (792 µg heparin/mL gel, Sigma, St Louis, USA). The heparin–Sepharose was washed with a NaH2PO4/Na2HPO4 buffer (12.5 mM, pH 7.4) containing 1 M NaCl before use to remove unbound heparin. The column was equilibrated with PBS pH 7.4, after which the reaction mixture was applied to the column. The column was eluted with a NaH2PO4/Na2HPO4 buffer (12.5 mM, pH 7.4) initially containing 0.4 M NaCl, which was increased to 1.8 M NaCl. The protein content of the eluent continuously was monitored with a spectrophotometer (UV1 LKB, Uppsala, Sweden) at 280 nm. The radioactivity of the eluent was measured using samples obtained after different elution times. The radioactivity was determined with a scintillation counter (1219 Rackbeta, LKB–Wallac Turku, Finland) using a mixture of 2 mL LumaSolve, 18 mL LumaGel (Lumac LSC, Olen, Belgium) and 200 µL 6 N HCl. ATIII fractions were pooled and dialyzed against distilled water and PBS at 4°C. The protein content was measured using Coomassie Plus Protein Reagent (Pierce, Rockford, USA) at 595 nm. The incorporation of label resulted in a specific activity between 1600–2100 CPM/µg protein. The purity of the starting material and the ATIII fractions obtained after purification were studied using SDS gel electrophoresis (Phast System LKB, Uppsala, Sweden).

Characterization of 14C-labeled ATIII

The biological activity of labeled ATIII was compared to that of unlabeled ATIII in an endpoint thrombin inhibition assay using a chromogenic substrate (S 2238, Kabi Vitrum, Stockholm, Sweden).19 To detect any change in binding between ATIII and heparin upon labeling, labeled and unlabeled ATIII were eluted on a heparin–Sepharose column using a NaCl gradient ranging from 0.15–1.8 mol/L in a NaH2PO4/Na2HPO4 buffer (12.5 mM pH 7.4).

Preferential binding of labeled ATIII on a surface containing covalently coupled alb-hep conjugates was investigated by mixing labeled and unlabeled ATIII in different ratios to a final concentration of 150 µg/mL. The ATIII mixtures, dissolved in PBS pH 7.4, containing 40 mg/mL albumin, were exposed to the heparinized surface for 1 h at 37°C. After incubation the beads were washed 3 times with PBS, transferred into a scintillation vial containing 2 mL LumaSolve, and incubated for 1 h at 55°C to dissolve the ATIII. After the vials were cooled, 18 mL LumaGel and 200 µL 6 N HCl were added. The radioactivity was measured and plotted against the ratio of labeled and unlabeled ATIII.

Preparation of plasma containing labeled ATIII

Eluted at high ionic strength in the purification procedure, and thus having a high affinity for heparin, 14C-labeled ATIII was added to ATIII-free plasma to a final concentration of 130 µg/mL. ATIII-free plasma was prepared by mixing 80 mL of citrated fresh frozen human plasma with 50 mL heparin–Sepharose for 15 min at 4°C. The heparin–Sepharose was removed from the plasma by filtration using a fritted glass filter with a pore size of 20 µm. The clear plasma filtrate was considered to be ATIII-free, based on a
thrombin inhibition assay in which no ATIII activity could be detected. A drawback of this depletion procedure is that in addition to ATIII, probably other heparin-binding proteins are removed or their concentrations decreased. Aliquots of 1 mL of labeled ATIII in both PBS and plasma were snapfrozen at −80°C and stored at −30°C.

Preparation and characterization of albumin–heparin surfaces

Carboxylated polystyrene (PS) beads (diameter 0.918 μm ± 0.020 μm, 0.12 meq COOH/g) were purchased from Polysciences Inc., Warrington, U.S.A. Albumin–heparin conjugates, based on bovine albumin, were purchased from Holland Biomaterials Group BV, Enschede, The Netherlands. The heparin contents of these conjugates were 14.5, 16.5, and 21.5% (w/w). These conjugates differ in their affinity for ATIII and are further referred to as Low Affinity (LA), Unfractionated (Un) and High Affinity (HA) alb-hep conjugates, respectively. Bovine albumin (Sigma) was treated with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC, Merck, Darmstadt, Germany) analogous to reaction conditions used for the preparation of the alb-hep conjugates.20 In short, albumin was added to a NaH₂PO₄ solution of pH 4.4 and dissolved overnight at 4°C. EDC was added in aliquots over a period of 3.5 h to a total of a 10 times molar excess over albumin. In contrast with the alb-hep conjugates EDC-treated albumin was not chromatographically purified but was dialyzed against water and freeze dried. EDC-treated albumin and untreated bovine albumin were used as controls throughout the experiments and henceforth will be referred to as Alb-EDC and Alb, respectively.

Alb-hep conjugates were immobilized onto the carboxylated polystyrene beads via preactivation of the surface with EDC. Before immobilization the beads were washed once with a NaHCO₃/Na₂CO₃ buffer (0.1 M, pH 9.6) and twice with a NaH₂PO₄ buffer (0.02 M, pH 4.5) and then suspended in the same phosphate buffer. Aliquots of 100 μL of EDC solution were added to heparin reference samples (168.5 U/mg, Bua Chemie, Castricum, The Netherlands) and different amounts of alb-hep beads. After 2 h of incubation at room temperature the precipitated heparin–toluidine complex or the beads were removed by centrifugation. Depletion of the toluidine blue from solution was measured at 595 nm. The potentiating effect of alb-hep beads (3–5 cm²) on the inhibition of thrombin by ATIII was measured using a chromogenic substrate (S 2238) in an endpoint assay as described by Chandler et al.19 Loss of thrombin activity due to adsorption of thrombin to the heparinized material during the assay did not significantly affect the observed activity of the heparinized surfaces. Similar values for the activity/cm² were obtained when the activity of the heparinized surfaces was determined using different amounts of surface per assay.

Stability of the alb-hep-coated surfaces

The stability of the albumin or alb-hep conjugate coating was investigated using 14C-labeled albumin and 14C-labeled alb-hep conjugates. The labeling was carried out using 14C-formaldehyde according to the procedure as described for the labeling of ATIII. The conjugates were freeze dried after dialysis against distilled water. The labeled conjugates were covalently immobilized onto the polystyrene beads preactivated with EDC, as described earlier. Control surfaces were prepared by immobilization of 14C-labeled albumin or 14C-labeled (Un) alb-hep conjugate without preactivation of the surface with EDC. The surface concentration of conjugate or albumin was determined by measuring the radioactivity on the beads and by performing a toluidine blue assay. The stability of the different coatings upon storage of the beads in PBS at 5°C was investigated by measuring the radioactivity of the supernatant after 10 weeks. The stability in a 2% SDS solution in PBS was measured by adding 1 mL SDS solution to 100 cm² of coated beads. After 16 h of incubation at room temperature, the desorption of conjugate or albumin was examined by determining the radioactivity of the supernatant.

ATIII binding kinetics

Interaction of 14C-labeled ATIII with beads coated with different alb-hep conjugates in albumin or
plasma solutions was measured at 37°C under stirring using an orbital shaker (Ika-Labortechnik, Staufen, Germany). ATIII and albumin in PBS pH 7.4 were mixed in 1-mL tubes after which alb-hep beads (60 cm²) were added. The final concentrations of the proteins were 100 µg/mL and 27 mg/mL for ATIII and albumin, respectively. ¹⁴C-ATIII containing plasma was diluted with the bead suspension and PBS, resulting in a 67% (v/v) dilution of all proteins present and thus in a concentration of ATIII of 100 µg/mL. After different incubation times (1–90 min) the beads were centrifuged and washed 3 times with PBS. The beads were transferred into a scintillation vial, and the radioactivity on the beads was determined as described earlier.

**Exchange of preadsorbed ¹⁴C-labeled ATIII by unlabeled ATIII**

The exchange of radiolabeled ATIII preadsorbed on alb-hep beads was studied by exposing these beads to a solution of unlabeled ATIII in PBS. Beads bearing a coating of (Un) alb-hep conjugates were incubated in a PBS solution containing labeled ATIII (100 µg/mL) and albumin (27 mg/mL) or in a 67% (v/v) plasma dilution for 30 min at 37°C. The beads were washed 3 times with PBS and resuspended in 0.5 mL of a PBS solution of unlabeled ATIII, pH 7.4 (500 µg/mL). The unlabeled ATIII was from the same source as described for the labeled protein and was purified in the same way. Samples of the supernatant were taken during incubation at 37°C at different time intervals to determine the release of labeled ATIII.

**ATIII binding isotherms**

ATIII binding isotherms were measured to determine the affinity of ATIII for the alb-hep surfaces. Radiolabeled ATIII in albumin or plasma solutions was diluted with PBS in a range from 10⁻⁶ to 10⁻⁹ M. The ratio between ATIII and albumin and all the other plasma proteins was kept constant. Alb-hep beads were added to the ATIII solutions and incubated for 1 h at 37°C. The beads were washed 3 times with PBS and the radioactivity on the beads was measured. Control experiments were carried out to determine the affinity of ATIII for beads having an Alb-EDC coating. The ATIII binding isotherms were modeled using two different equations (i.e., the classical Langmuir equation and a summation of two Langmuir equations) to distinguish between ATIII binding sites on the surface with different affinities for ATIII. An iteration program for curve fitting (MultiFit 1.5, Day Computing, Cambridge, U.K.) was used to calculate the association constants and the maximal ATIII surface concentrations for the different isotherms.

**RESULTS**

**Purification and characterization of ¹⁴C-labeled ATIII**

A typical chromatogram of the elution of labeled ATIII from a heparin–Sepharose column showed three peaks (Fig. 1). From SDS–gel electrophoresis it became clear that the first small peak contained IgG, probably traces against hepatitis added by the supplier. The second peak consists of ATIII molecules eluted at relatively low ionic strength. After about 150 mL of eluent was collected, the NaCl concentration was increased to 1.8 M. This resulted in a third peak consisting of ATIII molecules with a high affinity for heparin–Sepharose. Both ATIII peaks gave one single band at about 60 kD in a SDS–gel electrophoresis experiment.

The radioactivity of the eluent also showed three peaks with a pattern corresponding to that shown in Fig. 1 (not shown). The third fraction was pooled, dialyzed and stored at -30°C. In all further experiments ATIII from this fraction having a high affinity for heparin was used. The column was checked for possible overloading, but appeared not to be overloaded. Moreover a similar elution pattern was observed when unlabeled ATIII was used. To detect any change in the affinity of ATIII for the heparin–Sepharose upon labeling, labeled ATIII was eluted on a heparin–Sepharose column applying a NaCl gradient (Fig. 2). An increase in the applied ionic strength is followed by elution of a fraction of the bound ATIII. An identical elution pattern was observed using un-
labeled ATIII. When the absorbances at 280 nm of the fractions of the labeled ATIII and the unlabeled ATIII were plotted against each other, a straight line was obtained, indicating no detectable change in the binding between ATIII to heparin upon labeling.

The labeled ATIII was further characterized in a thrombin inhibition assay. No statistical difference could be observed using labeled and unlabeled ATIII in this assay. Possible preferential binding of labeled ATIII to an albumin-heparin coated surface was checked by mixing unlabeled and labeled ATIII in different ratios and exposing these mixtures to alb-hep beads. The result is shown in Figure 3. The amount of ATIII on the alb-hep surface is proportional to the ratio between labeled and unlabeled ATIII, indicating that no preferential binding occurred.

Characterization and stability of the alb-hep-coated surfaces

The characteristics of the alb-hep and control beads are reported in Table I. The amount of immobilized conjugate according to the toluidine blue assay ranged from 0.20 to 0.29 µg/cm². The surface concentration of conjugate appeared to be dependent on the fraction of heparin in the conjugate. A higher fraction of heparin results in a lower surface concentration.

The biological activity displayed by the heparinized beads is dependent on the type of immobilized conjugate: higher ATIII affinities of the surface immobilized conjugates resulted in a higher activity displayed by the beads. The activity of all control beads was approximately zero. The binding of toluidine blue to Alb-EDC-beads was negligible. In contrast Alb–beads and especially bare polystyrene beads did bind significant amounts of toluidine blue. The binding of toluidine blue to these control beads was expressed in terms of µg Un alb–hep conjugate/cm².

The validation of the toluidine blue assay and the stability of the alb–hep coating was investigated by using 14C-labeled conjugates. The surface concentration according to the toluidine blue assay of the labeled conjugates are somewhat lower but in the same range as found for the unlabeled conjugates (Table II). The values obtained with the toluidine blue test are in good agreement with the results found by radioactivity measurements. Both labeled and unlabeled conjugates show a similar trend in surface concentration: a higher heparin content in the alb–hep conjugate results in a lower surface concentration. The surface concentration of labeled albumin (0.14 µg/cm²) is below a monolayer coverage (0.2–0.8 µg/cm²) of protein.

The majority of the alb–hep conjugates immobilized using EDC is covalently coupled onto the surface. Less than 2% of these conjugates was found in the supernatant after 10 weeks storage at 5°C in PBS or after treatment of these surfaces with a 2% SDS solution. Conjugates that are immobilized without preactivating the surface with EDC are physically adsorbed and not covalently attached to the surface. These coatings are not stable.

Figure 2. Elution profile of 14C-labeled ATIII from heparin-Sepharose using a NaCl gradient in phosphate buffer.

Figure 3. The surface concentration of 14C-ATIII on polystyrene beads coated with alb–hep (HA) conjugates as function of the ratio between labeled and unlabeled ATIII in solution.

**TABLE I**

<table>
<thead>
<tr>
<th>Type of Coating</th>
<th>Hep Content of Conj. (w/w (%))</th>
<th>Thromb. Inh. Assay (mU hep/cm²)</th>
<th>Tol. Blue Assay (µg conj/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>alb-hep (HA)</td>
<td>21.5</td>
<td>1.48 ± 0.178</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>alb-hep (Un)</td>
<td>16.5</td>
<td>0.681 ± 0.105</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>alb-hep (LA)</td>
<td>14.5</td>
<td>0.360 ± 0.051</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>Alb</td>
<td>0</td>
<td>~0</td>
<td>0.10* ± 0.02</td>
</tr>
<tr>
<td>Alb-EDC</td>
<td>0</td>
<td>~0</td>
<td>0.01* ± 0.01</td>
</tr>
<tr>
<td>PS</td>
<td>—</td>
<td>~0</td>
<td>0.40* ± 0.02</td>
</tr>
</tbody>
</table>

Experimental values: n = 4–7, ±SD.

*Expressed in terms of µg Un alb–hep conjugates/cm².
TABLE II
Stability and Surface Concentration of 14C-labeled Albumin–Heparin Conjugates Coupled onto Functionalized Polystyrene Beads

<table>
<thead>
<tr>
<th>Type of Coating</th>
<th>Surface Activation*</th>
<th>Radioactivity (µg/cm²)</th>
<th>Tol. Blue (µg/cm²)</th>
<th>2% SDS Storage (%)</th>
<th>Storage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>alb-hep (HA)</td>
<td>yes</td>
<td>0.179 ± 0.012</td>
<td>0.18 ± 0.01</td>
<td>1.9 ± 0.1</td>
<td>0.64 ± 0.1</td>
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<tr>
<td>alb-hep (Un)</td>
<td>yes</td>
<td>0.201 ± 0.010</td>
<td>0.22 ± 0.01</td>
<td>1.9 ± 0.1</td>
<td>0.67 ± 0.1</td>
</tr>
<tr>
<td>alb-hep (LA)</td>
<td>yes</td>
<td>0.210 ± 0.012</td>
<td>0.24 ± 0.01</td>
<td>1.9 ± 0.2</td>
<td>0.68 ± 0.1</td>
</tr>
<tr>
<td>Alb</td>
<td>yes</td>
<td>0.143 ± 0.007</td>
<td>0.128 ± 0.01</td>
<td>3.3 ± 0.2</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Alb–EDC</td>
<td>yes</td>
<td>0.194 ± 0.020</td>
<td>nd</td>
<td>3.0 ± 0.3</td>
<td>nd</td>
</tr>
<tr>
<td>alb-hep (Un)</td>
<td>no</td>
<td>0.191 ± 0.009</td>
<td>0.24 ± 0.01</td>
<td>58.2 ± 2.5</td>
<td>61.1 ± 1.2</td>
</tr>
<tr>
<td>Alb</td>
<td>no</td>
<td>0.158 ± 0.012</td>
<td>nd</td>
<td>84.3 ± 4.0</td>
<td>8.9 ± 0.3</td>
</tr>
</tbody>
</table>

Experimental values: n = 3–6, ±SD.
*Reactivation of the carboxylated polystyrene surfaces with EDC.
†Desorption of conjugates after 16 h at RT in 2% SDS.
‡Desorption of conjugates after 10 weeks storage at 5°C in PBS.
§Expressed in terms of µg Un alb-hep conjugate/cm².

ATIII binding kinetics

The results of the binding kinetics of ATIII are presented in Figures 4 and 5. The adsorption of ATIII from albumin solutions is fast: more than 80% of the total amount of bound ATIII is adsorbed within the first minute. The surface concentration of ATIII reached a constant value after about 5–10 min. The plateau values of ATIII adsorbed on the different surfaces correlate with the affinity of the immobilized conjugates for ATIII. The plateau values are 3.1, 4.1, and 7.5 ng/cm² for beads containing LA, Un, and HA alb-hep conjugates, respectively.

ATIII adsorption from plasma solutions shows a high initial ATIII surface concentration after one minute of incubation, followed by a slight decrease at longer incubation times (Fig. 5). A constant surface concentration is reached after about 5–10 min of incubation. The plateau values for ATIII adsorbed from plasma solutions are 2–3 times less than the values obtained from corresponding experiments where ATIII is adsorbed from albumin solutions.

Exchange of preadsorbed 14C-labeled ATIII by unlabeled ATIII

The release of labeled ATIII molecules from beads that were preincubated in 14C-ATIII containing plasma or in albumin/14C-ATIII solutions is shown in Figure 6. After contacting the beads with a solution of unlabeled ATIII in PBS, the amount of labeled ATIII at the surface starts to decrease. A constant value is reached after approximately 10 min. About 70% of the initial amount of labeled ATIII molecules is released independent of the medium used for preincubation.

![Figure 4](image-url)  
**Figure 4.** Kinetics of the binding of ATIII from an albumin (26.7 mg/mL)–ATIII (100 µg/mL) mixture in PBS onto polystyrene beads containing different alb-hep conjugates. (■) HA alb-hep conjugates, (▲) Un alb-hep conjugates, (●) LA alb-hep conjugates.

![Figure 5](image-url)  
**Figure 5.** Kinetics of the binding of ATIII (100 µg/mL) from a 67% plasma dilution with PBS onto polystyrene beads containing different alb-hep conjugates. (■) HA alb-hep conjugates, (▲) Un alb-hep conjugates, (●) LA alb-hep conjugates.
**Figure 6.** Decrease of the surface concentration of $^{14}$C-ATIII adsorbed on polystyrene beads containing immobilized Un alb-hep conjugates after contacting the surface (100 cm$^2$) with a solution of unlabeled ATIII in PBS (500 µg/ML) for different time periods. (□) preadsorbing from an albumin-ATIII solution, (△) preadsorbing from a plasma dilution.

**ATIII binding isotherms**

ATIII-binding isotherms obtained using albumin solutions do not reach plateau values under the experimental conditions used (Fig. 7). Binding isotherms using plasma solutions show a high affinity character and seem to reach a plateau value at equilibrium concentrations of ATIII exceeding $10^{-6}$ M in solution (Fig. 8). The isotherms for the binding of ATIII onto the various surfaces were fitted with the classical Langmuir equation (1). An iteration program for curve fitting was used to calculate both the association constant and the maximal ATIII surface concentration.

![Graph](image1)

**Figure 7.** Isotherms for the binding of ATIII onto polystyrene beads coated with different alb-hep conjugates or Alb-EDC. (■) HA alb-hep conjugates, (▲) Un alb-hep conjugates, (●) LA alb-hep conjugates. ATIII binding took place from plasma dilutions with PBS, pH 7.4 at 37°C. $C_{\text{eq}}$ is the equilibrium concentration of ATIII in solution.

where: $C_s$ = ATIII surface concentration (ng/cm$^2$)

$C_{\text{max}}$ = maximal ATIII surface concentration (ng/cm$^2$)

$K$ = binding constant (L/mole)

$C_{\text{eq}}$ = ATIII equilibrium concentration in solution (mole/L)

Scatchard plots were obtained using values for $C_{\text{max}}$ calculated with the curve-fitting program. The slopes of these plots, based on the isotherms for ATIII using albumin solutions, change with the fraction of bound protein (Fig. 9). Scatchard plots based on isotherms obtained using plasma dilutions result in straight lines with a steep slope (Fig. 10). Small deviations from these lines are observed when the surface coverage is very low and when $C_s/C_{\text{max}}$ approaches 1. The adsorption of ATIII from plasma or albumin solutions onto control beads containing an Alb-EDC

![Graph](image2)

**Figure 8.** Isotherms for the binding of ATIII onto polystyrene beads coated with different alb-hep conjugates or Alb-EDC. (■) HA alb-hep conjugates, (▲) Un alb-hep conjugates, (●) LA alb-hep conjugates, (□) Alb-EDC. ATIII binding took place from plasma dilutions with PBS, pH 7.4 at 37°C. $C_{\text{eq}}$ is the equilibrium concentration of ATIII in solution.

![Graph](image3)

**Figure 9.** Scatchard plots for the binding of ATIII from albumin-ATIII mixtures in PBS onto polystyrene beads containing different alb-hep conjugates. (□) HA alb-hep conjugates, (△) Un alb-hep conjugates, (●) LA alb-hep conjugates. $v = C_s/C_{\text{max}}$. 

![Graph](image4)
coating was very small (Figs. 7 and 8). In this case no Langmuir isotherms of ATIII adsorption could be established.

The isotherms of ATIII using albumin solutions also were fitted with a summation of two Langmuir equations to discriminate between binding sites on the surface with different binding constants for ATIII, Equation (2). Each term describes a Langmuirian binding of ATIII to a set of binding sites with a certain affinity for ATIII. These two sets of sites are henceforth referred to as type 1 and type 2 binding sites. In this case four variables were used in the iteration program to fit the isotherms. These variables are two binding constants and two maximal surface concentrations for the two different sets of binding sites. A simplification of Equation (2) was used for the isotherms obtained using plasma dilutions. The contribution of the second Langmuir equation in Equation (2) using plasma dilutions appeared to be very small. No maximal surface concentration could be obtained with the iteration program. Therefore a term that describes a proportional increase of the ATIII surface concentration with an increasing equilibrium concentration of ATIII in solution is used [Eq. (3)].

\[
C_s = \frac{C_{\text{max1}}K_1C_{\text{eq}}}{1 + K_1C_{\text{eq}}} + \frac{C_{\text{max2}}K_2C_{\text{eq}}}{1 + K_2C_{\text{eq}}}
\]

(2)

\[
C_s = \frac{C_{\text{max1}}K_1C_{\text{eq}}}{1 + K_1C_{\text{eq}}} + AC_{\text{eq}}
\]

(3)

where: \(C_{\text{max1}}\) = maximal ATIII surface concentration on type 1-binding site (ng/cm²)

\(K_1\) = ATIII binding constant to type 1-binding site (l/mole)

\(A\) = constant (ng L/mole cm²)

Table III shows the results of the modeling of the ATIII-binding isotherms with Equations (1) and (2) or (3) in terms of the residual sum of squares (RSS) and the calculated values for the different binding constants.

From the RSS values in Table III it is clear that a summation of two equations results in a better description of the isotherms than a single Langmuir equation. This is most pronounced for the binding of ATIII from albumin solutions onto the different alb-hep surfaces. When Equation (2) is used, two average binding constants are obtained for the two sets of binding sites for ATIII. \(K_1\) describes the binding of ATIII to binding sites with a rather high affinity for ATIII, while \(K_2\) accounts for binding of ATIII to sites with a lower affinity for the protein. The values for the constant \(A\) are small, which indicates that there is only a minor contribution of the binding of ATIII from plasma solutions to low-affinity binding sites on the surface.

The \(K_1\) binding constant for the binding of ATIII adsorbed from albumin solutions to LA alb-hep beads is rather small. In this case the division into "high" and "low" affinity binding sites by the iter-
tion program results in a $K_1$ value of $4.85 \times 10^6$ M$^{-1}$, which is smaller than the other $K_1$ binding constants. A consequence of this fact would be that the maximal ATIII surface concentration of high affinity binding sites on LA alb-hep beads is higher than on beads bearing Un alb-hep conjugates, which is very unlikely. An average value of $K_1$ using the data for both media was calculated in which the $K_1$ value of LA alb-hep beads was not taken into account. This average $K_1$ value of $9 \times 10^6$ L/mol was further used in Equations (2) and (3) to compare the maximal amounts of available binding sites on the different alb-hep surfaces in the two incubation media. The results are given in Table IV and in Figures 11 and 12.

The RSS values were not, or were minimally, increased when a fixed value of $9 \times 10^6$ L/mol for $K_1$ was used compared to the results obtained when $K_1$ was variable (Table III). The average value of the binding constant for the interaction of ATIII using albumin solutions with sites having a relatively low affinity for ATIII is $0.33 \times 10^6$ L/mol. The maximal ATIII surface concentrations for the two sets of binding sites using albumin solutions ($C_{s_{\text{max}1}}$ and $C_{s_{\text{max}2}}$) are both dependent on the type of immobilized conjugate. More ATIII was bound to both sets of sites when the affinity of the immobilized conjugate for ATIII increases ($\text{HA} > \text{Un} > \text{LA}$). When ATIII is adsorbed from plasma dilutions, a similar trend is observed for $C_{s_{\text{max}}}$. Comparison of the values of $C_{s_{\text{max}}}$ for the binding of ATIII from plasma and albumin-ATIII solutions reveals that the values of $C_{s_{\text{max}}}$ for the binding of ATIII from plasma solutions are about 30% lower than the corresponding values for the binding from albumin solutions.

Figure 11 shows the results of the modeling for the isotherm obtained with albumin solutions onto surfaces containing HA alb-hep conjugate using Equation (2) and $K_1$ fixed at $9 \times 10^6$ L/mol. Besides the fit through the data points, curves are drawn using the data in Table IV. The curve fitted through the experimental data points is the summation of the two Langmuir isotherms with a high and a low binding constant for ATIII. Figure 12 shows the result of the modeling using Equation (3) and a fixed $K_1$ value of $9 \times 10^6$ L/mol for the binding of ATIII onto HA immobilized alb-hep conjugates using plasma dilutions. In this case the curves represent the two terms in Equations (3); the Langmuir equation and a proportional increase of $C_s$ with $C_{eq}$, respectively. The most striking difference between Figures 11 and 12 is the contribution of the second term, which describes the binding of ATIII to binding sites with a rather low affinity for ATIII. It becomes clear that the contribution of the binding of ATIII to binding sites with a low

### Table IV

**Modeling of the ATIII Binding Isotherms with a Summation of Two Langmuir Equations and a Fixed $K_1$ Value**

<table>
<thead>
<tr>
<th>Type of Coating</th>
<th>Incubation Medium</th>
<th>$C_{s_{\text{max}1}}$ (ng/cm$^2$)</th>
<th>$K_1 \times 10^6$ (L/mole)</th>
<th>$C_{s_{\text{max}2}}$ (ng/cm$^2$)</th>
<th>RSS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equation 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alb-hep (HA)</td>
<td>alb</td>
<td>2.91</td>
<td>0.330</td>
<td>9.88</td>
<td>0.0554</td>
</tr>
<tr>
<td>alb-hep (Un)</td>
<td>alb</td>
<td>1.42</td>
<td>0.333</td>
<td>7.00</td>
<td>0.0373</td>
</tr>
<tr>
<td>alb-hep (LA)</td>
<td>alb</td>
<td>1.05</td>
<td>0.338</td>
<td>4.23</td>
<td>0.0264</td>
</tr>
<tr>
<td>Equation 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alb-hep (HA)</td>
<td>Plasma</td>
<td>2.09</td>
<td>0.137</td>
<td>—</td>
<td>0.0095</td>
</tr>
<tr>
<td>alb-hep (Un)</td>
<td>Plasma</td>
<td>0.950</td>
<td>0.038</td>
<td>—</td>
<td>0.0042</td>
</tr>
<tr>
<td>alb-hep (LA)</td>
<td>Plasma</td>
<td>0.655</td>
<td>0.074</td>
<td>—</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

$K_1 = 9.10^6$ L/mol.

*RSS = residual sum of squares.
Figure 12. Modeling of the binding of ATIII from plasma dilutions onto polystyrene beads coated with HA alb-hep conjugates using Equation 3. (A) represents the fit through the data points. (B) and (C) represent the Langmuirian binding of ATIII to two different sets of binding sites on the surface having a high (C) or low (B) affinity for ATIII.

affinity for ATIII to the total amount of bound ATIII is much smaller when ATIII is adsorbed from plasma dilutions than when albumin solutions are used.

DISCUSSION

In this study 14C-labeled and purified ATIII was used to investigate in detail the different interactions of ATIII with a heparinized surface. The labeled ATIII showed no preferential binding over unlabeled ATIII for binding to polystyrene beads containing HA alb-hep conjugates and therefore was regarded as biologically active in binding to heparin.

The immobilization of alb-hep conjugates on carboxylated polystyrene beads by means of preactivation of the surface with EDC resulted in a stable coating with a surface concentration of 0.2-0.3 μg/cm². The heparin activity per surface area was dependent on the type of the immobilized conjugate. The anticoagulant activity of the surfaces increased in the order LA, Un, HA while the surface concentration of conjugate decreased in the same order. The differences in surface concentration (0.20, 0.25, and 0.29 μg/cm² for HA, Un, and LA alb-hep surfaces, respectively) may be explained by the occurrence of electrostatic repulsion during coupling. An increased heparin content in the alb-hep conjugates results in an increased repulsion during coupling, first between negatively charged conjugates in solution and bare spots on the negatively charged surface, and, second, between conjugates in solution and spots already covered with conjugates. This leads to a lower surface concentration for conjugates having a higher heparin content. The depletion of the toluidine blue solution by bare polystyrene beads can be explained by the anionic character of the polymer surface. Coloring of beads containing a coating of albumin (0.14 μg/cm²) is probably caused by binding of toluidine blue to bare spots on the surface. The toluidine blue assay to determine the surface concentration of alb-hep conjugates therefore can be applied only with polystyrene beads having a monolayer coverage of conjugate on the surface.

ATIII binding kinetics

The binding of ATIII to alb-hep surfaces was very fast. Within a few minutes a plateau value was reached. When ATIII was adsorbed from a 67% plasma dilution in PBS, an initial increase of the ATIII surface concentration was followed by a slight decrease. This phenomenon may be explained by the "Vroman" effect.22-24 The "Vroman" effect involves the complex change of the protein composition on a solid surface as a function of time, due to differences in protein concentration, adsorption energy, and diffusion coefficients. In time a fraction of the initially bound ATIII, a relatively small protein of 58.2 kDa, is desorbed by other plasma proteins. Desorption of initially bound ATIII molecules in combination with direct competition of ATIII with other plasma proteins for binding sites at the surface results in plateau values of ATIII that are about 2-3 times lower using plasma dilutions than the surface concentrations found for ATIII adsorbed from albumin solutions. The capacity of the alb-hep surfaces to bind ATIII from plasma ranged from 0.8 to 2.2 ng/cm², which is only a few percent of a theoretical monolayer of ATIII. These values correspond well with values for the binding of ATIII from plasma to different polymethacrylate surfaces coated sequentially with albumin and heparin as reported by Lindon et al.5

Only a very small fraction of the immobilized heparin is able to bind ATIII. The ATIII surface concentrations, combined with the surface concentration of (HA) alb-hep conjugates reported in Table I, result in an alb-hep/ATIII ratio (mole/mole) on the surface of 20:1 to 65:1 using albumin or plasma solutions, respectively. The low ATIII binding capacity of the alb-hep surfaces may be attributed to the immobilization procedure of heparin. Heparin is covalently attached to the albumin in the presence of an excess of EDC. This may lead to more than one covalent bond between heparin and the protein, which would result in chemical modification of the heparin and in a reduced segmental flexibility that would affect the binding of ATIII. A further decrease in the ATIII binding capacity of the alb-hep conjugates can be expected after coupling onto the polystyrene surface.

The ATIII binding capacity and the anticoagulant activity displayed by the heparinized surfaces are a
function of the type of immobilized conjugate; hence a correlation exists between the binding capacity of the surfaces for ATIII and their biological activity. Similar observations were made by Hennink et al., who studied the interaction of ATIII with polystyrene surfaces preadsorbed with different alb-hep conjugates.\(^{16,25}\)

**ATIII binding isotherms**

The ATIII binding isotherms were modeled using the Langmuir equation. One of the conditions for the use of a Langmuir equation is reversibility of binding. Although the interaction between heparin and ATIII can be regarded as a kind of ligand–receptor binding, about 30% of initially surface bound (labeled) ATIII could not be exchanged by unlabelled ATIII. Thus care has to be taken in the interpretation of the results of the modeling because not all of the conditions are met for using the Langmuir equation. Scatchard plots of the isotherms obtained from the adsorption of ATIII from albumin solutions showed a concave up plot. This may be explained either by the presence of a variety of binding sites on the surfaces having different binding constants for ATIII or by lateral repulsion between surface-bound ATIII molecules. The latter is not very likely because of the low surface occupancy of ATIII. The presence of different binding sites for ATIII may be explained by the heterogeneity of the immobilized heparin, the influence of the immobilization procedure on the mobility of heparin, and the accessibility of heparin for ATIII. These findings can be compared with the binding of ATIII on heparin–Sepharose at different ionic strengths. Figure 2 shows that heparin–Sepharose contains binding sites from which ATIII could be eluted at different ionic strengths. This also may be explained by the presence of binding sites with different affinity for ATIII. Scatchard plots based on isotherms obtained from the adsorption of ATIII from plasma dilutions show straight lines, with only small deviations at low and high surface occupancy by ATIII. This indicates that ATIII in the presence of plasma proteins is bound to binding sites with an almost uniform affinity for ATIII. The binding constant is rather high, \(7 \times 10^6\) L/mol. In contrast, the average binding constant for the binding of ATIII using albumin solutions onto the same alb–hep coated surfaces is \(\pm 1 \times 10^6\) L/mol. These differences may be explained by the presence of high and low affinity binding sites for ATIII together with binding of other plasma proteins onto the heparinized surface. Plasma proteins will occupy potential ATIII binding sites resulting in a lower surface occupancy of ATIII. Only binding sites on the surface with a high affinity for ATIII retain their capacity to bind ATIII in the presence of adsorbed plasma proteins.

ATIII binding isotherms for the adsorption of ATIII from PBS solutions containing albumin and ATIII also were modeled using a summation of two Langmuir equations' Equation (2). Equation (3) was used to describe the ATIII adsorption from plasma dilutions. The binding constant that describes the binding of ATIII to binding sites with a rather high affinity for ATIII \((K_1)\) was about \(9 \times 10^6\) L/mol. This interaction may be regarded as the specific interaction between heparin, containing the pentasaccharide sequence, and ATIII. The value for \(K_1\) comes close to literature values of \(\pm 1 \times 10^7\) L/mol for binding of ATIII from plasma heparin–Sepharose.\(^26\) The binding constant for the binding of ATIII to binding sites with a low affinity for ATIII was about \(0.3 \times 10^6\) L/mol when albumin solutions were used. A similar value was found by Fougnot et al. for the binding of ATIII from buffer solutions to sulphonated polystyrene and sulphonate–glutamic acid sulphamide polystyrene, which are regarded as “heparin–like” surfaces.\(^27\) The maximal binding capacity of nonspecific ATIII binding sites \((C_{\text{max}})\) on alb–hep surfaces increases with increasing affinity of the immobilized conjugates for ATIII. Thus the binding of ATIII from albumin solutions to binding sites with a rather low affinity for ATIII can be attributed to nonspecific interaction between ATIII and the immobilized heparin. There is some adsorption of ATIII onto the Alb–EDC beads using albumin solutions. A small amount of the surface bound ATIII on the alb–hep surface therefore probably is adsorbed to sites not covered with heparin.

The nonspecific binding of ATIII to the HA alb–hep surfaces using plasma solutions is very small (Fig. 12) and is comparable to the adsorption of ATIII from plasma solutions to the Alb–EDC control surface (Fig. 8). These data suggest that nonspecific binding of ATIII on alb–hep surfaces in the presence of plasma proteins is low and not mediated by immobilized heparin on the surface.

The number of specific binding sites \((C_{\text{max}})\) that can bind ATIII in the presence of plasma proteins is about 30% less than those that can bind ATIII only in the presence of albumin. This must be ascribed to the plasma proteins on the heparinized surface partly neutralizing the interaction between ATIII and high affinity binding sites of the immobilized heparin.

In summary, the binding of ATIII to nonspecific binding sites on the surface is almost completely inhibited in the presence of plasma proteins and the interaction between ATIII and surface-immobilized heparin containing specific ATIII binding sites is inhibited to some extent. In this study a fixed amount of 200 μL (diluted) plasma is contacted with 60 cm\(^2\) heparinized surface. During incubation a depletion of
proteins that influence the ATIII-surface interaction may occur. It therefore can be speculated that the inhibition of the specific interaction between ATIII and surface-immobilized heparin may be higher when the surface is contacted with an excess of plasma.

Two different processes may contribute to the neutralization effect. First, the outcome of the competition of ATIII with other heparin binding proteins for the surface-immobilized heparin depends on the affinities of the involved proteins for heparin and their relative concentrations. The affinities of heparin-binding proteins such as HRGP and vitronectin for heparin at physiological pH and ionic strength are both high \((\pm 10^9 \text{ L/mole})\),\(^{9,13}\) whereas the affinity of ATIII for heparin is \(\pm 10^7 \text{ L/mole}\).\(^{22,28}\) The concentrations of heparin-binding proteins in this study are unknown because the depletion of ATIII from plasma using heparin-Sepharose probably also resulted in decreased concentrations of these proteins. The neutralization of the ATIII binding to the surface-immobilized heparin thus may be even larger at physiological concentrations of heparin-binding proteins. A second process that may influence the binding of ATIII is non-specific adsorption of plasma proteins on the surface. For instance, adsorption of plasma proteins onto sites at the surface not covered with heparin may have an effect on the accessibility of heparin for ATIII. Further, it may be speculated that the albumin molecule is denatured during preparation and immobilization of the conjugate. Adsorption of plasma proteins onto the albumin moiety of the conjugate therefore cannot be excluded.

The identification of the proteins involved and the impact of the neutralization of the ATIII binding on the anticoagulant activity displayed by the surface-immobilized heparin currently are being investigated.

CONCLUSIONS

Covalent coupling of alb-hep conjugates onto carboxylated polystyrene surfaces resulted in a stable coating that displayed anticoagulant activity. The interaction between ATIII and these alb-hep surfaces either in the presence of albumin or in plasma was investigated. It was demonstrated that three different interactions played a role in the binding of ATIII onto the heparinized surfaces. First, ATIII could bind with high affinity to immobilized heparin containing specific ATIII binding sites. Second, ATIII was bound to the heparinized surface mediated by nonspecific interactions between ATIII and the immobilized heparin. Third, a small fraction of the surface bound ATIII molecules probably was adsorbed to sites on the heparinized surface not covered with heparin. Plasma proteins were able to desorb initially bound ATIII, and they inhibited, to some extent, specific and non-specific interactions between ATIII and the immobilized heparin. Binding of ATIII to nonspecific binding sites having a rather low affinity for ATIII was almost completely inhibited in the presence of plasma proteins. Binding of ATIII to immobilized heparin containing specific ATIII binding sites with a high affinity for ATIII decreased 30% in the presence of plasma proteins in comparison with the binding of ATIII from albumin containing PBS. This reduction in ATIII binding is probably higher when more plasma is applied per surface area and when the plasma contains physiological concentrations of heparin-binding proteins.

It is concluded that the accessibility of immobilized heparin for ATIII is decreased by adsorption of plasma proteins onto the heparinized surface and/or by competition of ATIII with other heparin-binding proteins for the surface-immobilized heparin. The neutralization of the ATIII binding to surface-immobilized heparin in plasma may affect the biological activity and performance of the heparinized material when a correlation exists between the binding of ATIII to the heparinized surface and the anticoagulant activity displayed by the surface in plasma.

References


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