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Study of haemodialysis materials: physico-chemical and biological characterization of EVALVA, EVAPA and heparinized EVAPA

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Partially hydrolyzed ethylene/vinyl acetate copolymers were modified by the covalent binding of a heparin-complexing polymer and further heparinized in order to improve their blood compatibility. These heparinizable polymeric materials (EVAPA) were obtained by a two-step reaction between an ethylene/vinyl alcohol/vinyl acetate (EVALVA) terpolymer, and the heparin complexing polymer N_2LL. The physico-chemical characterization of EVALVA, EVAPA and heparinized-EVAPA was carried out through thermal analysis, SEM, contact angle, potentiometric measurements, water uptake and FT-IR spectroscopic measurements. The biocompatibility of the above-mentioned samples was evaluated using *in vitro* methods, through the determination of heparin release in phosphate buffer solution (PBS) and in human plasma, and with the investigation of hemostasis activation.

1. Introduction

The interaction between blood and artificial devices results in the activation of a number of humoral and cellular processes involved in non-specific and specific recognition of foreign surfaces by the host. Among these, activation of the complement system appears to be a key event which renders many materials unsuitable for hemodialysis. Nevertheless commercial membranes are still frequently used in spite of their poor biocompatibility.

For instance, it is well known that during hemodialysis with first use cellulose derivatives, activation occurs via the alternative pathway. Nevertheless cellulosic membranes are the most generally utilized because of the relatively low costs, the extensive clinical experience ground, the good ultra-filtration characteristics and the natural reluctance of manufacturers to change materials [1].

Thus the need for studies of alternative materials is mandatory, considering that a change in raw material is a decision to be made only after careful consideration. The ability of heparin-bound surfaces to inhibit alternative pathway activation may be of particular interest for the design of biocompatible surfaces [2]. Many researches appear, in the literature, to improve existing materials by linking heparin to their surfaces [3].

Recently ethylene vinyl alcohol copolymer (EVAL) membranes have been used for hemodialysis and seem very promising given their good blood compatibility. Nevertheless, a completely heparin-free dialysis with EVAL membranes is not possible [4].

Our study concerns the synthesis of a new material (EVAPA) consisting of EVALVA (a terpolymer of ethylene vinyl alcohol vinyl acetate) crosslinked with a heparin-complexing polymer the polyamidoammine N_2LL, and its physico-chemical characteristics and biological properties, in both native and heparinized form.

2. Experimental procedures

2.1. Materials

Ethylene/vinylacetate (EVA) copolymer (MW = 110 000, vinylacetate content 40 wt %) was purchased from Janssen Chimica (The Netherlands). Ethylene/vinylalcohol (EVAL) copolymer (MW = 10 000; 85/15 wt %) was purchased from M.P. Dajac (USA).

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2.2. EVALVA synthesis

Ethylene/vinylalcohol/vinylacetate terpolymer (EVALVA) was prepared by a homogeneous saponification process, performed using toluene as inert solvent and a methanol–potassium hydroxide solution as a reagent for the ester → alcohol transformation, starting from an ethylene/vinylacetate (EVA) copolymer as previously reported [5].

The percentage of hydrolysis of EVALVA terpolymer was varied by changing the quantity of saponification agent and was determined by analytical and spectroscopic (NMR) methods. Differences of about 0.5% were found between the analytical and spectroscopic determination of EVALVA hydroxyl content.

EVAPA material was obtained by a two-step reaction (Fig. 1) between an ethylene/vinyl alcohol/vinylacetate (EVALVA) terpolymer and a poly(amidoamine) (N_2LL) using 1,1'-carbonyldiimidazole (CDI) as bifunctional agent, as described in [5]. EVAPA films were also obtained by casting the solution on to a glass plate and by slow evaporation of the solvent at room conditions. EVAPA films were extracted with methanol at 40 °C for 72 h. The films were then dried under vacuum (2×10^3 Pa) at 40 °C for 24 h.

2.3. Thermal analysis

The thermal behaviour of the materials were investigated by a Mettler DSC-30 (Differential Scanning Calorimeter). The sample, about 10 mg, was heated from − 100 to 300 °C at a heating rate (HR) of 20 °C/min.

2.4. SEM analysis

The surfaces of the film were analysed after coating by Au–Pd evaporation using a Philips 505 scanning electron microscope (SEM) at 30 kv. Both surfaces of each film were analysed.

2.5. Potentiometric measurements

Potentiometric titrations were carried out in a thermostat glass cell at 25 °C, at a constant ionic strength of 0.1 M NaCl. A digital Radiometer PHM-84 potentiometer equipped with a Ross glass electrode (Orion, mod. 80-05) was used together with a Metrohm Multidosimat piston burette connected to an Olivetti M20 computer. All the titrations were carried out using a cell filled with approximately 100 ml of 0.1 M NaCl solution.

EVAPA films were cut in such a way as to obtain a fine dispersion of the material in 0.1 M NaCl aqueous medium, as previously reported [6].

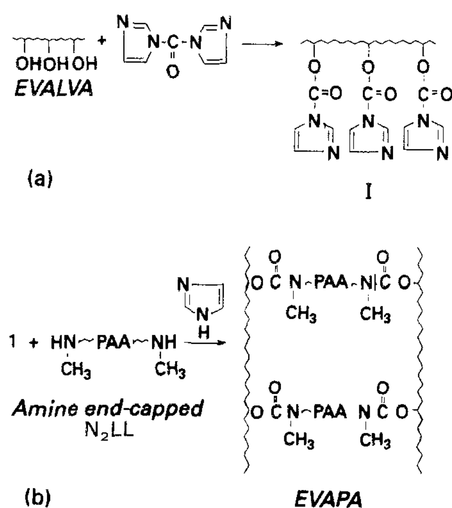

Figure 1 Two-step reaction showing how EVAPA material was obtained.

2.6. Water uptake

Water uptake (WU) studies were carried out by soaking films of EVALVA 41% and EVAPA into 0.1N NaCl or 1N HCl solutions. They were periodically removed, wiped with absorbent paper to eliminate surface liquid and weighed on a Mettler AC 100 digital balance. After 8 and 5 days in saline and acid solution, respectively, the specimens approached a limiting weight which, within the precision of the balance, did not change significantly over a further period of 1 day. The water uptake percentage was calculated from

$$WU\,(\%) \;=\; (M - m)/m \times 100$$

where M = wet sample weight and m = dry sample weight.

2.7. FT-IR spectroscopic measurements

The FT-IR spectra were obtained using a Perkin–Elmer M 1800 spectrophotometer connected to a Data Station 7500 professional computer. The ATR spectra were recorded using a KRS-5 crystal with an incident angle of 45°. A MTC detector was used and the apparatus was purged with N_2. The frequency scale was internally calibrated, with a helium–neon reference to an accuracy of 0.01 cm. Typically 300 scans for resolution of 2 cm^{-1} were averaged and the spectra stored on a floppy disk for further manipulation.

EVAPA samples were sliced by a microtome and the ATR spectra of the different slices were obtained using the KRS-5 crystal.

2.8. Heparinization

The heparinization of EVAPA samples was performed by dipping the EVAPA films in a 0.5% solution of heparin (sodium salt, from Roche, containing 5000 units = 50 mg/ml) in distilled water for 48 h.

2.9. Total heparin bound to the EVAPA surface

Thrombin time (TT) and Toluidine Blue (TB) tests were carried out on the heparin solution before and after the heparinization procedure and the heparin adsorbed on EVAPA surface was estimated from the difference between the two values.

Venous blood from healthy individuals was placed in plastic tubes containing sodium citrate (9:1, v/v). The samples were centrifuged for 15 min at 3500 rpm,

to obtain platelet poor plasma. A pool of human plasma was obtained by collecting plasma from multiple donors. Small aliquots (8 ml) were stored in plastic tubes at − 80 °C.

TT was determined by incubating 0.2 ml of human plasma at 37 °C for 2 min with 0.2 ml of heparin solution, after which 0.2 ml thrombin (0.6 NIH; from Boehringer Mannheim) were added. The clotting time was revealed by an Automatic Elvi Digiclot 2 Coagulometer (Logos S.p.A).

TB tests were performed according to Smith *et al.* [7].

2.10. Heparin release in phosphate buffer solution (PBS) and in Human plasma

Heparin release in PBS (phosphate buffer solution) and in human plasma from heparinized EVAPA-coated polyethylene and heparinized EVAPA films, respectively, was evaluated at room temperature using a flow system.

Samples (~ 9 cm^2) were positioned in a flow chamber within a loop consisting of a peristaltic pump (ISMATE SA with 3 mm inner diameter Silastic tubing) and a polystyrene tube. The pump speed was adjusted to obtain a shear rate of 100 s^{-1} which is the average shear rate of the venous system. Aliquots of human plasma or PBS were taken off at different times and the amount of released heparin was determined by TT tests.

After 2 h the release plateau was reached, the circuit was drained off and new human plasma or PBS was used for the second washing. The test was carried out twice with two samples obtained by the same synthesis.

2.11. Hemostasis activation

Glass tubes coated with EVAL, EVALVA 20%, EVALVA 41%, EVAPA, heparinized EVAPA and glass and plastic tubes as controls, having a surface area of about 40 cm^2, were filled with human blood. The ratio sample/blood was approximately 4 $cm^2\,ml^{-1}$.

The samples were tested with non-anticoagulated blood from two different volunteer donors. 90 ml of blood were continuously collected, directly into the test tubes. Using these conditions, 10 ml of blood were in contact with the sample. The coagulation process was observed and coagulation times were noted when a blood clot was apparent.

Simultaneously, 1 ml of blood was taken from each tube after a period of 0, 3, 5, 7, 9, 11, 15, 20 min. Each 1 ml aliquot was placed in a tube containing an anticoagulant able to inhibit any further proteasic activity.

Fibrinopeptide A (FpA) determinations were performed using an immunoenzymatic assay (Asserachrom® technique).

3. Results and discussion

3.1. Thermal analysis

The thermogram of an EVA sample (Fig. 2a) shows the presence of the glass transition temperature (T_g) at about − 20 °C and a little endothermic peak centred at about 55 °C. The endothermic peak is probably due

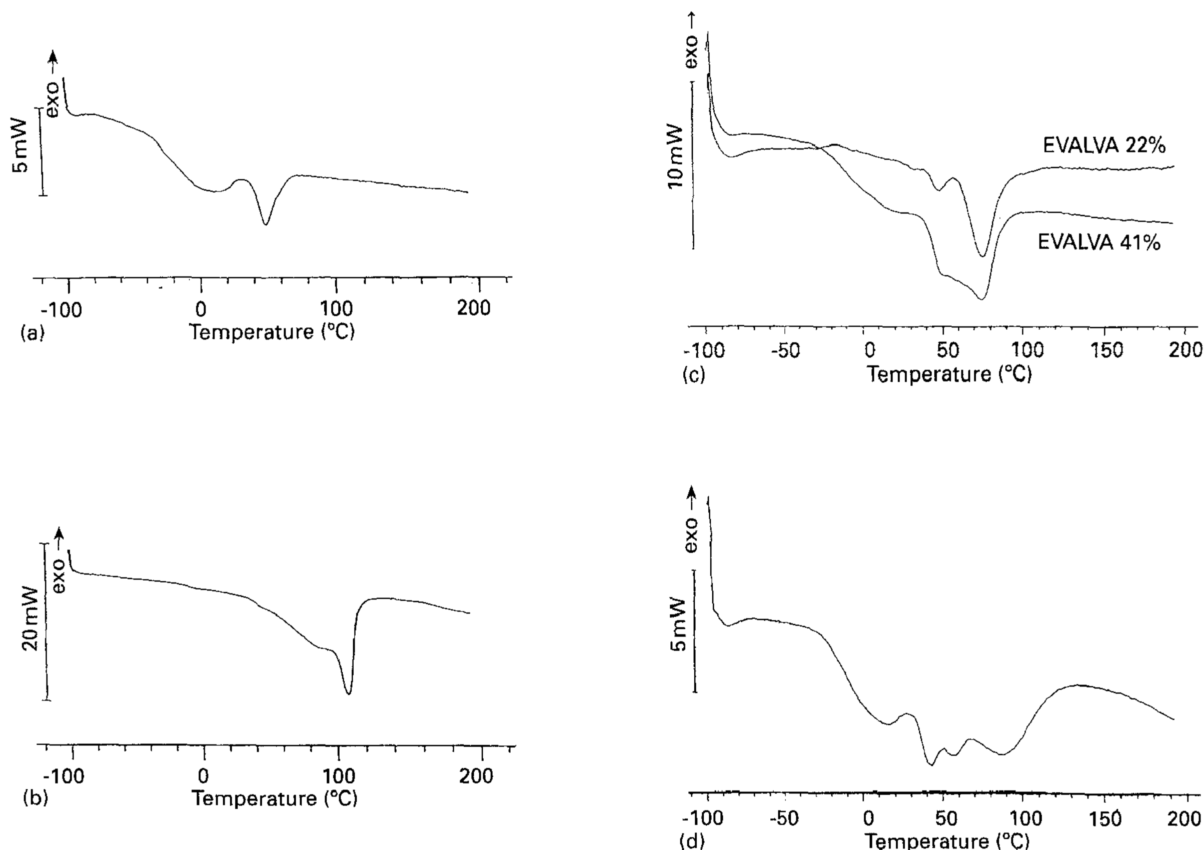

Figure 2 DSC thermograms of: (a) EVA sample; (b) N_2LL sample; (c) EVALVA 41% and 20% samples; (d) EVAPA sample.

to the presence of the relatively long sequences of ethylene units that could form small crystals with low melting point value (compared to the value of high-density polyethylene). This hypothesis is in agreement with the T_g value found for the EVA. In fact if the copolymer were completely amorphous, the T_g value would be expected to be about $-50\,°C$ (assuming random mixing of the ethylene and vinylacetate units and setting the T_g of polyethylene at $-90\,°C$ and that of polyvinylacetate at $+44\,°C$). Since some ethylene units are in succession and so able to crystallize, the real amorphous phase of EVA has a composition different from the nominal (60% in weight of ethylene and 40 wt % of vinylacetate) with less units of ethylene in the amorphous phase, which produces an increase of T_g value.

The thermogram of N_2LL, Fig. 2b, shows the presence of an endothermic peak whose maximum is at about 105 °C and a glass transition temperature at about $-15 \pm 5\,°C$. The analysis of the thermogram clearly indicates that N_2LL contains a high percentage of crystallinity.

In Fig. 2c the DSC thermograms of the EVALVA 41% and 20% samples are shown. It is found that the increase of hydrolysis percentage produces a merging of the two separated peaks present in EVALVA-20%: the first at about 50 °C is due to melting of the ethylene sequences, the second ($+75\,°C$) is probably due to the melting of vinylalcohol sequences crystallized because of hydrogen bonds.

In Fig. 2d the EVAPA thermogram is shown. The analysis of the EVAPA thermogram clearly indicates the presence of poly(amido-amine). The EVAPA film has a glass transition temperature of about $-20\,°C$, like the EVA sample. Moreover, at about 50 °C EVAPA shows an endothermic peak attributed to melting of the ethylene sequences present in the starting material (i.e. EVA). Another endothermic peak is present at 90 °C. This peak is attributed to N_2LL present in the sample.

3.2. SEM analysis

One surface of the EVAPA film presents a dune morphology. The other surface is generally smooth with the presence of small particles embedded in the matrix. Both surfaces of EVALVA 41% and EVALVA 20% films present a waviness morphology, while the surface of the EVAL film is characterized by the presence of small cavities (average diameter 1 to 5 μm) uniformly distributed.

3.3. Potentiometric titrations

Only two protonation constants were sufficient to simulate the titration curves of EVAPA. The protonation constants and n values of EVAPA agree with those reported for PUPA [8] and attributed to N_2LL present in the materials. A substantial difference is observed in the waiting time to reach the steady voltage after the addition of 0.1 ml of NaOH titrant: for PUPA 15 min; for EVAPA 60 min. By reading the voltage after 15 min, a different curve is obtained, shifted to lower voltage. At this point by backtitrating with hydrochloric acid and using the same time (15 min) for reading the voltage, a large potentiometric hysteresis is formed (Fig. 3), while the equilibrium curve lies in the middle.

Generally the weaker the interaction, the shorter the lag time (if the permeability to protons is the same for both materials). Thus we can conclude that the interaction of N_2LL with EVALVA is stronger than that with PU. In fact EVALVA is more hydrophylic than PU.

3.4. Water uptake

Fig. 4 shows the water uptake of EVAPA and EVALVA 41% samples in 0.1 N NaCl solution and in acidic medium (1 N HCl).

The water uptake of the EVAPA sample is greater in acidic medium than in a saline solution, while the water uptake of EVALVA 41% is almost zero in both the solutions.

A maximum in the EVAPA water uptake is obtained after 8 days in 0.1 N NaCl solution and after 5 days in 1 N HCl solution. Further soaking of the samples in both the above solutions for a period of 6 days does not change significantly the sample weight.

If the EVAPA sample, previously immersed in acidic medium, is soaked in alkaline solution (1 N NaOH) the water uptake decreases and returns to the value obtained in 0.1 N NaCl.

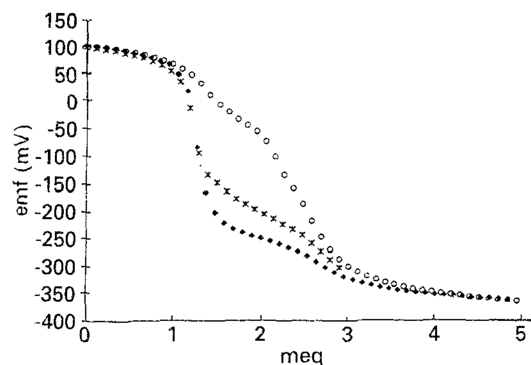

Figure 3 Potentiometric titration curves of EVAPA samples. (◆) 0.1 M NaOH (stabilization time 15 min.), (○) 0.1 M HCl (stabilization time 15 min.) and (*) 0.1 M NaOH (equilibrium curve).

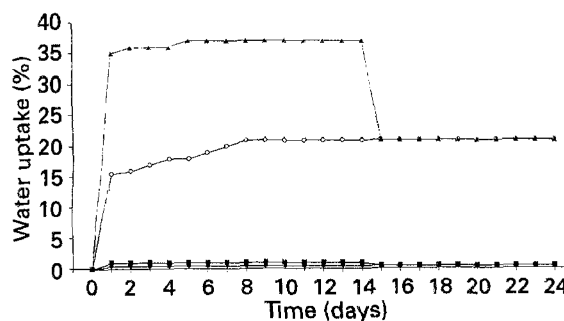

Figure 4 Water up take of EVAPA and EVALVA 41% samples in 0.1 N NaCl solution and in acidic medium (1 N HCl). ◆ EVALVA 41% in saline solution; ○ EVAPA in saline solution; ■ EVALVA 41% in acidic and alkaline medium; ▲ EVAPA in acidic and alkaline medium.

3.5. FT-IR spectroscopic measurements

The i.r. spectrum of the EVAPA sample shows the characteristic bands of both EVALVA and N_2LL, together with those of the urethane group due to the binding of N_2LL chains to those of the functionalized EVALVA.

The i.r. spectrum of EVAPA shows the characteristic absorption of the amide C=O stretching vibration of N_2LL at around $1650\ cm^{-1}$. The covalent bond between the poly(amido-amine) and EVALVA chains is shown by the presence of the shoulder at $1760\ cm^{-1}$, which is attributed to the C=O stretching of the tertiary urethane group formed at the end of the reaction.

The absorption of the tertiary urethane C=O stretching occurs at the same wavenumber as the urethane groups formed between carbonyldiimidazole (CDI) and EVALVA in the first step of the reaction.

Although the reaction is carried out with a shortage of poly(amido-amine), this absorption is not influenced by the contemporary absorption of the unreacted carbonylimidazolyl groups, since they are hydrolysed to the free hydroxyl groups during the hydrolytic work up at the end of the reaction. Moreover, the decrease of the OH stretching absorption (centred at $3450\ cm^{-1}$) in the spectrum of EVAPA with respect to that of EVALVA, demonstrates that the N_2LL chains are covalently bonded to the EVALVA chains.

3.6. Heparin release in PBS and in human plasma

The total amount of heparin adsorbed on EVAPA surface was estimated from differences between the heparin solution before and after the heparinization procedure, using TT and Toluidine Blue tests.

The amount of total heparin bound to the EVAPA surface determined by these different methods is $0.7\ mg/cm^2$. By studying the release heparin in PBS in a flow chamber, it was noted that after 5 min of washing the heparin release reached a plateau and it was possible to evaluate the amount of heparin released (0.34 IU, corresponding to $2.4 \times 10^{-4}\ mg/cm^2$ of heparin).

The amount (mg) of released heparin in human plasma against time (min), is reported in Fig. 5.

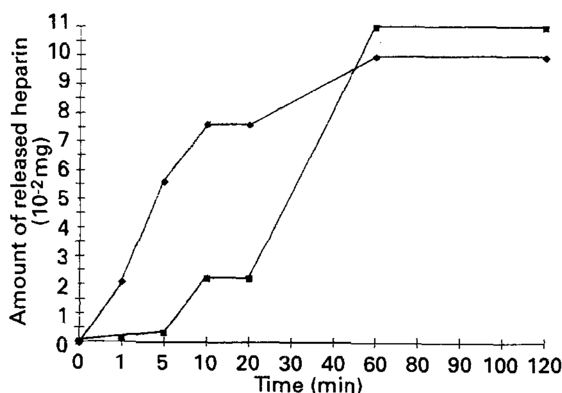

Figure 5 Amount (mg) of released heparin in human plasma against time (min.).

A first plateau is obtained between 10 and 20 min of washing and a second plateau after 60 min.

By washing the material again with fresh plasma, no heparin was released or at least the quantity determined lies within the range of error for the method.

The difference in the heparin release between the first and second sample is obtained in the first 10 min of washing. In fact, for EVAPA# the amount of released heparin at the first plateau ($2.4 \times 10^{-3}\ mg/cm^2$) is much lower than for EVAPA* ($8.4 \times 10^{-3} mg/cm^2$), while the amount of heparin released at the second plateau is, more or less, the same (1.1–1.2 $\times 10^{-2}\ mg/cm^2$).

3.7. Hemostasis activation

For evaluating the hemocompatibility of the EVAPA-coated tubes, two parameters were considered:

- The coagulation time which is a global parameter of the coagulation process.
- The sequential generation of fibrinopeptide A (FpA) on human whole blood which is a quantitative parameter of hemostasis activation.

Blood coagulation results in the formation of a blood clot consisting of fibrin, blood cells and platelets, whichever blood coagulation pathway is involved (intrinsic or extrinsic). The final step of the clot formation is the transformation of fibrinogen into fibrin and fibrinopeptides by thrombin. The measurement of fibrinopeptide A released is a quantitative expression of this final reaction and specific of thrombin activity.

The results of coagulation time (min), measured in each tube for each volumeter, are shown in Table I.

TABLE I Coagulation time with different materials

Tubes	Coagulation time (min)
Glass tube + EVAL	15
Glass tube + EVALVA 41%	19
Glass tube + EVALVA 20%	31
Glass tube + EVAPA	22
Glass tube + Heparinized EVAPA	67
Glass tube control	10
PST tube control	28

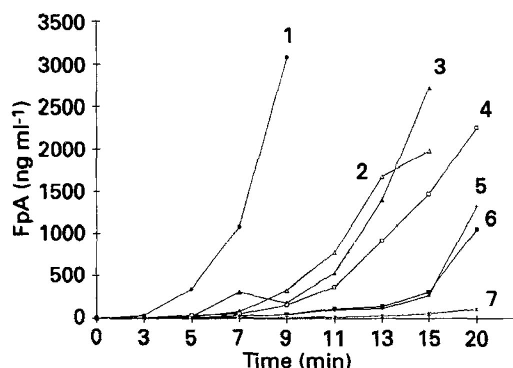

Figure 6 The kinetics of FpA generation. 1 glass tube; 2 EVAL; 3 EVALVA 41%; 4 EVAPA; 5 PST tube; 6 EVALVA 20%; 7 Hep-EVAPA.

They show that although blood clotted very rapidly in glass tube, the EVALVA treatment on glass increased blood compatibility of glass to almost that of the reference polystyrene.

The efficacy of Heparin-EVAPA treatment was largely demonstrated since coagulation times increased to three times those observed in polystyrene tubes and to 6–7 times those observed in glass tubes. The kinetics of FpA generation, reported in Fig. 6, confirmed the results of the clotting time. In fact the observed trend for both these tests is the following:

$$\text{Glass tube} > \text{EVAL} > \text{EVALVA } 41\% > \text{EVAPA} > \text{PST tube} > \text{EVALVA } 20\% > \text{Hep. EVAPA}$$

The kinetics showed a rapid increase of FpA in contact with glass tubes and a tendency for EVAL and EVALVA to reduce hemostatic activation. Among all the treatments tested EVALVA 20% has the most interesting behaviour, transforming glass to a polystyrene-like reactive surface. Finally, glass tubes coated with Heparin-EVAPA and subjected to three washings during a 25 h period retain a strong ability to inhibit thrombin, since after 20 min a very low level ($100\ ng\ ml^{-1}$) of FpA was generated.

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