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Heparin-like functionalized polymer surfaces: discrimination between catalytic and adsorption processes during the course of thrombin inhibition

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Thrombus formation on blood-contacting artificial surfaces is a major problem. Antithrombogenic polymer surfaces have been obtained either by heparin binding, or by grafting sulphonate and/or amino acid sulphonamide groups on insoluble polystyrene. In addition to their capacity to adsorb thrombin, such surfaces were shown to be able to catalyse its inhibition by antithrombin III (AT), i.e. they are endowed with heparin-like activity. The results were mainly obtained by using clotting assays. In many cases, delineating adsorption and catalytic processes by such assays is not possible when evaluating anticoagulant polymer surfaces. To overcome this problem, the kinetics of thrombin adsorption and inhibitions by AT and heparin cofactor II (HC) in the presence of such surfaces have been measured by using an assay performed with a thrombin-specific chromogenic substrate. A simple kinetic model of thrombin consumption is proposed. The relevant calculations, carried out with the help of a computer program, lead to determination of relative second order rate constants of thrombin adsorption and inhibitions by AT and HC in the presence of the polymers. In addition to thrombin adsorption, polystyrene surfaces bearing only sulphonate groups catalyse inhibition by AT, whereas polystyrene surfaces bearing either aspartate, glycinate or isophthalate sulphonamide groups catalyse both inhibitions by AT and HC.

Keywords: Anticoagulants, polystyrene, heparin, thrombin, adsorption, catalytic inhibition

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The biocompatibility of the vascular system reflects the favourable interactions occurring between blood and a healthy vascular wall. On the contrary, the biocompatibility of the artificial surfaces reflects the different events occurring at their interface with blood[1]. Among these, formation of thrombus whether adherent or generating emboli on artificial material surfaces contacting non-anticoagulated blood, is a major problem. One way to solve this problem was proposed by Gott *et al.*[2] by introducing ionically bound heparin coatings. Since then many attempts have been made based on the concept that the surface contacting blood should become antithrombogenic. This was achieved by locally increasing the inactivation rate of the coagulation serine proteinases by their circulating inhibitors. This is generally the case when heparin is ionically bound to the surface, but as it is released, the use of such a surface is time-limited.

The inactivation rate of serine proteinases can also be increased when heparin is covalently bound to the surface but it depends on the method used for heparin binding, i.e. mainly upon the accessibility of its active sites[3–5]. Even if bound heparin is active, it is susceptible to biodegradation. To overcome these problems, the development of artificial 'heparin-like' polymer surfaces has been proposed. Among these, different surfaces derived from insoluble chlorosulphonated polystyrene bearing sulphonate and amino acid sulphonamide groups, have been prepared as beads[6] or modified tubings[7]. Interactions of such surfaces with thrombin and Factor Xa have been detected in the presence and absence of plasma or antithrombin III (AT)[8–11]. Such a concept has also been developed by different authors using different modified materials[12–14].

In a previous work[15], we have shown that heparin

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cofactor II (HC) can contribute to some extent to the anticoagulant properties of some functionalized polystyrene surfaces. In the present paper, we will show first that the relative contribution of HC on thrombin inhibition, measured in plasmatic suspensions of such polymers, depends upon their variable compositions. Such determinations performed by means of clotting methods reflect the global interactions between the polymer surface, fibrinogen, thrombin and its inhibitors. Thus, they are not sufficient to delineate the relative contributions of true catalytic, i.e. heparin-like, and simple adsorption phenomena occurring on polymer surfaces which delay thrombin-induced clotting. With this aim in view, the kinetics of thrombin adsorption and/or inactivation by its inhibitors was measured in the presence of functionalized polystyrene surfaces by means of an assay performed with a thrombin-specific chromogenic substrate. Using a computer program capable of simulating kinetic models, second order rate constants of the reactions of thrombin adsorption and inactivation were calculated in a simple but realistic model. The use of such a method can contribute strongly to the discrimination between simple thrombin or factor Xa adsorption which readily occurs in the presence of negatively charged polymer surfaces and catalytic processes involving the inhibitors, which can occur simultaneously and less frequently.

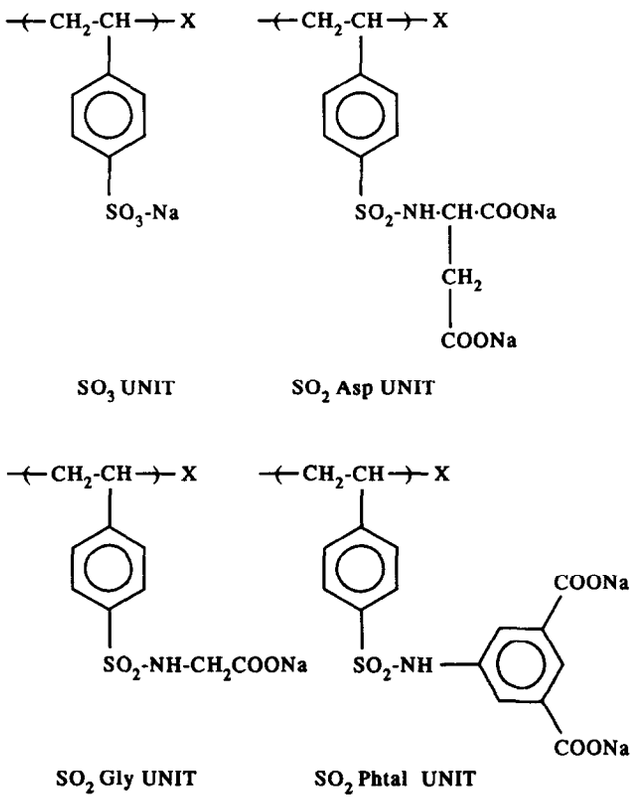

Figure 1 Chemical structures of the elementary units present in the polymers. The polymers are cross-linked (×). $PSSO_3$ is polystyrene beads including only SO_3 units; $PSSO_2Asp$, $PSSO_2Gly$ and $PSSO_2Phthal$ are polystyrene beads that include SO_3 and either SO_2Asp, SO_2Gly or $SO_2Phthal$ units respectively, randomly distributed.

MATERIALS AND METHODS

Polymers

The following polymers were synthesized from cross-linked polystyrene beads (Biobeads® SX2 from Biorad, Ivry, France) as previously described[6,16,17]: (a) polystyrene beads bearing only sodium sulphonate groups ($PSSO_3$), (b) polystyrene beads bearing sulphonate groups and an average of 0.56, 0.72, 0.78 sodium aspartate sulphonamide groups per phenyl ring (respectively $PSSO_2Asp0.6$, $PSSO_2Asp0.7$, $PSSO_2Asp0.8$), (c) polystyrene beads bearing sulphonate groups and an average of 0.59, 0.71, 0.78 sodium glycinate sulphonamide groups per phenyl ring (respectively $PSSO_2Gly0.6$, $PSSO_2$-$Gly0.7$, $PSSO_2Gly0.8$) and (d) polystyrene beads bearing sulphonate groups and an average of 0.40 sodium isophthalate sulphonamide groups per phenyl ring ($PSSO_2Phthal$) (*Figure 1*). Briefly, the synthesis needed three steps: (i) chlorosulphonation of polystyrene by chlorosulphonic acid, (ii) binding of the amino acid (only in the case of 5-amino isophthalic acid) or its dimethylester, (iii) saponification of the esters with sodium hydroxide. The polymers were isolated as sodium salts and dried under vacuum at 50°C. The chemical composition (*Table 1*) was assessed by elemental analysis of

Table 1 Chemical composition of the polymers

Polymer	Composition (mmol g^{-1})		Substitution degree (mol%)	
	SO_2AA*	SO_3Na	SO_2AA	SO_3Na
$PSSO_3$	0	4.29	0	100
$PSSO_2Asp0.6$†	1.73	1.37	56	44
$PSSO_2Asp0.7$	1.96	0.78	72	28
$PSSO_2Asp0.8$	2.30	0.65	78	22
$PSSO_2Gly0.6$	1.83	1.27	59	41
$PSSO_2Gly0.7$	2.67	1.11	71	29
$PSSO_2Gly0.8$	2.81	0.77	78	22
$PSSO_2Phthal$	1.44	2.16	40	60

*AA is a general abbreviation for amino acids.
† The number following the denomination of the polymer corresponds to the degree of substitution expressed in molar fraction of polystyrene units bearing one amino acid sulphonamide.
$PSSO_3$ = polystyrene beads bearing only sodium sulphonate groups.
$PSSO_2Asp.X$ = polystyrene beads bearing sulphonate groups and an average of X sodium aspartate sulphonamide groups per phenyl ring.
$PSSO_2GlyY$ = polystyrene beads bearing sulphonate groups and an average of Y sodium glycinate sulphonamide groups per phenyl ring.
$PSSO_2Phthal$ = polystyrene beads bearing sulphonate groups and an average of 0.40 sodium isophthalate sulphonamide groups per phenyl ring.

sulphur, sodium and nitrogen in every cases (Service Central de Microanalyse du CNRS, Vernaison, France) and by potentiometric titration of the carboxylic groups with an automatic titrator (Tacussel-Solea, Villeurbanne, France). After several treatments with 1.5 M sodium chloride, 1.0 M sodium citrate and buffer A, as described by Charef *et al.*[15], the samples were crushed to increase their surface:volume ratio. The fines were discarded to allow further separation of particles from plasmatic suspensions by only one short centrifugation before optical density determination. Suspensions of the polymers were prepared in buffer A and the content of polymers in the suspensions was calculated from the weight of the particles after drying aliquots at 50°C under vacuum and subtracting the weight of salts present in the suspension volume. Before testing the polymer anticoagulant activity, it was ensured that supernatants of the suspensions contained no activity.

The specific surface areas of the polymer samples were deliberately not determined. Comparison between polymers was performed as described further, by reference to their capacity to adsorb thrombin from solutions of similar thrombin concentration, i.e. determination of the number of available thrombin adsorption sites involved in such conditions.

Reagents and buffers

Buffer A was prepared from 26 mM sodium barbital, 26 mM sodium acetate and 0.1 M sodium chloride, to pH 7.3. Buffer B was prepared from 0.05 M tris (hydroxymethyl) aminomethane, 0.1 M sodium chloride at pH 8.4, 0.5 g bovine serum albumin (Sigma, St Louis, MO, USA) extemporaneously added per litre. Buffer B–ethylenediaminetetraacetic acid (EDTA) was prepared from buffer B by adding EDTA to make its final concentration 7×10^{-6} M. Platelet-poor plasma (PPP) was prepared from human citrated blood (1 volume 0.13 M sodium citrate for 9 volume blood) by centrifugation, stored at −80°C and thawed just before use. Antithrombin-depleted plasma (P-AT) was prepared from the above fresh plasma by affinity chromatography on an anti-AT Sepharose column as previously described[18].

Human purified thrombin (1100 NIH U mg^{-1} in coagulation experiments or 1600 NIH U mg^{-1} in kinetics experiments), chromogenic substrate (Chromo-Thrombin®) and human purified HC (10 U mg^{-1}) were provided by Diagnostica Stago (Asnières, France). Human purified AT (5 U mg^{-1}) was from Centre Régional de Transfusion Sanguine (Lille, France). Polybrene® was provided by Sigma.

Relative contribution of HC to thrombin consumption in plasmatic suspensions of polymers of variable compositions

Polymer suspensions (0.1 ml) in buffer A at increasing concentrations ranging from 0 to 400 mg polymer ml^{-1} suspension, were incubated for 30 min at 37°C with 0.2 ml of either PPP or P-AT. Then 0.1 ml thrombin (20 NIH U ml^{-1}) were added and the clotting time recorded. Residual thrombin amount was calculated by reference to a calibration curve obtained in the same test systems with either PPP or P-AT and with thrombin at different concentrations, in the absence of polymer. The amount of thrombin consumed was calculated and plotted versus polymer amount. The initial slopes defining antithrombic activity were determined for P-AT and PPP and the slopes ratio calculated.

Kinetics of thrombin inhibition and adsorption measured by an assay performed with a thrombin-specific chromogenic substrate

The experimental conditions were chosen according to the following rationale:

- As the catalytic process takes place on the surface of the polymers, a suitable surface area of polymer has to be introduced for the catalytic effect to be significant when compared with the reaction occurring in the absence of polymer.
- In order to allow a comparison between the polymers, the surface areas of contact between the polymers and the proteins have to be as close as possible to each other, and are evaluated by the number of available thrombin adsorption sites able to adsorb thrombin from solutions of similar thrombin concentration.
- The concentrations of antiproteinases have to be high enough to be efficient and low enough to obtain relatively low reaction rates of thrombin inhibition in the absence of polymers. In addition, comparison of plasma versus purified proteins implies the use of EDTA diluted plasma — which contains fibrinogen — in the presence of thrombin.
- As the kinetics of thrombin inhibition by AT and HC follows a second order equation, the initial concentrations of thrombin and antiproteinases are chosen equal or closely related on a molar basis.

A polymer suspension (0.15 ml) at given concentrations (11 mg ml^{-1} for $PSSO_3$ and 28 mg ml^{-1} for $PSSO_2$Asp0.8, $PSSO_2$Gly0.8 and $PSSO_2$Phthal) was added to 0.15 ml of either HC (0.10 U ml^{-1} in buffer B), AT (0.056 U ml^{-1} in buffer B), PPP (dilution 1/18 V/V in buffer B–EDTA) or buffer B. After 5 min at 37°C, 0.15 ml thrombin (18 NIH U ml^{-1} in buffer B) were added. The suspensions were regularly shaken and incubated for variable lengths of time (0.5; 1; 2; 3; 5 min); then 0.15 ml of either Polybrene solution (50 mg ml^{-1}) or buffer B were added. The mixture was centrifuged for 2 min at 5000 g in a centrifuge TH21 (MLW, Leipzig, Germany). An aliquot of 0.15 ml supernatant was added to a mixture of 0.30 ml buffer B and 0.15 ml chromogenic substrate (0.5 mg ml^{-1}), directly in the cell of a spectrophotometer 'Response' (Gilford, Ciba-Corning Diagnostics, Eragny sur Oise, France) thermostated at 37°C. The extent of substrate amidolysis, measured by the initial rate of the optical density increase at 405 nm, is proportional to the concentration of residual thrombin as determined on a calibration curve.

Calculation of the second order rate constants of thrombin inhibition

The model designed to calculate the constants from the experimental data should meet the following requirements:

- The model should take into account only thrombin concentration measurements because antiproteinases concentrations cannot easily be measured simultaneously and independently. Accordingly, antiproteinase adsorption onto polymer surfaces is not assumed to be a limiting step and is therefore not taken into account in the equations.
- The model should be as realistic as possible, with the simplest one fitting the data, including the initial thrombin concentration. As the reactions of thrombin inhibition by AT[19] and HC[20] are of a second order and as the initial concentrations of the reactants are similar or closely related, the result is that a simple logarithmic decay may not be retained.
- The reaction rate of thrombin spontaneous inhibition by AT cannot be neglected — contrary to that owing to HC — when compared with the reaction rates occurring on the polymer surfaces.
- Adsorption of thrombin occurring on either catalytic or noncatalytic polymer sites simultaneously with its inhibition is reversible in experiments of short duration and is a limiting step in the global mechanism of thrombin consumption.
- The polymers are compared on the basis of thrombin adsorption capacity in similar conditions of thrombin concentration.

A detailed rationale of the calculations is given in the Appendix to this paper. Briefly, four steps are necessary to calculate the normalized rate constants:

1. In the presence of thrombin and antiproteinases only, the rate constants k_{0AT} and k_{0HC} are determined.
2. In the presence of polymer and thrombin only, the number of available adsorbing sites P_0 and the adsorption rate constant k_{ads} are calculated (see *Figure 3* and *Table 2*.
3. In the presence of polymer, thrombin and antiproteinases, the apparent catalytic constants k_{catAT} and k_{catHC} are calculated (see *Figures 4* and *5*, and *Table 3*).
4. The apparent constants are normalized by taking into account the amount of polymer sites able to adsorb half of the initial amount of thrombin present in the assay (see *Table 3*).

RESULTS

Relative contribution of HC to thrombin consumption in plasmatic suspensions of polymers of variable compositions

In the presence of increasing amounts of polymers present in plasmatic suspensions to which a constant amount of thrombin is added, the clotting times are increasingly delayed, indicating that increasing amounts of thrombin are consumed. The antithrombic activity of each polymer is calculated as defined in 'Materials and Methods'. As shown in our previous paper[15], the antithrombic activity of $PSSO_3$ determined in P-AT is half of that determined in PPP. In contrast, the antithrombic activities of $PSSO_2Asp$ determined in both plasmatic mediums are similar when the substitution degree in amino acid is *ca* 80%. A similar result is found in the case of $PSSO_2Gly0.8$. In the presence of polymers bearing variable proportions of aspartate or glycinate sulphonamide and sulphonate groups, the ratio of the antithrombic activities respectively measured in P-AT and in PPP varies between these extreme values (*Figure 2*). In the presence of $PSSO_2Phthal$, similar antithrombic activity is measured in both media (not shown). The results indicate that the variable compositions of the polymers can modulate their antithrombic activity in a plasmatic medium through different interactions with both heparin cofactors. Nevertheless, the relative contributions of the catalysed reactions and of thrombin adsorption on the polymers, in thrombin consumption, cannot be delineated in such a clotting assay.

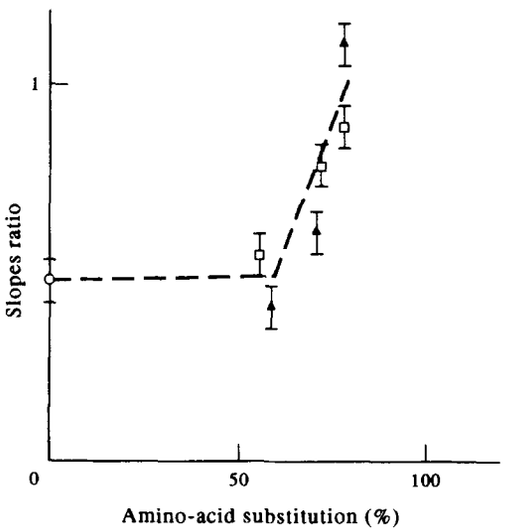

Figure 2 Relative antithrombic activity of polymers measured in the presence of either antithrombin-depleted plasma or platelet-poor plasma and expressed as slope ratios, as a function of their degree of substitution in amino acid: ○, polystyrene containing only SO_3 ($PSSO_3$); □, polystyrene containing sulphonate and aspartate sulphonamide ($PSSO_2Asp$); ▲, polystyrene containing sulphonate and glycinate sulphonamide ($PSSO_2Gly$). Each point is the mean of at least four determinations (± standard deviation).

Kinetics of thrombin inhibition and adsorption measured by an assay performed with a thrombin-specific chromogenic substrate

The relative contributions of the catalysed thrombin inhibition, i.e. heparin-like activity, and of thrombin adsorption on the polymers have been quantified in the presence of heparin cofactors by using an assay performed with a thrombin-specific chromogenic substrate. A constant amount of thrombin is added to polymer suspensions prepared in solutions of either purified AT, purified HC, diluted PPP or buffer. After variable periods of incubation followed by addition of Polybrene or buffer and centrifugation to remove the polymer, the concentration of residual thrombin in the supernatants is measured by its ability to lyse a specific chromogenic substrate.

In order to make a comparison between the polymers

and to determine thrombin adsorption parameters, thrombin adsorption on the polymers is first determined in the absence of antiproteinases. After a given duration of contact with polymers suspended in buffer and centrifugation, the concentration of thrombin remaining in the supernatants is measured and plotted against time (*Figure 3*). After calculations as detailed in 'Materials and Methods', the rate constant for thrombin adsorption (k_{ads}), the number of adsorbing sites on the polymers per litre of suspension (P_0), the concentration (T_∞) of thrombin remaining in solution at equilibrium and the amount (n_{Tads}) of thrombin adsorbed at equilibrium are determined for each polymer sample and are reported in *Table 2*. It was verified that the treatment with Polybrene was sufficient to recover the initial amount of thrombin by desorption from the polymers.

After incubation of thrombin with the polymers in the presence of antiproteinases, followed by addition of Polybrene and centrifugation, the concentration of thrombin remaining in solution is plotted against time in every case and the results reported on *Figure 4* for HC and on *Figure 5* for diluted PPP.

When Polybrene is replaced by buffer added after a nominal incubation time, thrombin consumption is the sum of the reactions of thrombin with its inhibitors, whether catalysed or uncatalysed, and of the adsorption of thrombin on the polymer surface in the presence of other proteins present in the mixture.

When Polybrene is added, the catalysed reaction is quenched and the adsorbed thrombin is desorbed from the polymer surface. Thus thrombin consumption measured in this case, approximately results only from the reactions of thrombin with its inhibitors. Comparison of the data obtained in the presence or absence of Polybrene clearly shows that thrombin adsorption represents a large part of thrombin consumption in the experimental conditions that were used (*Figure 6*).

The hypothesis that thrombin decrease resulted only from the sum of the adsorption process and the spontaneous inhibition of thrombin by antiproteinases, i.e. without catalysis, was simulated by calculation. *Equation (A5)* (see Appendix) was used with k_{catHC} and $k_{catAT} = 0$ and 1/18 diluted plasmatic concentrations of antiproteinases. The resulting curves generated were compared with the experimental data obtained in the presence of Polybrene and the hypothesis was then rejected.

Table 2 Thrombin adsorption on polymer samples in the absence of other proteins

Polymer	k_{ads} (μM^{-1} min^{-1})	P_0 (μM)	T_∞* (μM)	n_{Tads}† (pmol)
$PSSO_3$	2.00	0.310	0.0152	18.4
$PSSO_2Asp0.6$	1.85	0.231	0.0190	16.65
$PSSO_2Asp0.7$	0.857	0.267	0.0171	17.5
$PSSO_2Asp0.8$	5.82	0.082	0.0348	9.5
$PSSO_2Gly0.8$	2.93	0.27	0.0169	17.6
$PSSO_2Phthal$	0.61	1.13	0.0048	23.1

* T_∞ is calculated from the *Equation (A1)*: $K = (T_0 - T_\infty)/(P_0 - T_0 + T_\infty)$. T_∞
† $n_{Tads} = (T_0 - T_\infty)$. V, with $V = 0.45 \times 10^{-3}$ l
$PSSO_3$ = polystyrene containing SO_3 only.
$PSSO_2AspX$ = polystyrene containing SO_3 and X aspartate sulphonamide.
$PSSO_2GlyY$ = polystyrene containing SO_3 and Y glycinate sulphonamide.
$PSSO_2Phthal$ = polystyrene containing SO_3 and 0.40 isophthalate sulphonamide.

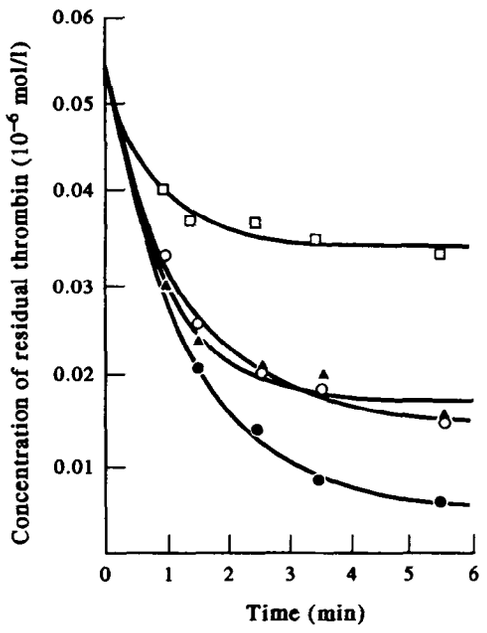

Figure 3 Kinetics of thrombin adsorption on either ○, 1.65 mg polystyrene containing only SO_3 ($PSSO_3$); □, 4.2 mg polystyrene containing sulphonate and 0.8 aspartate sulphonamide ($PSSO_2Asp0.8$); ▲, 4.2 mg polystyrene containing sulphonate and 0.8 glycinate sulphonamide ($PSSO_2Gly0.8$) or ●, 4.2 mg polystyrene containing sulphonate and 0.40 isophthalate sulphonamide ($PSSO_2Phthal$) in the absence of other proteins. Each point is the mean of at least two determinations. The curves are calculated from *Equation (A3)* (see Appendix) with t = (incubation time + 0.5 min).

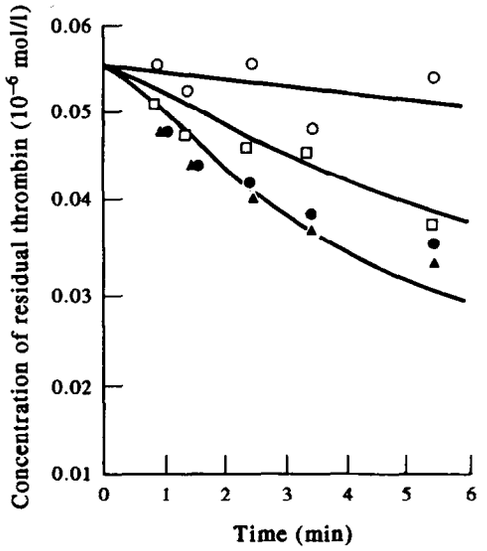

Figure 4 Kinetics of thrombin inactivation by HC in the presence of either ○, 1.65 mg polystyrene containing only SO_3 ($PSSO_3$); □, 4.2 mg polystyrene containing sulphonate and 0.8 aspartate sulphonamide ($PSSO_2Asp0.8$); ▲, 4.2 mg polystyrene containing sulphonate and 0.8 glycinate sulphonamide ($PSSO_2Gly0.8$) or ●, 4.2 mg polystyrene containing sulphonate and 0.40 isophthalate sulphonamide ($PSSO_2Phthal$), followed by incubation with Polybrene. Each point is the mean of at least two determinations. The curves are calculated from *Equation (A4)* (see Appendix).

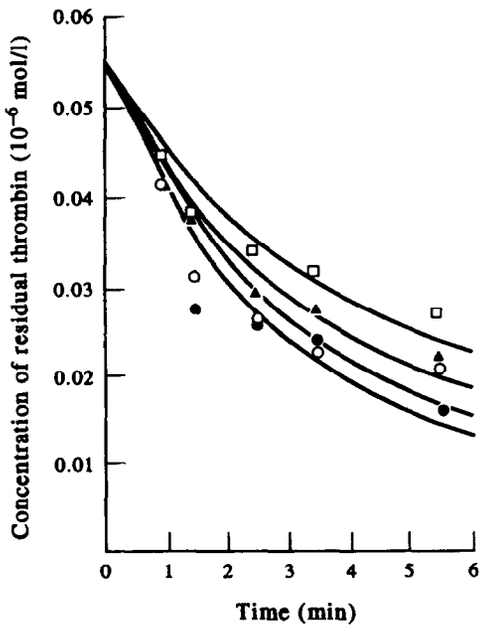

Figure 5 Kinetics of thrombin inactivation by diluted platelet-poor plasma in the presence of either ○, polystyrene containing only SO_3 ($PSSO_3$); □, polystyrene containing sulphonate and 0.8 aspartate sulphonamide ($PSSO_2Asp0.8$); ▲, polystyrene containing sulphonate and 0.8 glycinate sulphonamide ($PSSO_2Gly0.8$) or ●, polystyrene containing sulphonate and 0.40 isophthalate sulphonamide ($PSSO_2Phthal$), followed by incubation with Polybrene. Each point is the mean of at least two determinations. The curves are calculated from *Equation (A4)* (see Appendix).

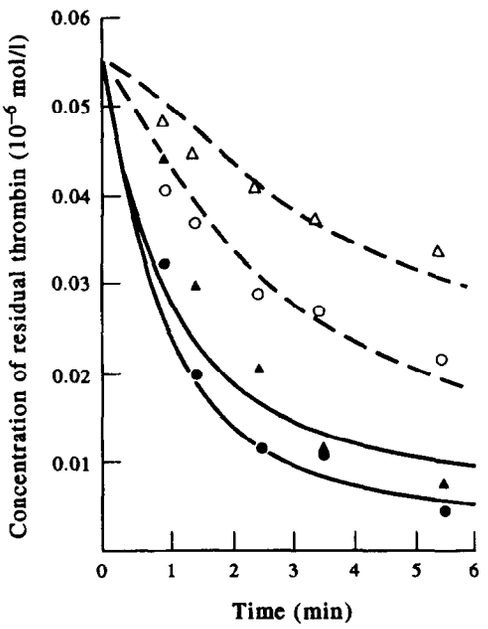

Figure 6 Kinetics of thrombin inactivation by heparin cofactor II (triangles) or by platelet-poor plasma (circles) in the presence of polystyrene containing sulphonate and 0.8 glycinate sulphonamide ($PSSO_2Gly0.8$), followed by incubation with Polybrene (open symbols and dashed lines), or with buffer (closed symbols and solid lines). The curves are calculated from *Equation (A4)*, (see Appendix).

The decrease of residual thrombin concentration after incubation of the polymers with HC and then thrombin is reported in *Figure 4*. The fit between the experimental points and the simulated curves generated from *Equation (A4)* (see Appendix) is good concerning the data obtained after Polybrene addition, as opposed to some data obtained without Polybrene. It can be seen that $PSSO_3$ has no significant catalytic effect on the inhibition of thrombin by HC. On the other hand, the reaction is catalysed by the polymers bearing amino acids. The apparent second order rate constants k_{HCapp}, calculated as described in detail in the Appendix, are reported in *Table 3*.

The decrease of residual thrombin concentration after incubation of the polymers with diluted PPP and then thrombin is reported in *Figure 5*. The same experiment made by using AT instead of PPP in the presence of either $PSSO_3$, $PSSO_2Asp0.8$ or $PSSO_2Gly0.8$ shows no significant difference between the results obtained with either AT or PPP. After calculation of the rate constants k_{ATapp}, it is clear that all the polymers tested increase the rate of inhibition of thrombin as reported on *Table 3*.

The amount of thrombin which can be adsorbed on the polymer samples in the absence of other proteins (n_{Tads}) is proportional to the number of sites which are active towards thrombin (both by catalysis and by simple adsorption), present in the assay on the surface of the polymers. This amount is being used to compare the effects of the different polymers by normalizing the constants. The concentration of thrombin remaining in

Table 3 Apparent second order rate constants of thrombin inhibition

Polymer	k before normalization, (μM^{-1} min^{-1})		Adsorbed thrombin (p mol)	k after normalization* (μM^{-1} min^{-1})	
	k_{HCapp}	k_{ATapp}	n_{Tads}	k_{HCnorm}	k_{ATnorm}
Buffer	<0.2	3.3	0	<0.2	3.3
$PSSO_3$	0.5	12.5	18.4	0.3	8.6
$PSSO_2Asp0.6$	3.0	7.5	16.65	2.3	5.7
$PSSO_2Asp0.7$	6.0	15	17.5	4.3	10.8
$PSSO_2Asp0.8$	6.0	7.5	9.5	8.0	10.0
$PSSO_2Gly0.8$	8.0	8.5	17.6	5.7	6.1
$PSSO_2Phthal$	7.0	12.5	23.1	3.8	6.8

* $k_{norm} = k_{app} \times 5.6 \times 10^{8} \times 0.45 \times 10^{3}/2 \times n_{Tads}$.
$PSSO_3$ = polystyrene containing SO_3 only.
$PSSO_2AspX$ = polystyrene containing sulphonate and X aspartate sulphonamide.
$PSSO_2GlyY$ = polystyrene containing sulphonate and Y glycinate sulphonamide.
$PSSO_2Phthal$ = polystyrene containing sulphonate and 0.40 isophthalate sulphonamide.

solution in the presence of the polymers is reported in *Figure 3*. The amount of thrombin adsorbed at equilibrium (n_{Tads}) is calculated from these data and *Equation (A3)*, and reported in *Table 3*. Given the amounts of polymers of different unknown granulometric characteristics that are used, it can be seen that n_{Tads} determined for $PSSO_2Asp0.8$ is inferior to half of the initial amount of thrombin present in the assay, whereas n_{Tads} exceeds this quantity for the other polymers. Concerning the calculation used for normalization, the result is that k_{norm} for $PSSO_2Asp0.8$ is above k_{app}, whereas the reverse is true for the other polymers.

After normalization, it is clear that although $PSSO_3$ has no catalytic effect towards HC, it increases the rate of thrombin inactivation by AT, while the other polymers increase both reaction rates at variable extents.

DISCUSSION AND CONCLUSIONS

Covalent binding of heparin onto polymer surfaces has been proposed as an appropriate way to prepare long-term antithrombogenic surfaces. On the one hand, unfavourable physical and biochemical changes can result from turning soluble heparin into a water-swollen but insoluble material surface. On the other hand, physical, chemical and biological degradations of this polysaccharide can occur. These reasons have led to the search for biostable 'heparin-like' polymer surfaces. Concerning coagulation, a surface with heparin-like activity should be able to increase (i.e. to catalyse) the rate of inactivation of at least thrombin and/or other activated coagulation factors by their circulating inhibitors, i.e. AT and/or HC.

To mimic heparin activity, different anionic groups have been bound to surfaces. However, evaluation of the actual catalytic capacity of such polyanionic surfaces is complicated by the fact that:

- they are able to readily adsorb significant amounts of thrombin and sometimes other proteases, e.g. Factor Xa, which can remain active when adsorbed or after desorption;
- they are able to readily adsorb significant amounts of AT and/or HC which can, however, remain inactive.

If adsorption of the reactants is a requirement for heterogeneous catalysis, it is far from sufficient to supply the surface with a catalytic capacity, but both adsorptions and catalysis occur simultaneously. Moreover, polymerization of fibrinogen into fibrin is usually modified by the presence of such charged surfaces. Because clotting time delay can be due to several of the above causes, the result is that clotting tests are not sufficient to delineate the relative contributions of heparin-like and adsorption properties of polymer surfaces, as shown further. More specific tests are available to measure thrombin activity independently of fibrinogen, but they require at least separation of the solid phase.

In order to determine the kinetics of thrombin consumption in the presence of each polymer surface, the following kinetic experiments were performed:

- with thrombin alone to determine the amount of thrombin adsorption sites which can be occupied on the surface at this thrombin concentration and the adsorption second order rate constant;
- with diluted PPP and thrombin to measure total thrombin consumption in the presence of the other plasmatic proteins;
- the same experiment except that Polybrene was added at the end to quench thrombin inactivation and adsorption on the surface and to desorb thrombin, in order to remove thrombin adsorption from total thrombin consumption;
- similar experiments with purified HC or AT instead of PPP.

The model chosen to calculate the second order rate constants from the data and compare the polymers obviously does not reflect the actual molecular mechanisms completely, because the fit with some data obtained without Polybrene addition is sometimes bad. However, the consistency between the calculated curves and the experimental data is good in the case of reactions with final Polybrene addition, which evidence heparin-like properties. A more sophisticated model would be more likely to be realistic but should need simultaneous and independent determinations of antiproteinases concentrations. In addition, the rather high scattering of data, which is linked to the heterogeneous nature of the system, limits the validation of a model when compared with another one. A simplest one, e.g. semi-logarithmic decay, does not comply with the requirements of second order reaction conditions. Therefore, it cannot be retained.

Despite its defects, the present model needs only thrombin concentration measurements after separation and is able to discriminate between thrombin adsorption and thrombin inhibition by AT and/or HC in the presence of different polymer surfaces of unknown specific surface areas. Of course our model could be improved by systematically varying some parameters such as concentrations of thrombin, antiproteinases, polymers or the nature of the coagulation activated factor. Some relationships between the composition of polymers and their antithrombic activities can be evidenced by bringing together the results obtained from clotting assays (shown on *Figure 2*) and those obtained from kinetic experiments (reported in *Figures 4–6* and *Table 3*).

$PSSO_3$ is not a catalyst of thrombin inhibition by HC (k_{HC} is similar to k_{0HC}). Thus it can be concluded that the important antithrombic effect evidenced in P-AT for $PSSO_3$ is owing only to thrombin adsorption occurring even in the presence of plasmatic proteins. It means that no plasmatic protein can compete with thrombin on its adsorption sites, as previously noted[21]. Very significant thrombin adsorption is also evidenced in kinetic experiments. Such a response is likely for $PSSO_2Asp$ or $PSSO_2Gly$ substituted at *ca.* 60% and below by amino acid sulphonamides. Indeed, in the presence of these polymers, thrombin consumption determined by using P-AT in the clotting assay is half that determined in PPP. First it means that these surfaces are active through AT catalysis, as already demonstrated, and reported in *Figure 5* and *Table 3*. It

also means that a possible weak activity mediated by HC on such surfaces cannot be evidenced by clotting assays, because it cannot be discriminated from a considerable thrombin adsorption. In fact, k_{HC} determined from kinetic experiments for $PSSO_2Asp0.6$ is significantly different from k_{0HC} but is still weak when compared with k_{AT}.

Above 60% substitution by amino acid sulphonamides, both clotting assays and kinetic experiments show that HC contributes to the inhibition of thrombin. It is noteworthy that similar results are obtained for PPP and P-AT in clotting assays in the case of the most substituted $PSSO_2Asp0.8$ or $PSSO_2Gly0.8$, but also in the case of $PSSO_2Phthal$ which is only 40% substituted by amino acid. These results most likely reflect the fact that the values of k_{HC} determined for these polymers are close or similar to the values of k_{AT}: the polymers are as active towards HC and AT. But other limiting factors could be involved, as there is no additivity of the activities in PPP, in which AT and HC concentrations are different. This is also the case in kinetic experiments when AT and PPP are used at similar dilution (1/18). An explanation for this lack of additivity was examined via simulation with our model. Curves representing thrombin decay were generated from *Equation (A5)* (see Appendix) by using the concentrations of AT and HC in diluted PPP and the kinetic constants determined for $PSSO_2Asp0.8$. No significant difference was found between the simulated curves obtained in AT and PPP. This result can be attributed to the fact that:

(a) plasmatic concentration of HC is lower than AT concentration,
(b) both antiproteinases compete for the same amount of thrombin,
(c) thrombin adsorption could limit the whole rate.

In conclusion, the results obtained by both methods for the measurement of antithrombic activity lead to complementary information. The clotting assays give global answers under plasmatic conditions, i.e. in the presence of all plasmatic proteins at rather high concentration. The great advantage of the kinetic measurements performed with the chromogenic assay is that catalytic processes are evidenced at least when the uncatalysed reaction is not too fast, as is the case with HC. In the case of AT, more discriminatory conditions have to be chosen. However, direct comparison between both types of experiments should be considered carefully because the experimental conditions are different; for instance, concerning the concentrations of the proteins and the very short duration of interactions between the polymers and the proteins in clotting assays.

Concerning structure/activity relationships with, at least the polystyrene backbone, our conclusions are that:

(a) the sites implied in the catalysis of the reaction between HC and thrombin include the carboxylic groups present in $PSSO_2Phthal$, $PSSO_2Asp$ or $PSSO_2Gly$, but does not include the sulphonate groups.
(b) the reaction between AT and thrombin is catalysed by sulphonate and these amino acid sulphonamides groups, but does not include isolated carboxylic groups directly borne by phenyl rings as previously observed on carboxylated polystyrene[16].

The catalytic capacity of these surface-borne groups on both antiproteinases is a promising feature with regard to their possible use for the design of antithrombogenic surfaces in contact with blood.

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APPENDIX

Calculation of the second order rate constants of thrombin inhibition

The initial concentrations of thrombin (T), antithrombin (AT) and heparin cofactor II (HC) were respectively:

$T_0 = AT_0 = 5.6 \times 10^{-8}$ M and $HC_0 = 4.0 \times 10^{-8}$ M in the systems with purified proteins

$T_0 = AT_0 = 5.6 \times 10^{-8}$ M and $HC_0 = 2.2 \times 10^{-8}$ M in the systems with diluted plasma.

In the absence of polymer

The rate of thrombin inhibition in solutions of purified proteins AT or HC, corresponding to the following schemes:

$$AT + T \longrightarrow AT - T \text{ or } HC + T \longrightarrow HC - T$$

This is given, respectively, by the equations:

$$-dT/dt = k_{0AT} \cdot AT.T \text{ or } -dT/dt = k_{0HC} \cdot HC.T$$

AT, T and HC are the respective concentrations of the free reactants in solution at time t, and k is the rate constant.

In the presence of polymer (P) and T

The rate of thrombin decrease in the supernatant (see *Figure 3*) depends on the respective concentrations of thrombin and adsorbing sites on the polymer and on their affinity for thrombin, according to the scheme:

$$T + P \rightleftharpoons PT$$

As the number of available adsorbing sites depends on the type and surface area of each polymer, the adsorption rate constant k_{ads} and the desorption rate constant k_{des} are apparent. At equilibrium, these constants are linked by the equilibrium constant K:

$$K = k_{ads}/k_{des} = (T_0 - T_\infty)/(P_0 - T_0 + T_\infty).T_\infty \qquad \text{(A1)}$$

with:

$$T_0 = T + PT$$
$$P_0 = P + PT$$

T_∞ being the concentration of free T at equilibrium. Thus:

$$-dT/dt = k_{ads} \cdot T.P - k_{des} \cdot P - T$$
$$= k_{ads} (T.P - 1/K.P - T) \qquad \text{(A2)}$$

For T and $PSSO_3$, K determined by Fougnot *et al.*[22] was found to be 10^7 M^{-1}, a value used to link the rate constants k_{ads} and k_{des}. After integrating *Equation (A2)* a final *Equation (A3)* is obtained:

$$T = T_0 - \frac{(b^2 + \Delta).[\text{Exp}\,(k_{ads} \cdot t.\Delta^{0.5}) - 1]}{2[(b + \Delta^{0.5}).\text{Exp}\,(k_{ads} \cdot t.\Delta^{0.5}) - (b - \Delta^{0.5})]} \qquad \text{(A3)}$$

where $b = T_0 + P_0 + 1/K$ and $\Delta = b^2 - 4T_0P_0$

In addition to the values of P_0 and k_{ads} which are unknown, it is clear that during the centrifugation process following the nominal incubation time, the adsorption process of T on P is not stopped. This results in a difference (t_{cent}) between the nominal time and the actual time corresponding to the end of the adsorption. In the first calculation step, the best values of (P_0 and k_{ads}), reported in *Table 2*, have been determined by least square fitting between the experimental data and *Equation (A3)*, for different arbitrary values of t_{cent}. The best fit has been found for 0.5 min. This value has been added to nominal times of incubation in the following results.

In the presence of polymer P, AT (and/or HC) and T

Decrease of thrombin concentration in the supernatant can result from the sum of different reactions. In the absence of a catalytic process, the simplest system is:

in solution: $AT + T \rightarrow AT - T$
with rate constant k_{0AT}

on the polymer: $P + T \rightleftharpoons P - T$

In the presence of a catalytic process, we add:

$P - T + AT \rightarrow P + AT - T$ with rate constant k_{catAT}.

A similar system can be written with HC instead of AT. In the presence of purified AT (or HC), we put $x = P - T$ and $y = AT - T$ (or $HC - T$), with $x_0 = y_0 = 0$. The rates of P – T and AT – T increases are given by *Equation (A4)*:

$$dx/dt = k_{ads}.[(T_0 - x - y)(P_0 - x) - 1/K\,x] - k_{catAT}.x.(AT_0 - y)$$

$$dy/dt = k_{0AT}.(T_0 - x - y)(AT_0 - y) + k_{catAT}.x.(AT_0 - y) \quad \text{(A4)}$$

In the presence of diluted plasma, we put $x = P - T$, $y = AT - T$ and $z = HC - T$, with $x_0 = y_0 = z_0 = 0$. The rates of P – T, AT – T and HC – T increases are given by *Equation (A5)*:

$$dx/dt = k_{ads}[(T_0 - x - y - z)(P_0 - x) - 1/K\,x] - k_{catAT}.x.(AT_0 - y) - k_{catHC}.x.(HC_0 - z)$$

$$dy/dt = k_{0AT}[(T_0 - x - y - z)(AT_0 - y) + k_{catAT}.x.(AT_0 - y)$$

$$dz/dt = k_{catHC}.x.(HC_0 - z)$$

$$\text{with } k_{0HC} = 0 \quad \text{(A5)}$$

These equation systems were introduced in a computer (Sun Sparc II) able to generate model curves by using a software, Mathematica®[23]. After simulations using arbitrary values for k_{catHC}, and comparison of the calculated curves of T decrease with the experimental data (*Figure 4*), the values of k_{catHC} were deduced. A similar process was achieved in the case of AT (*Figure 5*).

As the surface areas of contact between the proteins and the polymers are not similar, the amount of thrombin which can be adsorbed on the polymers at equilibrium, in the absence of antiproteinases [$n_{Tads} = (T_0 - T_\infty).V$], is used to normalize the apparent rate constants in order to compare the catalytic activities of the polymers. This amount is calculated for each polymer from the data reported on *Figure 3* using *Equations (A1)* and *(A3)* (*Table 2*). The catalytic activity of the polymers expressed by the apparent rate constants (k_{app}) is reported to the amount of polymer sites able to adsorb half of the initial amount of thrombin present in the assay, $n_{T0} = T_0.V$. In this equation, V is the volume of the reactants in the system including (polymer suspension + antiproteinase solution + thrombin solution); k_{norm} results from the calculation. Thus

$$k_{norm} = k_{app}.n_{T0}/2.n_{Tads}$$

The calculated values are reported in *Table 3*.