J Mater Sci: Mater Med (2008) 19:713–719
DOI 10.1007/s10856-007-3191-6

Blood interactions with plasticised poly (vinyl chloride): influence of surface modification

Xiaobin Zhao · James M. Courtney · Hua Q. Yin · Robin H. West · G. D. O. Lowe

Received: 20 July 2006 / Accepted: 22 May 2007 / Published online: 10 July 2007

Abstract Surface modification of plasticised poly (vinyl chloride) (PVC), with di-(2-ethylhexyl) phthalate (DEHP) as plasticiser, for the improvement of blood compatibility in potential clinical use such as cardiopulmonary bypass was achieved by heparinisation. The influence of surface modification on blood compatibility was assessed in terms of the influence on fibrinogen and factor XII adsorption in vitro, and the generation of thrombin-antithrombin III complex (TAT) and the complement component C3a, in vitro and ex vivo. Electron spectroscopy for chemical analysis (ESCA) was used to characterise the heparinised surface in order to correlate the surface properties with the blood response. Results indicate that at the plasticised PVC surface there is a higher content of heparin than that of the PVC and the DEHP content is lower than that present at the surface of standard plasticised PVC. The blood compatibility assessment confirms the importance of surface modification for the improvement of blood compatibility.

X. Zhao · J. M. Courtney · H. Q. Yin
Bioengineering Unit, University of Strathclyde, Glasgow, UK

X. Zhao (✉)
Technical Department, NovaThera Ltd, Minerva Building, Babraham Research Campus, Cambridge CB2 4AT, UK
e-mail: x.zhao@novathera.com

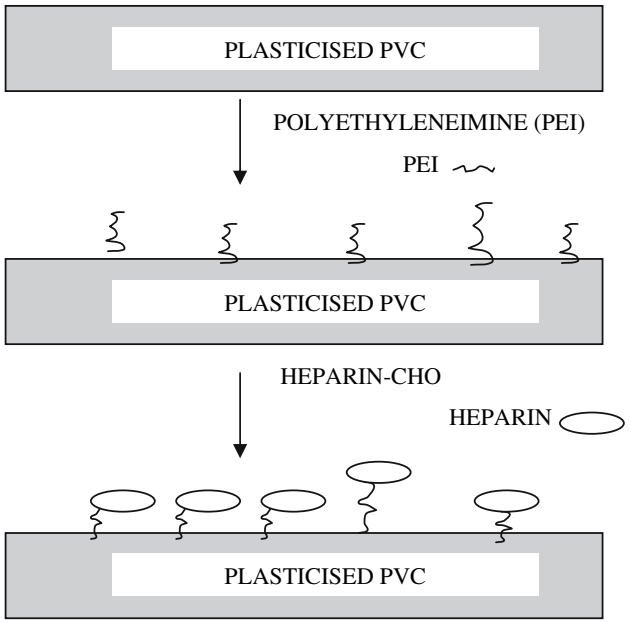
R. H. West
CSMA Ltd, Manchester, UK

G. D. O. Lowe
University Department of Medicine, Royal Infirmary, Glasgow, UK

Introduction

Contact of blood with a foreign surface, such as the wall of an extracorporeal circulation tube in cardiopulmonary bypass, in the absence of an antithrombotic agent, generally initiates a series of reactions leading to thrombus formation [1]. In cardiopulmonary bypass, the influence on the blood response comes not from a particular biomaterial but from the combined effect of biomaterials, the blood oxygenation device, circuit components and patient status [2, 3]. However, modification of the surface of polymeric blood-contacting biomaterials is considered to be an important approach for the improvement of blood compatibility. This approach makes it possible to utilise the advantages of existing biomaterials and obtain improved biocompatibility [4]. Surface modification for improvement of blood compatibility can be achieved by physical, chemical and biological approaches [5, 6]. Surface immobilisation of biological anticoagulants, such as heparin or heparin-like bioactive substances [7–9] and surface coating with heparin and its poly (ethylene oxide) (PEO) conjugates have achieved clinical use [10, 11]. The Carmeda BioActive Surface (CBAS) approach is considered to be able to create a biological active surface based on the coating of blood contacting surfaces with heparin. The key technology of the *end-point attachment of heparin*, developed by Carmeda, has been described as being able to successfully bind the heparin molecule to artificial materials with retained anticoagulant activity and thus yield highly thromboresistant surfaces [12–14].

In this study, medical grade plasticised poly (vinyl chloride) (PVC) in tubing form was selected for surface modification by utilisation of the CBAS heparinisation technology as shown in Scheme 1. For understanding the relationship between the artificial surfaces and blood,



Scheme 1 Surface modification of PVC-DEHP using end-point attachment of heparin

electron spectroscopy for chemical analysis (ESCA) technology [15] was employed to determine the surface chemical composition and bonding structure. In respect of the blood compatibility assessment, protein adsorption, the measurement of thrombin-antithrombin complex (TAT) and the generation of the complement C3a were assessed in vitro and ex vivo to evaluate the improvement of blood compatibility by surface modification.

Materials and methods

Materials

Two types of medical grade plasticised PVC tubing were evaluated with the internal diameter of 6.25 mm. The comparison was between a control PVC plasticised with di-(2-ethylhexyl) phthalate (DEHP) (PVC-DEHP) and this material heparinised by the method of end-point attachment (PVC-DEHP-H), as shown in Scheme 1 [12]. The two materials were supplied by Carmeda AB, Sweden. The modified surface was achieved by end-point attachment of heparin. In summary, the DEHP-PVC tubing was subjected to a rapid surface cleaning and immersed into polyethyleneimine (PEI) solution to obtain a PEI-modified surface. To the surface, the heparin, which had been subjected to $NaNO_2$ oxidation to obtain an aldehyde end, was added to enable the formation of a heparin-DEHP-PVC stable surface with reduction off the Schiff base. After further purification, PVC-DEHP-H tubing was obtained.

Surface characterisation with electron spectroscopy for chemical analysis

Internal surfaces of each PVC tubing, in size of about 3×3 mm, were removed with fresh scalpel blades and mounted on the standard sample holder with small squares of double-sided adhesive tape. A nickel mesh was placed 1–2 mm above the sample surfaces to control electrostatic charge build-up. The analysis was performed with the Fisons SSI M-probe XPS instrument (VG Scientific Ltd., UK). About 200 w monochromatised AlK_α were focussed into an elliptical spot size of $400 \times 1{,}000$ μm on the material with a standard take-off angle of 35° to the sample surface. Survey scan analysis and high resolution of C1s, O1s, N1s and S2p regions were recorded. All spectra were referenced to the C1s peak at 285.0 eV binding energy. Composition tables were derived for each surface by peak area measurements followed, by the application of Scofield based sensitivity factors. High-resolution data were subject to Shirley background subtraction prior to peak synthesis using the instrument software.

In vitro assessment

In vitro fibrinogen and factor XII (FXII) adsorption

The exposure of the proteins to PVC tubing was achieved by a syringe pump system. About 20 mL of the protein solution with the concentration of 0.031 μg/mL for fibrinogen and 0.416 μg/mL for FXII were placed into a 30 mL syringe, which was then mounted in the syringe pump (Model 915A, Harvard Apparatus). The solution was perfused through the tubing (15 cm in length) at a flow rate of 1.2 mL/min for 15 min. After that period, the system was rinsed with PBS solution (0.5 M, pH = 7.4) for 10 min at the same flow rate and three segments (1.0 cm each) of the tubing were cut at the beginning, middle and end part. The radioactivity of the working solution and the segments was measured with a gamma counter (Panax Ltd. UK).

In vitro blood response assessment

In vitro blood-material contact was achieved by a syringe pump system. A length of the tubing material was fixed at each end within a rigid plastic cylinder, which in turn was mounted onto the cross-head of a syringe pump (Model 915A, Harvard Apparatus). A large polypropylene syringe (30 mL) was attached to one end to push the blood and two small syringes (5 mL) were attached to the other end via a 3-way stopcock for sample collection. By fixing the large syringe plunger to the pump body, blood is perfused through the tubing by displacement of the cross-head. The flow rate can be controlled by changing the speed of the

pump and the size of the syringe. This method of perfusion eliminates blood–air interfaces that may occur with discrete reservoir/roller pump circuitry and any blood trauma induced by roller pump application. Unsterilised PVC tubing with a length of 15 cm was tested. The system was filled with physiological saline prior to contact with blood. Blood was taken from healthy donors who had not taken any medication for the preceding 14 days. PVC-DEHP and PVC-DEHP-H were exposed to blood without anticoagulant. This was achieved by single pass flow at a rate of 1.2 mL/min. Blood samples were collected before perfusion and at 3, 6, 9 and 12 min after commencement of perfusion. All the experiments were performed at room temperature.

Ex vivo blood response assessment

An *ex vivo* system employing human blood [16, 17] was modified to permit testing of tubing. Blood was obtained from an antecubital vein using a special designed catheter, which allows the blood to be heparinised (0.5 IU/mL) immediately when it enters the catheter tip. Blood flow from the catheter was diverted via a Y-piece to two channels of a peristaltic pump (Ismatec-Switzerland) to allow simultaneous perfusion of tubing segments located at the outlet of the pump. Blood flow rate was monitored periodically by timed volume collection at the outlet of each segment. One-metre length segments of unsterilised PVC tubing were tested. Prior to the test, the system was rinsed with 1.0 L of sterilised physiological saline. The other components (i.e. cannula and heparin infusion line) were supplied pre-sterilised by the respective manufacturers. Blood from healthy donors was perfused through the tubing at a flow rate of 10 mL/min for 20 min. Blood samples were taken before the perfusion (with 0.5 IU/mL heparin) and at the outlet of the tubing at 5, 10, 15 and 20 min after blood-material contact.

Assays

Thrombin-antithrombin III complex (TAT)

Blood samples were collected into tubes containing trisodium citrate and centrifuged at 2,000 × *g*, 4 °C for 15 min. Aliquots of the plasma obtained were snap frozen in dry ice and stored at –50 °C until assay. TAT was measured by an enzyme-linked immunosorbent assay (ELISA) with commercially available kits (Behringwerke AG, Germany).

Complement C3a

Blood samples were collected into tubes containing disodium EDTA and centrifuged at 2,000 × *g*, 4 °C, for 15 min. Aliquots of the plasma obtained were snap frozen in dry ice and stored at –70 °C until assay. C3a was measured by radioimmunoassay kits (Amersham International Plc, UK).

Statistics

Statistical analyses were performed with the Minitab package (version 8.0). Comparisons of the different groups were carried out by analysis of variance. All statistically significant differences are reported at 95% confidence intervals ($p < 0.05$).

Results

Surface analysis by ESCA

ESCA survey scanning provided quantitative information on the elements, which were present at the surface of the PVC-DEHP and heparinised PVC, PVC-DEHP-H as shown in Figs. 1 and 2. The chemical elemental composition of samples at 35° take-off angle is summarised in Table 1.

ESCA results show that there is a much higher content of oxygen in heparinised plasticised PVC and the sulphur (S) detected in heparinised plasticised PVC indicates the successful attachment of heparin onto the plasticised PVC surface while the decrease of chlorine (Cl) demonstrates a formation of heparin-rich surface after the surface modification of plasticised PVC.

The high-resolution spectra results show there are two states of nitrogen present at the heparinised PVC surfaces. C–N–S was from heparin and C–NH_2 was from the PEI layer, which is applied as a spacer for end-point linkage of heparin. For sulphur, 26.1% was bonded in the N–SO_3

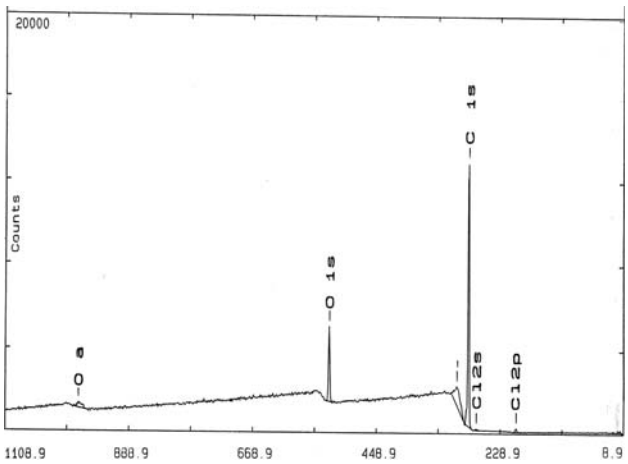


Fig. 1 ESCA survey scan record of PVC-DEHP

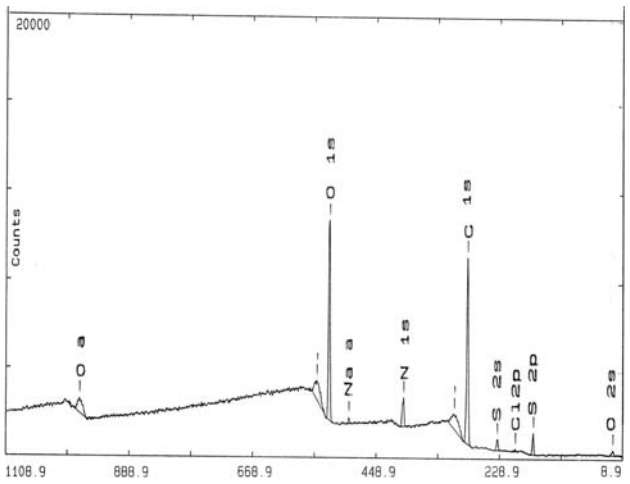


Fig. 2 ESCA survey scan record of PVC-DEHP-H

form and 73.9% in O–SO_3 form and its ratio is in agreement with that reported by West et al. [15]. The high-resolution spectra results are summarised in Table 2.

From data on the surface composition, taken in association with the standard theoretical elemental composition, the molecular distribution at the surface can be determined as shown in Fig. 3. The information derived from the ESCA surface characterisation indicates that the heparinisation leads not only to the presence of heparin at PVC surface but also a marked reduction of plasticiser level at the surface (from 75% reduced to 38%), which resulted from the surface modification process. The higher level of heparin (20%) relative to PEI (16%) at the surface of heparinised PVC is regarded as a confirmation of the suitability of the Carmeda^{TM} surface modification with heparin.

In vitro protein adsorption

Two indicators of blood coagulation, fibrinogen and FXII, were selected for protein adsorption on PVC tubing with surface modification. The results are presented in Figs. 4 and 5. Results show that heparinised PVC tubing reduced both fibrinogen and FXII adsorption. As two important initial indices for assessment of blood compatibility, the decrease of the adsorption of these two coagulation proteins on the heparinised PVC tubing surface may contribute to the reduction of its thrombogenicity, which is in agreement with data obtained in the blood tests.

In vitro blood response study

An effective technique to illustrate the efficient thrombin inhibitory properties of the end-point attached heparin

Table 2 ESCA high-resolution spectra results of PVC-DEHP and PVC-DEHP-H

	Chemical state	Binding energy (eV)	Elemental composition	
			PVC-DEHP	PVC-DEHP-H
% C	C–H			
	C–C	285.0	73.6	37.3
	CH_2			
	C–N	286.0	10.7	21.0
	C–O	286.5	5.3	29.0
	CHCl	287.0	3.9	–
	O–C–O			
	O=C–N	288.0	2.4	7.4
	O=C			
	O=C–O	289.0	4.1	5.2
% O	O–C–O	531.0	–	11.8
	O=C	532.0	54.9	39.0
	C–OH			
	C–O–C	533.0	30.7	38.3
	C–O–SO_3	533.5	–	10.9
	H_2O	534.0	14.4	–
% N	C–NH–SO_3	400.0	–	59.0
	C–NH_2	402.0	–	41.0
% S	C–NH–SO_3	168.0	–	26.1
	C–O–SO_3	169.0	–	73.9

Table 1 ESCA elemental composition of PVC-DEHP and PVC-DEHP-H

Theoretical composition[a]						ESCA elemental composition	
%	PVC	DEHP	Heparin	PEI[b]	HC[c]	PVC-DEHP	PVC-DEHP-H
C	67	86	34	67	100	82.9	64.4
O	–	14	54	–	–	10.1	25.0
N	–	–	3	33	–	–	6.3
Cl	33	–	–	–	–	6.4	0.3

[a] The theoretical composition was from proposed chemical structure

[b] PEI: Polyethyleneimine, as spacer for immobilisation of heparin

[c] HC: carbon-hydrogen as factor of surface contamination during process/measurement

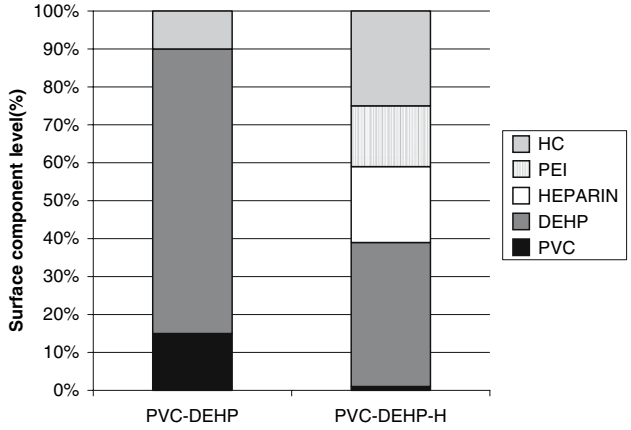


Fig. 3 Component distribution (%) at the surfaces of PVC-DEHP

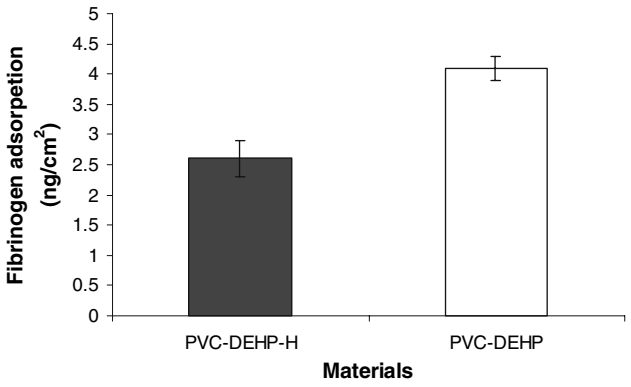


Fig. 4 Fibrinogen adsorption on PVC-DEHP and PVC-DEHP-H (^{125}I-fibrinogen working concentration is 0.031 μg/mL, total volume is 20 mL in the circulating system)

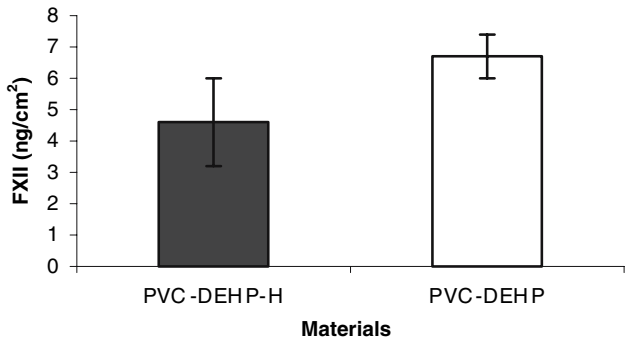


Fig. 5 FXII adsorption on PVC-DEHP and PVC-DEHP-H (^{125}I-FXII working concentration is 0.416 μg/mL, total volume is 20 mL in the circulating system)

surface is to follow the formation of the enzyme–inhibitor complex, i.e. the TAT complex [18]. Moreover, the influence of surface modification on complement activation can be monitored by measurement of the generation of

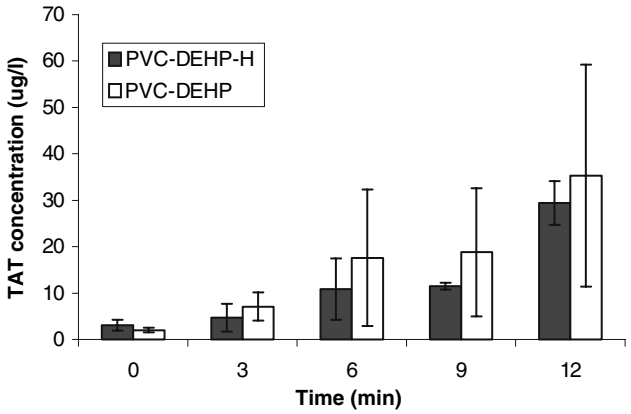


Fig. 6 *In vitro* TAT levels induced by PVC-DEHP and PVC-DEHP-H in blood without anticoagulant

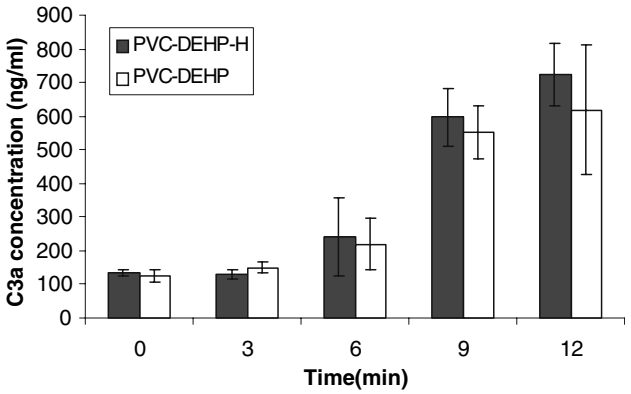


Fig. 7 *In vitro* C3a levels induced by PVC-DEHP and PVC-DEHP-H in blood without anticoagulant

complement component C3a. *In vitro* study results of TAT and C3a for PVC-DEHP and PVC-DEHP-H are shown in Figs. 6 and 7 respectively. The mean TAT values for surface modified plasticised PVC were lower than those for the control at all time intervals. However, no statistically significant differences were found. In addition, there were no differences between C3a levels induced by heparinised PVC and the control.

Ex vivo blood response study

The *ex vivo* tests of TAT and C3a show a strong reduction in activation of the thrombogenicity (TAT) and complement system (C3a) by surface modification, which is shown in Figs. 8 and 9. With respect to heparinised PVC tubing, all the mean TAT values were lower than those for the control at all time intervals with a significant difference at 20 min. Mean C3a values for heparinised PVC at 5, 15 and 20 min were lower than the corresponding values for the control, with a statistically significant difference at 15 min.

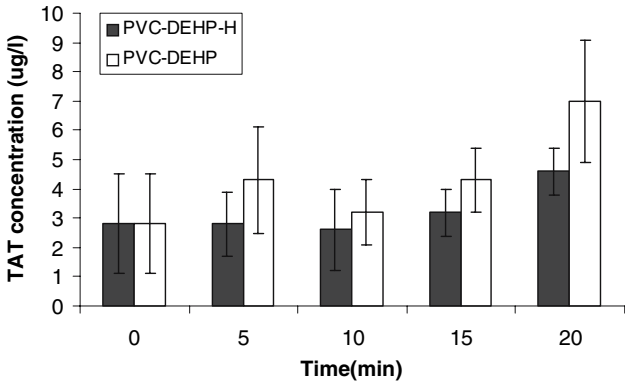


Fig. 8 *Ex vivo* TAT levels induced by PVC-DEHP and PVC-DEHP-H

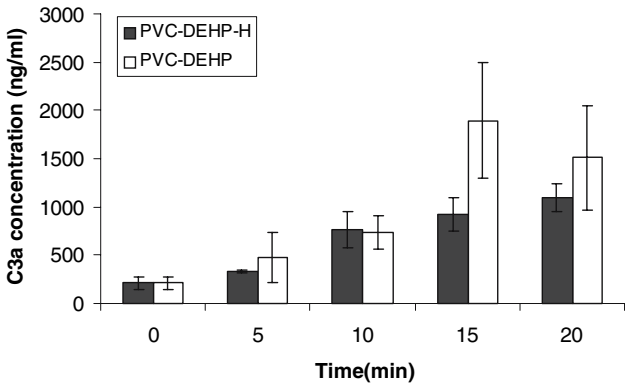


Fig. 9 *Ex vivo* C3a levels induced by PVC-DEHP and PVC-DEHP-H

Discussion

Utilisation and development of blood-contacting biomaterials would benefit from an improved understanding of the relationship between a characteristic of the biomaterial and a relevant feature of the biological response [19]. Additionally, since the blood–biomaterial interactions occur at the outmost layer of the artificial surface, surface modification for improved blood compatibility is of great importance. In this study, surface modification of plasticised PVC tubing with heparin resulted in an identified surface characteristics, which correlated with the in vitro and ex vivo biological response and led to an enhanced understanding of the nature of the biomaterial.

After the surface modification with the end-point attachment process, a rearrangement of molecular distribution at the plasticised PVC was confirmed by ESCA surface analysis. In comparison with the original surface of DEHP plasticised PVC tubing, the heparinised modification leads to a concentrated heparin layer with a reduced DEHP level. The marked reduction of DEHP plasticiser at the surface might be an additional factor in the improvement of blood compatibility, since we have demonstrated that the plasticiser level at the surface of plasticised PVC plays a very important role in its reactivity towards blood compatibility [20–22].

Certainly, however, this can not exclude the function of surface immobilised heparin since *in vitro* and *ex vivo* evaluation confirmed a definite influence of heparinisation, in that TAT and C3a values in most cases were lower with the heparinised surfaces, although in some cases it was not significant. Complement activation induced by artificial surfaces has been of interest since Craddock et al. [23] discovered that the reduction in white blood cell count during haemodialysis was associated with complement activation. The component C3 plays a very important role in activation of complement via two pathways: the classical pathway and the alternative pathway. The measurement of the generation of C3a component can provide an indication of the level of complement activation. The influence of heparin either acting as an anticoagulant or a surface modifier on complement activation in vitro is not fully understood. There is evidence that heparin in blood interacts with complement components to diminish or augment the activities with dose-dependency [24, 25]. It is also reported that antithrombin III may inhibit the generation of C3a [26] and an antithrombin complex with heparin certainly will affect this process. Both decreases and increases in in vitro complement activation by DEHP-PVC in the presence of heparin have been reported [27]. In our in vitro test, there is no significant difference between the control and heparinised surface without the presence of heparin. This is in agreement with DEHP-PVC film tested in the presence of heparin [27] and also other reports on this method of heparinisation [28–30]. Ex vivo testing shows a significant difference. However, this could be due to the physical adsorption of C3a on the PVC surface rather than the reduction of the complement activation. TAT concentrations tested both *in vitro* and *ex vivo* indicate there is a significant difference between control and the modified at certain point of time during blood–material interaction. This is due to complexation of surface heparin with antithrombin in the blood while the physical adsorption of TAT also cannot be ruled out.

Conclusions

The work presented here has investigated the relationship between the surface modification with heparin and its blood response. The surface modification of DEHP-PVC by heparinisation has altered the surface chemical composition with an increase in heparin level and a reduction in DEHP plasticiser level, which ultimately changes the surface physical and biological properties. This demonstrates

that in the heparinisation of PVC, while the attachment of heparin can be considered as the primary effect, there are secondary effects, such as a reduction on plasticiser surface level. This should be recognised when interpreting the clinical performance of heparinised PVC since plasticiser surface level plays a very important role in determination of the blood compatibility [31].

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