Chemical Functionalisation of Vinyl Polymers to Obtain Heparin-Like Materials

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Summary: Vinyl polymers such as poly(ethylene-co-vinyl alcohol) (EVAL) and polyallylamine (PALA) both commercially available were chemically modified by introduction of carboxylic and sulfonate groups to obtain polymeric materials with improved haemocompatibility. The introduction of carboxyl groups was carried out by reaction of EVAL's hydroxy groups with acrylonitrile followed by basic hydrolysis of –CN groups. Amino groups of PALA were transformed into sulfonate groups by reaction with pyridine-SO_3 complex. Influence of reagents molar ratio, temperature and reaction time on the carboxylation degree was evaluated. In particular, yields of 86% (EVAL-CN 0.52) and 30% (EVALCOOH 0.16) were obtained for the cyanoethylation and the hydrolysis reaction of the –CN groups, respectively, whereas a sulfonation of 24% of the PALA amino groups was found. The functionalised polymers were characterized by physicochemical measurements. Preliminary biological tests proved the importance of strong acidic groups on the anticoagulant properties of the polymeric materials.

Keywords: haemocompatibility; heparin-like polymers; polymer functionalisation; vinyl polymer

Introduction

The development of suitable medical devices requires the use of materials with no toxic or injurious effects on biological systems. In these years, a deeper knowledge of complex biological systems and improvements in the physicochemical characterisation of materials and their processing, have made possible the development and diffusion of the so-called ''biomaterials'', giving rise to a great therapeutic revolution. Particular attention was devoted to polymeric biomaterials that are used for therapeutic applications due to their good mechanical properties that make them intrinsically suitable for the substitution of parts of the human body. They are now available in many compositions with different properties and can be formed into complex structures. However, while the mechanical properties are bulk characteristics of a material, the type of interaction when in contact with a biological system depends on the nature of its surface. For this reason and since the biocompatibility of biomaterials is to date far from being satisfactory, their surface modification is essential in view of real medical application. In particular, in the fields of cardiovascular prostheses, artificial hearts, and central venous catheters, the research is primarily focused on the development of antithrombogenic polymers.[1–6] Indeed, the hypothesis that substances able to exert an antithrombotic action in the human body could act in the same way when immobilized onto polymer surface prompted several research groups to develop different immobilization techniques of biologically active molecules, and in particular

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heparin.[7] Since biological tests with the modified surfaces gave good results, a development of this research line consisted in chemically modifying the surfaces, by introducing onto them the specific functional groups (sulfate, sulfamic and carboxyl), that are considered responsible for the anticoagulant activity of heparin. These polymers miming heparin are called "heparin like" [8,9] materials. Besides, since it has been seen that these groups can influence the adhesion, mobility and proliferation of various cell types, these materials could be also used as *scaffolds* for tissue engineering. Indeed, they could have the advantage of the biological recognition since these polymers are similar to the extracellular matrix (ECM) components.[10,11]

Thus, to develop antithrombogenic polymers, carboxylic and sulfonate groups were introduced in two commercial vinyl polymers, ethylene-vinyl alcohol 40/60 (EVAL) and polyallylamine (PALA), already employed in the medical field. Previous results obtained by our group showed the EVAL polymer as a promising material both for the ionic and covalent immobilization of heparin [12] and for the synthesis of heparin-like materials [13]. For this reason, we continued the research by introducing carboxylic and sulfonate groups in this polymer and in a similar vinyl polymer.

The resulting polymers were characterized by titration of acidic groups, infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR), thermogravimetric analysis, swelling and contact angle measurements and dynamic-mechanical analysis (DMA). The anticoagulant activity of polymers was evaluated by preliminary *in vitro* tests of Activated Partial Thromboplastin Time (APTT).

Experimental

Functionalisation of Polymers

Carboxylic groups were introduced in EVAL (ethylene/vinyl alcohol 40/60, Dajac) through two consecutive reactions: a cyanoethylation reaction with acrylonitrile (EVAL-CN) followed by basic hydrolysis of the –CN groups, resulting in a polymer called EVAL-COOH. Parameters influencing the reactions as reagent/functional group ratio, temperature and reaction time have been investigated (Table 1). In particular, the EVAL cyanoethylation reaction was carried out in basic medium (1/1 NaOH/EVAL hydroxy groups ratio) by adding acrylonitrile (ACN, see molar ratio in Table 1) to a 3 wt-% EVAL solution in dymethylformamide (DMF). Trimethylbenzyl ammonium hydroxide (TRITON) was also added as a catalyst in a 5% amount with respect to EVAL hydroxyl groups.

As far as the introduction of sulfonate groups is concerned, the amino groups of polyallylamine (PALA, Aldrich) were reacted with the pyridine-SO_3 complex ($PySO_3$, Fluka), at a 2/1 reagent/amino groups ratio. The reaction was performed first at 0 °C for 1 h, under nitrogen flow, employing a 3% (w/v) polymer solution in anhydrous DMF, and then for 6 h at room temperature.

Polymer Characterization

Infrared analysis was accomplished using a MATTSON GALAXY 5020 FT-IR spec-

Table 1.
Experimental conditions and molar fractions for the cyanoethylation reaction.

Molar ratio (ACN/OH)	Temperature (°C)	Time (h)	–CN molar fraction (χ)
4/1	25 °C	24	0.20
4/1	2h at 0 °C + 22h at 25 °C	24	0.22
8/1	25 °C	12	0.27
8/1	25 °C	24	0.29
8/1	2h at 0 °C + 22h at 25 °C	24	0.31
8/1	15 °C	24	0.52

trometer working at 2 cm^{-1} resolution. The ^{1}H-NMR spectra were recorded at 25 °C using a VARIAN XL 300 instrument and dimethylformamide-d_7 as solvent.

Differential scanning calorimetry (DSC) measurements were carried out by a METTLER TA 3000 calorimeter provided with a TC 10 A processor, by keeping the cell (DSC30) under N_2 flow. The explored temperature range was $-100 \div 300$ °C and the employed heating rate was 10 K/min.

The thermogravimetric analysis was carried out employing a METTLER TG 50 thermobalance, provided with the same TC 10 A processor. The explored temperature range was 25–600 °C, and the heating rate 10 K/min, under N_2 flow.

The contact angle measurements in water were performed by using a computerized CAHN DCA 312 dynamic contact angle analyzer. Samples were prepared by coating glass slides with DMF polymer solutions. For the experiments a stage speed of 50 μm/s was used. The advancing contact angles were averaged over 3–5 values measured in the immersion phase of the sample. In addition, to evaluate the possible kinetic hysteresis of polymer surfaces the contact angle was determined performing two immersion cycles.

The swelling experiments were performed by dipping polymer films in water at room temperature. At different times, films were blotted on filter paper to remove the excess water from surface and weighed.

A Rheometrics Scientific Analyzer RSA II was used to perform mechanical tests. Each sample was cut to a small rectangular film having the following dimensions: 30 mm length, 1 mm width and 0.2 mm thickness. A strain limit of 0.2% and a frequency of 6.28 rad/sec were applied in the temperature range from −150 to either 15 or 100 °C.

Biological Test

The activated partial thromboplastine time (APTT) tests were carried out by using an automatic coagulimeter (Koagulab MJ, Ortho Diagnostic System). This test consists of contacting the polymer sample (5–8 mg) and lyophilized human plasma (0.1 ml), by adding 0.1 ml of partial thromboplastin. After 120 sec incubation time, 0.1 ml of 0.025M $CaCl_2$ was added, and the coagulation time of the samples was recorded by the automatic coagulimeter.

Results and Discussion

As far as EVAL functionalisation is concerned, acid-base titration and ^{1}H-NMR measurements allowed us to evaluate the functionalisation degree (Table 1). As for the cyanoethylation reaction, a 86% yield (EVAL-CN 0.52) was obtained by performing the reaction at 15 °C with a 8/1 ACN/OH molar ratio.

Similarly, several NaOH/-CN molar ratios and reaction times were employed in the hydrolysis reaction (Table 2) thus obtaining a 30% maximum hydrolysis for EVAL-CN 0.52 (EVAL-COOH 0.16).

Since the haemocompatibility of the polymers is strongly influenced by their hydrophilicity, our materials were characterized by dynamic contact angle and swelling measurements in water. In Table 3, advancing and receding contact angles of polymers measured in water are reported.

The poor wettability of the functionalised surfaces, with the exception of EVAL-COOH 0.16, was supported by a high advancing angle that shows the existence of hydrophobic domains on the surface. On the other hand, the low receding angle displayed by the polymers indicates that there is also a high concentration of polar groups at the interface. The contact angle hysteresis evidences, therefore, both hydrophobic and hydrophilic feature of polymer surfaces.

Table 2.
Experimental conditions and molar fractions for the hydrolysis reaction of EVAL-CN 0.52.

Molar ratio (NaOH/CN)	Time (h)	–COOH molar fraction (χ)
1/1	24	0.06
2/1	24	0.10
2/1	48	0.16

Table 3.
Advancing and receding contact angles of polymers measured in water.

Polymers	θ_{adv}(°)	θ_{rec}(°)
EVAL	86 ± 1	18 ± 2
EVAL-CN 0.52	87 ± 3	33 ± 2
EVAL-COOH 0.06	91 ± 2	44 ± 2
EVAL-COOH 0.16	72 ± 2	44 ± 2

In particular, the lower contact angle hysteresis observed for EVAL-COOH 0.16 is probably due to a higher surface homogeneity of this polymer with respect to EVAL that, on the contrary, shows the existence of a microdomains separated structure. Besides to evidence surface homogeneity at the polymer/air/liquid interface, DCA measurements can be also employed to investigate the surface molecular mobility. The second immersion cycle performed on the samples showed, however, the poor surface mobility of functional groups since advancing contact angles were unchanged (data not reported in Table 3).

While contact angle values evidenced that the introduction of –COOH groups did not significantly change the surface hydrophilicity of polymers, swelling measurements (Figure 1) showed a greater ability of the functionalised polymers to adsorb water compared to their precursor (EVAL).

DSC and DMTA analysis showed that the introduction of functional groups in EVAL decreased the crystallinity degree

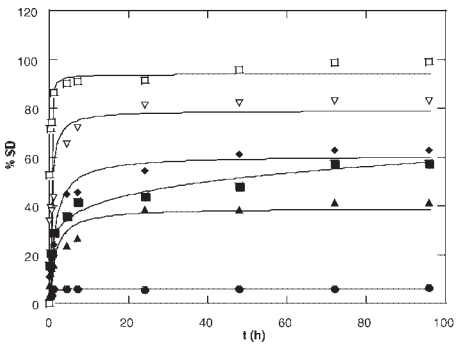

Figure 1.
Water swelling data of polymers: EVAL (●); EVAL-CN 0.22 (■); EVAL-CN 0.31 (□); EVAL-CN 0.52 (▲); EVAL-COOH 0.06 (□); EVAL-CN 0.16 (□).

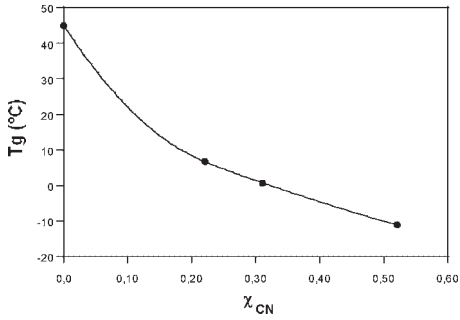

Figure 2.
Glass transition temperature (Tg) dependence on polymer degree of cyanoethylation.

changing, moreover, the mobility of polymer molecular segments. Indeed, polymers containing –CN groups displayed a decrease of the polymer glass transition temperature with increasing the functionalisation degree (Figure 2). Meanwhile, a greater number of –COOH groups in the polymer caused an increase of polymer stiffness due to the greater probability to establish hydrogen-bond interactions. In fact, the T_g value of EVAL-COOH 0.16 changed from −12 to 0 °C.

In addition to the glass transition, it was possible to observe by DMTA measurements chain movements of small segments. Indeed, the DMTA E″ curves showed two main transitions. As an example, the E″ vs. T curve of EVAL-CN 0.52 is reported in Figure 3.

A first peak at a higher temperature (T_α 48 and −8 °C for EVAL and EVAL-CN 0.52, respectively), corresponding to the glass transition, and a second one in the lower temperature region (T_β −30 and −90 °C for EVAL and EVAL-CN 0.52, respectively) corresponding to the β transition can be observed. This latter transition was attributed to the relaxation of small segments of the polymer chain.

In the sulfonation of PALA, the functionalisation degree, evaluated by titration and by elemental analysis, was of 24%. The obtained polymer was called PALAS.

Similarly to EVAL, a morphologic change of the polymer was evidenced after chemical functionalisation as shown by the

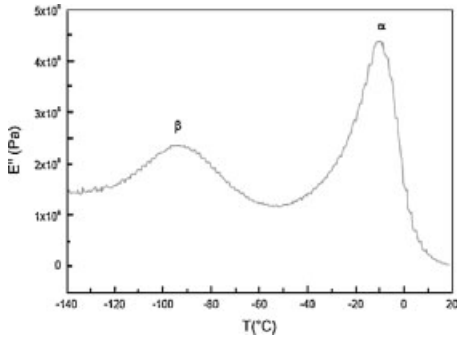

Figure 3.
DMTA E″ curve for EVAL-CN 0.52.

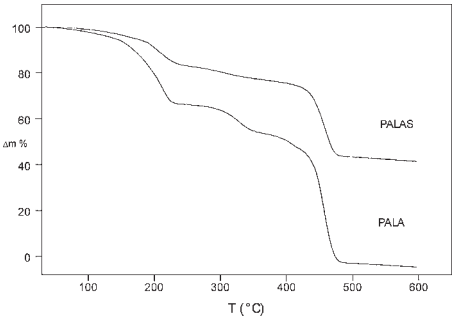

Figure 5.
PALA and PALAS thermogravimetric curves.

decrease of both the polymer glass transition temperature from 100 to 44 °C and the polymer crystallinity (Figure 4). This behavior can be due to the reduction of hydrogen bonds among PALA amino groups and the presence of strongly bulky sulfonate groups.

Differently from polymers resulting from EVAL functionalisation, thermogravimetric analysis indicated that the thermal stability of PALAS polymer is larger than that of PALA (Figure 5).

The antithrombogenic activity of materials was preliminary evaluated *in vitro* by the APTT test, by contacting samples with human plasma and by recording the time of plasma coagulation. Since PALAS resulted hydrosoluble, the polymer was crosslinked either by thermal treatment or by neutralization with calcium ions before the test. As it is shown in Table 4, the functionalised polymers are more haemocompatible than the pristine matrices. In addition, sulfonate groups seem to be more active than carboxylic groups in determining the anticoagulant properties of polymers.

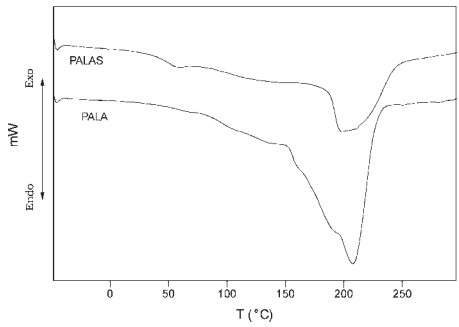

Figure 4.
DSC curves of PALA and PALAS polymers.

Conclusions

In order to develop polymers with enhanced blood compatibility, we prepared new heparin-like vinyl polymers possessing carboxylic and sulfonate groups. Carboxylic groups were introduced in the ethylene-vinyl-alcohol polymer (EVAL) by cyanoethylation reaction and hydrolysis of –CN groups, obtaining 86% and 30% yields respectively. Similarly, sulfonate groups were introduced in polyallylamine thus affording a polymer with a 0.24 molar fraction of sulfonate groups. Physical-chemical characterization of polymers evidenced both a lower stiffness and a greater hydrophilicity of the functionalised polymers as compared to the starting polymers.

Table 4.
Coagulation time of plasma in contact with polymers.

Sample	APTT (s)
glass	28 ± 1
EVAL	30 ± 1
EVAL-CN 0.52	29 ± 1
EVAL-COOH 0.06	50 ± 2
EVAL-COOH 0.16	85 ± 2
PALA	29 ± 1
PALAS (hydrosoluble)	>200
PALAS (thermally treated)	120 ± 1
PALAS (neutralized with Ca^{2+})	86 ± 1

Finally, preliminary biological tests showed that the presence of strong acidic groups together with a greater polymer hydrophilicity corresponded to an increase of the anticoagulant properties of polymers. Further study will be carried out to assess the anti-platelet activity of polymers as well as their possible use as scaffolds for tissue engineering.

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