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In vitro investigation of the effect of plasticizers on the blood compatibility of medical grade plasticized poly (vinyl chloride)

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Abstract This paper reports the results of an in vitro investigation into the blood response of medical grade poly (vinyl chloride) (PVC), and two types of plasticized PVC in tubing or sheet form, with di-(2-ethylhexyl)phthalate (DEHP) and di(isononyl) cyclohexane-1,2-dicarboxylate (HEXAMOLL® DINCH) as plasticizer, were selected for assessment of complement activation, coagulation system and platelet activation. The results of the study show that not only the plasticizers at PVC surface have an influence on complement activation, but also the incubation condition such as incubation time and the diameter of PVC tubing. Under static status, C3a, C5a and SC5b-9 concentration in the blood were higher after contacting with PVC plasticized with DEHP (PVC1) than after contacting with PVC plasticized with DINCH (PVC2). However, under dynamic circulation, the results were totally converse, which may be due to smaller diameter and higher shear rate of PVC2. In addition, there was a significant increase of activated partial thrombin time (APTT) and decrease of FIX concentration after plasma contacting with the PVC tubing, which indicated that the intrinsic pathway may be impacted when blood contacted with PVC tubing. However, there was no significant difference of APTT, FIX concentration and CD62p expression rate between the two materials. Moreover, the migration in the DINCH system was considerably lower than for DEHP, which indicates that DINCH could be a promising alterative plasticizer of DEHP.

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1 Introduction

Plasticized poly(vinyl chloride) (PVC) is the most widely utilized blood-contacting material, and is employed in applications including blood storage bags, and as blood tubing in extracorporeal circuits [1, 2], because it shows optimal inertness, durability, and resistance to heat and chemicals. Di-(2-ethylhexyl)phthalate (DEHP) has been the most common plasticizer to be incorporated into PVC at a level between 30 and 40 % to produce the required flexibility to PVC [3]. However, DEHP has been found to leach out from the containers such as blood bags and tubing, although many studies have confirmed that DEHP offers a benefit for RBC storage [4, 5], the risk of exposure to DEHP is acknowledged, which has deleterious effect particularly for newborns [6, 7]. In order to reduce the extractability of plasticizer, one of the non-phthalates di(isononyl) cyclohexane-1,2-dicarboxylate (HEXAMOLL® DINCH) has been used, which has similar technical properties as DEHP but lower migration. In the selected two types of plasticizer, DEHP and DINCH have a similar chemical structure, with the only difference in the benzene ring and six-membered ring. Figure 1 shows the chemical structures of the two plasticizers.

It was reported that DINCH has no indication of toxicity or genotoxicity; it is biodegradable; and it has low sensitizing properties [8]. Currently, it seems to be that DINCH has no effects on fertility up to the highest administered dose of 1,000 mg/kg bw/day [9]. In addition, DINCH has the Food and Drug Administration approval, and RBCs appear to be stable for 42 days in a Hexamoll DINCH-based blood bag [10].

Previous studies of the blood response to plasticized PVC indicate that the blood compatibility of plasticized PVC is influenced not only by the PVC itself, but also by

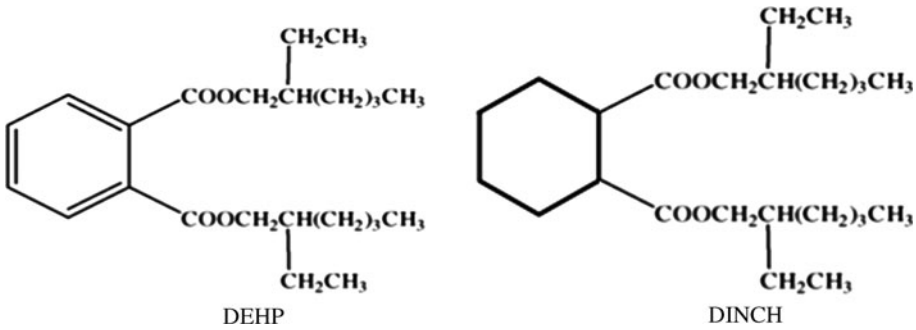

Fig. 1 The chemical structure of DEHP and DINCH

the nature of plasticizers employed and the concentration of plasticizer in the PVC formulation [11–13]. As an alternative to DEHP, it may be necessary to incorporate a higher proportion of DINCH to obtain the desired flexibility, which may lead to a change of surface characteristics of the materials and a more pronounced blood response. Therefore, further blood compatibility studies of PVC plasticized with DINCH needs to be evaluated.

In this study, the blood compatibility of the medical-grade plasticized PVC tubing for extracorporeal circulation devices was evaluated with respect to the plasticizer selection. In considering the blood response in the clinical utilization of this biomaterial is diverse and complex [14], initially complement activation was examined under static incubation and dynamic circulation status firstly. Complement activation has been thought to be responsible for triggering inflammatory responses. The relationship between surface-induced complement activation and thrombosis is not clear, but there have been claims that complement activation promotes thrombosis by enhancing cellular adhesion, platelet aggregation, and platelet activation [15–17]. Therefore, the influence of plasticizers on the coagulant system and platelet activation was also assessed.

2 Materials and methods

2.1 Materials

Two medical-grade plasticized PVC tubing and sheet materials were evaluated to achieve a comparison between PVC plasticized with DEHP (PVC1) and PVC plasticized with DINCH (PVC2). PVC1 and PVC2 were supplied by one of the Chinese Medical Polymer Company, with an internal diameter of 5 and 3 mm respectively.

2.2 Surface analysis

2.2.1 Wettability

The surface wettabilities were measured by the sessile water drop method. Water contact angles were measured directly on the PVC sheet surfaces by a contact angle meter (Model OCA 20, DataPhysics Instruments, Germany) using the sessile drop method. The diameter of a 10-μl droplet of Milli-Q water applied on the surfaces was measured.

2.2.2 Measurement of surface zeta-potential

The measurements of the surface zeta-potentials of both the PVC sheets were carried out in a 10 mM NaCl solution using an electrophoretic light-scattering spectrophotometer (DelsaTM Nano C, particle analyzer, Beckman Coulter, USA) with a plate cell. Three measurements were applied to each sample.

2.2.3 Migration behaviour of DEHP and DINCH from PVC in contact with blood

Blood was collected from volunteer donors at Chengdu blood center and anticoagulated with citrate–phosphate–adenine. The blood sampling and this study were approved by Chinese Academy of Medical Sciences and Peking Union Medical College Research Ethics Committee. To simulate the extreme conditions of clinical extracorporeal circulation, the 2.5 m length of PVC1 and PVC2 tubing were circulated with 715 ml blood by Roller pump at 37 °C water bath for 6 h respectively, and the flow rate of blood was 6 l/h. After circulation, the blood was extracted by ethyl acetate and by use of a rotary evaporator, then the PVC1 sample was dissolved in methanol and tested by high pressure liquid chromatography (HPLC) (Waters e2695-2489, Waters, USA) (detector: UV, 270 nm, mobile phase: methanol, flow rate: 1.0 ml/min) [18], while the PVC2 sample was dissolved in cyclohexane and tested by liquid chromatography-mass spectrometry (LC–MS) (Finnigan TSQ Quantum Ultra, Thermo, USA).

2.3 Blood analyses

2.3.1 Complement activation

Fresh whole human blood was collected from healthy volunteer donors using a vacuum tube technique for

Routine Venipuncture by Chengdu blood center, allowed to coagulate at room temperature for 30 min and centrifuged at 4 °C for 10 min to collect the serum. According to ISO 10993-12:1996, PVC tubing with total surface area of 9 cm^2 were incubated with 3 ml serum at 37 °C for 2 h. In addition, serum without any material incubated at 37 °C for 2 h was set as the native control (control I), and serum added with zymosan (7 mg/ml) was set as the positive control (control II). The serum was then stored below −70 °C until analysis. On the other hand, in order to simulate the clinical extracorporeal circulation, different lengths of PVC tubing (the same ratio of blood to material area as static incubation status) were circulated with 25 ml blood by Peristaltic pump for 2 h, and 3 ml blood samples were collected before circulation and at 15, 30, 60, 120 min intervals during circulation. This was achieved by flow at a rate of 16.7 ml/min. Then the blood was centrifuged at 3,500 rpm/min at 4 °C for 10 min to obtain plasma which was stored below −70 °C until analysis. Complement components of serum/plasma such as C3a, C5a and SC5b-9 were then analyzed using commercially available ELISA kits (Quidel corporation, San Diego, CA, USA).

2.4 Blood coagulation

In order to explore the effect of PVC tubing on the blood coagulation system, PVC tubing with an area of 9 cm^2 were incubated with 3 ml fresh frozen plasma (anticoagulation with citrate–phosphate–adenine) supplied by Chengdu blood center at 37 °C for 1 h, and the plasma without any material was set as the negative control. After incubation, the plasma sample was assessed for coagulation times, including activated partial thrombin time (APTT) and prothrombin time (PT). In addition, considering some intrinsic blood clotting factors were sensitive to be influenced by biomaterial, the concentration of FVIII, FIX and FXII of the plasma sample were also tested. The concentrations of the blood clotting factors were determined by measuring the ability of the sample plasma to correct for the coagulation time of plasma being deficient for the blood-clotting factor of interest. The clotting time was then converted to a concentration expressed as an international unit per ml, IU/ml, using a standard curve constructed using dilutions of normal plasma as sample. All the tests were determined using an automated blood coagulation analyzer (Instrumentation Laboratory ACL ELITE, USA).

2.4.1 Platelet activation

The ACD-blood (the blood anticoagulated with citric acid-sodium citrate-glucose) supplied by ChengDu blood center was centrifuged at 180 g for 10 min to obtain a platelet-rich plasma (PRP). Then the PVC tubing with area of 1.5 cm^2 were immersed into 0.5 ml PRP for incubation at 37 °C for 1 h. After incubation, the expression of P-selection (CD62p) activity of incubated PRP was assessed by flow cytometry (Becton–Dickinson) using anti-CD61- fluorescein isothiocyanate (FITC), anti-CD62p–phycoerythrin(PE) and IgG1(mouse)-PE(BD Pharmingen, BD Biosciences, San Jose′, CA, USA). Five microlitre of PRP was incubated with 5 μl antibodies for 15 min at room temperature, then 400 μl TBS buffer (0.15 mol/l NaCl, 0.02 mol/l Tris) was added for flow cytometric analysis. With the light scatter and fluorescence channels set at logarithmic gain, 10,000 platelets were counted for each sample. Data was analyzed using Cellquest Professional (Becton–Dickinson, USA).

2.4.2 Statistics

Statistical evaluation of the data was performed using paired t test and single factor analysis of variance. The probability (P) values < 0.05 were considered to indicate statistically significant differences and were calculated with the software assistance of Excel 2003. The results are expressed as mean ± SD.

3 Results

3.1 Surface properties

To investigate the surface wettability, the static contact angle (SCA) analysis was performed. The results indicated that both PVC1 and PVC2 sheets were hydrophobic as they had 86.36° and 94.46° static contact angle respectively.

The surface zeta-potentials of sample surfaces were measured to characterize their surface charge under the aqueous environment. As shown in Table 1, the zeta-potential of the PVC plasticized with different plasticizer was both negative, but the absolute values were different, while PVC1 was higher than PVC2. Table 2 shows the results of migration of DEHP and DINCH into blood after circulation under real condition. PVC1 was set as a negative control. It can be seen from the data that migration in the DINCH system is considerably lower than for DEHP.

3.2 Complement activation

C3 is cleaved into C3a and C3b upon contact activation of the complement system on artificial materials. Then the smaller 9-kDa anaphylatoxin C3a is released into the fluid phase and rapidly cleaved to form C3a-desArg. In addition, C3b is involved in mediating monocyte and macrophage phagocytosis and cell adherence, as well as contributing to the formation of C5 convertase, which causes the cleavage

Table 1 Static water contact angle and zeta potential of PVC1 and PVC2

Sample surface	Contact angle (°)	ζ potential (mv)
PVC1	86.36 ± 0.65	−23.00 ± 0.80
PVC2	94.46 ± 2.14	−11.47 ± 0.59

Table 2 Migration of plasticizers into blood under real application condition

	PVC1	PVC2
Concentration of plasticizer in blood (μg/ml)	58.2 ± 3.5	0.673 ± 0.084
Migration of plasticizer of per unit area (μg/cm^2)	55.8 ± 4.2	0.645 ± 0.076
Migration of plasticizer of one set of circulation tubing (mg)	249.68 ± 13.28	2.89 ± 0.12

of C5 into the potent anaphylatoxin C5a and C5b. Then the smaller 11-kDa anaphylatoxin C5a is released into the fluid phase and rapidly cleaved to form C5a-desArg, while the presence of C5b initiates the formation of the membrane attack complex (MAC, C5b-9), the common terminal complex for all pathways of complement activation. In the absence of a target cell membrane (that is, activation on artificial materials), it binds to regulatory proteins, like the S protein, and is released to serum as a non-lytic SC5b-9 complex [19–21]. Thus, the presence of C3a/C3a-desArg, C5a/C5a-desArg and SC5b-9 in the fluid phase indicates activation of the complement system.

Figure 2 shows the results for C3a, C5a and SC5b-9 levels in human serum applied to PVC plasticized with DEHP and DINCH. Results showed that after contacting with PVC tubing, the level of complement component fractions including C3a, C5a and SC5b-9 have a significant increase in contrast with the native control but much less than the positive control. In addition, the result of paired *t* test analysis indicated that the amount of C3a, C5a and SC5b-9 released into serum induced by PVC1 was significantly higher than PVC2 ($P < 0.05$), which means the more amount of C3a induced by PVC1, the more amount of C5a and SC5b-9 would be generated.

Complement activation was monitored over time by measuring C3a, C5a and sC5b-9 levels in plasma after whole blood contact with PVC1 and PVC2 tubing (Fig. 3). It was found that the amount of C3a, C5a and SC5b-9 in the plasma induced by the two materials increased with incubation time, and analysis of variance revealed that complement component fraction generation were significantly higher from 15 to 120 min when compared to the pre ($t = 0$) value. On the other hand, the value of C3a, C5a and SC5b-9 in plasma induced by PVC2 was higher than PVC1 from 30/60 min, although the result of paired *t* test analysis of C3a concentration showed that there was no significant difference between the two materials until 120 min, which was different from the results of static status. This reason may be due to the smaller internal diameter of PVC2, which may cause the higher shear rate according to the same velocity of blood flow. However, the analysis of C5a and SC5b-9 result was different from C3a, which showed that there was a significant difference of C5a concentration between the two materials from 30 min, while the significant difference of SC5b-9 concentration between the two materials was occurred from 15 min.

3.3 Blood coagulation

The blood coagulation system includes the intrinsic pathway, the extrinsic pathway and the common pathway. APTT and PT are used to examine mainly the intrinsic and extrinsic pathway, and FVIII, FIX, FXII are the most sensitive coagulation factors in the intrinsic pathway. Figures 4 and 5 show the results of these parameters of blood coagulation system before and after plasma contacting with PVC tubing. The single factor analysis of variance revealed that there was no significant difference of PT times between the plasma with and without contacting materials, while significant difference of APTT times were measured. The above result indicated that the intrinsic pathway was impacted when blood contacted the materials, which was also proved by experiments of activity of blood coagulation factors. Although no significant difference was measured between FVIII and FXII before and after contacting the PVC tubing, the concentration of FIX after contacting with the PVC tubing was significant lower than original plasma. The analysis indicated that the PVC tubing may bind FIX and deplete the protein from plasma. In addition, the paired *t* test analysis indicated that no significant difference of FVIII, FIX and FXII was tested between the two materials.

3.4 Platelet activation

Flow cytometry rapidly measures the specific characteristics of a large number of individual cells. The most widely studied type of activation-dependent monoclonal antibody (MoAbs) directed against granule membrane proteins are those directed against P-selectin (CD62P), which is only expressed on the platelet surface membrane after granule secretion. And mediates adhesion of activated platelets to neutrophils and monocytes [22]. The expression of CD62P of PRP contacted with PVC1 and PVC2 are presented in Fig. 6. The single factor analysis of variance revealed that there was no significant difference on the expression of CD62p between the plasma with and without contacting

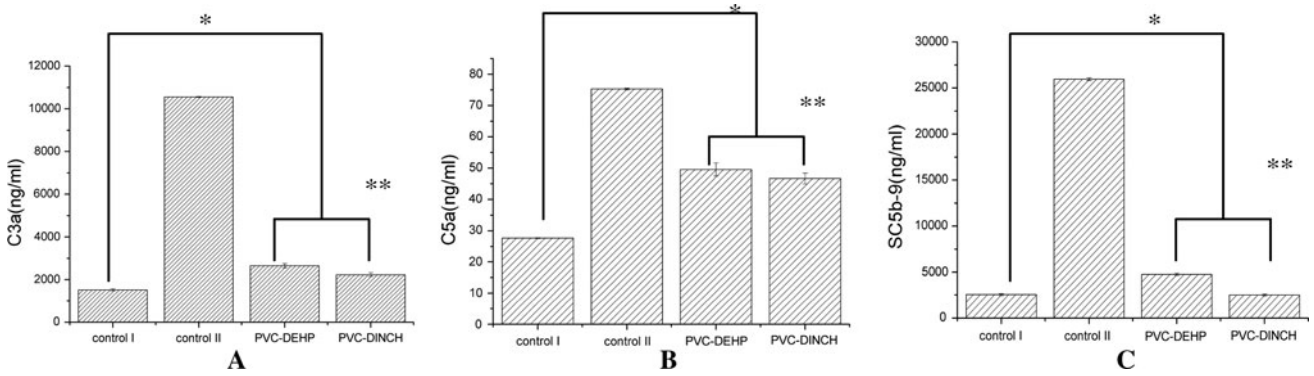

Fig. 2 Quantification of complement components in human serum incubated with PVC plasticized with DEHP and DINCH. Control I: native control, control II: positive control, **a** concentration of C3a, **b** concentration of C5a, **c** concentration of SC5b-9. Results expressed as mean ± standard deviation, $n = 6$. * C3a, C5a and SC5b-9 concentration of PVC1and PVC2 were significantly different from control, ** C3a, C5a and SC5b-9 concentration of PVC1 was significantly different from PVC2. ($P < 0.05$) for all statistics

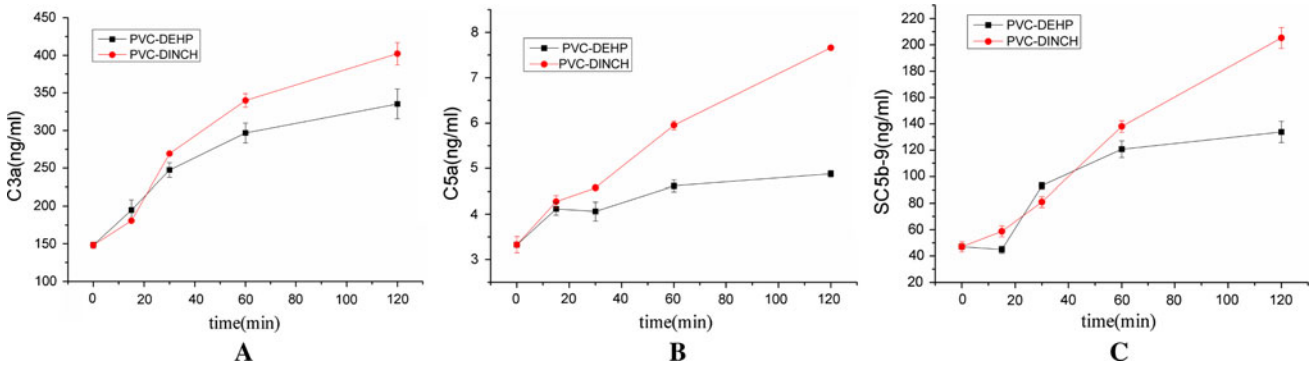

Fig. 3 QuantifIcation of complement component in human blood incubated with PVC1 and PVC2 under dynamic circulation, the total circulation time was 120 min. **a** concentration of C3a, **b** concentration of C5a, **c** concentration of SC5b-9. Results expressed as mean ± standard deviation, $n = 6$

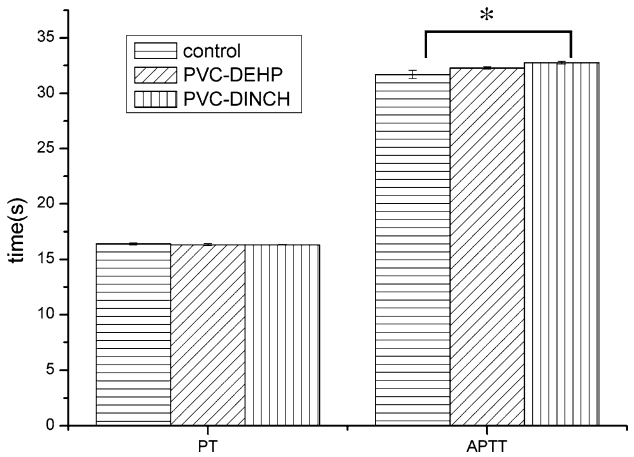

Fig. 4 Clotting time of plasma after incubating with PVC1 and PVC2 at 37° for 1 h. PT and APTT were respectively for native control (blank plasma without any material). The results are shown as mean values ± SEM ($n = 4$). *PVC1 and PVC2 were significantly different from control, ($P < 0.05$) for all statistics

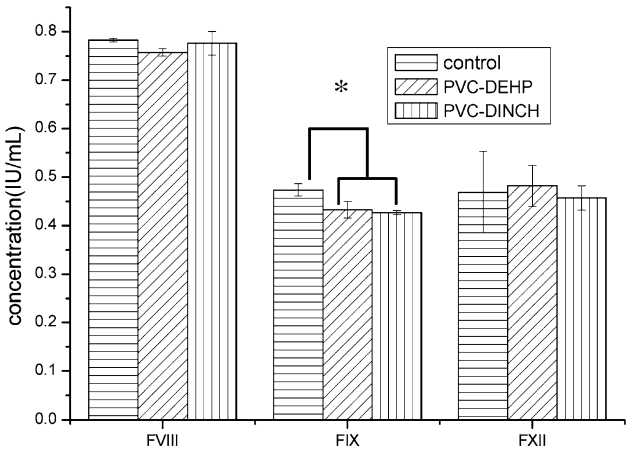

Fig. 5 Coagulation factors levels of plasma after incubating with PVC1 and PVC2 at 37° for 1 h. The results are shown as mean values ± SEM ($n = 4$). * PVC1 and PVC2 were significantly different from control, ($P < 0.05$) for all statistics

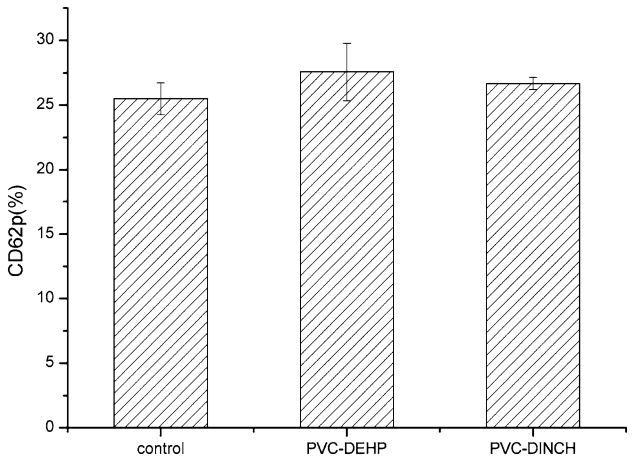

Fig. 6 The expression rate of CD62P of PRP after incubating with PVC1 and PVC2, measured with BD flow cytometry. The results are shown as mean values ± SEM ($n = 4$). In each experiment a minimum of 10,000 platelets were collected and analyzed

materials, and the paired *t* test analysis of the result indicated that there was also no significant difference between the two materials.

4 Discussion

The incorporation into PVC of a plasticizer is essential for the production of a flexible biomaterial, but the migration behavior of different plasticizer is different. The result of migration of plasticizer in this study indicated that considerable quantities of DEHP can transfer into blood and is much higher than DINCH, which suggested that DEHP at the surface of medical grade PVC may be richer than DINCH. However, the plasticizer not only determines the flexibility, but also must play an important role for its surface properties and blood compatibility. The results of static contact angle and zeta potential showed that PVC2 was more hydrophobic and less charged than PVC1, and thus, one may expect that the plasticizer has the potential to influence the blood response.

The use of blood-contacting biomaterials has been challenged by diverse and complex reactions of the blood components to the biomaterials [15, 23]. Complement activation induced by artificial surfaces has been of interest since Craddock et al. [24] discovered that the reduction in white blood cell count during haemodialysis was associated with complement activation. Furthermore, the complement system is interlinked with the coagulation system and with activation of platelets; hence complement is considered an important participant in material-induced thrombosis [15]. The importance of the complement system in the pathogenesis of blood-contacting medical device failure is emphasized by standard ISO 10993-4:2002 which identifies complement activation testing as a recommended procedure for demonstrating aspects of hemocompatibility [25, 26].

The component C3 plays a very important role in complement activation, which is initiated in the fluid phase with spontaneous and continuous generation of enzymes that cleave C3 to C3a and C3b through the alternative pathway. The measurement of the generation of C3a component can provide an indication of the level of complement activation. In this study, after contacting with PVC tubing, the level of C3a increased significantly, which is consistent with previous' reports that PVC1 tubing has high complement generating capacity. By comparing PVC1 tubing with two haemodialysis membranes such as Cuprophan and AN69S, Lamba et al. [27] found that PVC tubing plasticized with DEHP produced higher level of plasma C3a than Cuprophan, a membrane that is regarded as a high complement activator. Our results showed that in comparison with PVC1, the amount of C3a, C5a and SC5b-9 released into blood by PVC2 was significantly lower under static status, while the reason may be due to the different surface properties of the PVC tubing. Engberg et al. [28] synthesized 22 polymers with different physicochemical properties, for example, composition, surface area, pore size and wettability, then six of these polymers were screened for complement activating capacity. Data obtained from the study suggest that a complement-resistant surface should be hydrophobic, uncharged, and have a small available surface, accomplished by nanostructured topography. This conclusion may be partly consistent with our study. Since PVC1 had lower static contact angle and higher surface charge density, and PVC2 was more hydrophobic and less charged, PVC1 caused more complement activation fragments such as C3a, C5a and SC5b-9 released into blood.

The results of this study showed that not only the plasticizers at PVC surface had an influence on complement activation, but also the incubation condition such as incubation time and the diameter of PVC tubing. The time profile for complement activation is similar for PVC tubing, with levels of the soluble components C3a, C5a and SC5b-9 increasing with incubation time. The difference in complement activation between the two materials increased with time, showing a statistically significant difference after 60 min, and the concentration in blood induced by PVC2 was higher than PVC1, which is different from static incubation. However, considering the smaller inner diameter of PVC2, which could be the reason to cause the higher shear rate of blood flow with the same blood velocity under dynamic circulation. The shear rate of blood is higher; the shear stress of blood would be bigger, therefore, in this situation, blood Hydrodynamics may play more important role on complement activation than material or plasticizer.

The similar phenomenon could be observed in other studies. Helen et al. [29], evaluated blood compatibility of different kinds of tubes with inner diameters of 4 and 6 mm, and the results indicated that the thrombin–antithrombin (TAT) generation depended on the shear rate of the blood in the loop system; the higher the velocity, the greater the TAT generation. It has been shown that complement contributes significantly to thrombosis by directly enhancing blood clotting properties, for example, C5b-9 complex can activate endothelial cells to express tissue factor procoagulant activity [17] which could lead to the activation of coagulation and increase of TAT complex in plasma. On the other hand, lower concentration of complement component in blood may relate to the physical adsorption of C3a on the PVC surface rather than the reduction of the complement activation.

Blood coagulation involves a series of proteolytic reactions resulting in the formation of a fibrin clot. Initiation of clotting occurs either by surface-mediated reactions, or through tissue factor (TF) expression by cells. Activation of the coagulation cascade by blood-contacting biomaterials can lead to material failure via thrombin activation of fibrinogen and fibrin formation, which can occur via the intrinsic pathway or the extrinsic pathway [15]. Artificial surfaces activate the coagulation system through the intrinsic pathway, also referred to as contact phase activation. The contact phase is initiated through the interactions of FXII, prekallikrein (PK) high molecular weight kininogen (HMWK) and FXI: it is commonly said that these molecules require contact with (negatively charged) surfaces for zymogen activation in vitro [30]. The ability of plasma to clot through the intrinsic/contact pathway was analyzed by the APTT assay, and the result in this study showed that plasma incubated with PVC tubing had a significant prolonged clotting time in comparison with control plasma (Fig. 4), indicating the PVC tubing also decreased the ability to generate thrombin through the intrinsic/contact pathway. Kaolin, which is used in the APTT test, is a silicate mineral that has a high negative surface charge that activates the intrinsic/contact pathway. Another study [31] showed that carboxyl-modified nanoparticles (NPs) also had a negative charge and thus activated coagulation in a manner similar to that of kaolin. In this study, the zeta potential analysis of the two PVC tubing showed that they both had negative charge and thus activated coagulation in a manner similar to that of kaolin and NPs. In addition, the paired *t* test analysis indicated that no significant difference of FVIII, FIX and FXII was tested between the two materials; means different plasticizer such as DEHP and DINCH can have the same effect on blood coagulation.

Platelet activation and adhesion is known to occur during cardiopulmonary bypass, hemodialysis, as well as with vascular grafts and catheters [32, 33]. The antibody CD61 was used as a marker for a general identification and quantification of the platelets on the polymer spots as it recognizes GPIIb which is present on all platelet membranes [34]. In contrast, CD62P (P-selectin) is only present on the outside of the platelet membrane after activation. Therefore, it was used as a marker to check if the platelet activated by PVC tubing [22]. The statistical analysis revealed that there was no significant difference of the expression of CD62p between the plasma with and without contacting PVC tubing, and there was also no significant difference between the two materials, which indicated PVC tubing and plasticizer may have no effect on platelet activation. Complement activation may relate with coagulation system and platelet activation as previously reported [15–17]. In our study it was indicated that higher complement activation caused by PVC tubing may not result in significant coagulation system and platelet activation.

5 Conclusion

The work presented here describes the blood response of two types of plasticized PVC in order to correlate this with plasticizer selection and surface properties. Results indicate that the plasticizer type at the PVC surface may affect the surface properties such as wettability and charge and has a strong influence on complement activation, but has little effect on the coagulation system and platelet activation. As a promising alternative plasticizer for DEHP, DINCH shows better blood compatibility than DEHP, since it causes less complement activation and has a more prolonged clotting time. Furthermore, the migration rate of DINCH into blood is much lower than DEHP, which suggests that it will have less adverse events to the human body when it's utilized in real condition.

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