

# Vpeljava in optimizacija in-vitro metode za določanje hemokompatibilnosti modificiranih polietilenteraftalatnih površin

## Introduction and optimization of the in-vitro method for determining the hemocompatibility of modified poly(ethylene)terephthalate surfaces

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### Izvleček

**Namen:** Razvoj hemokompatibilnih biomaterialov je zelo pomembno področje znanosti o materialih, zato je nujno potreben znaten napredek v iskanju in razvijanju zanesljivih in standardiziranih metod za analizo hemokompatibilnosti (2).

V članku je predstavljena analiza metode sprostitve hemoglobina za določanje časa strjevanja krvi v stiku z obdelanimi površinami PET. Namen raziskave je bil uvedba in optimizacija metode za analizo hemokompatibilnosti različno kemijsko spremenjenih (PET) površin.

**Metode:** Za določanje hemokompatibilnosti različno obdelanih PET površin smo uporabili prilagojeno metodo detekcije prostega hemoglobina (3,4).

Analiziranih je bilo pet različno kemično obdelanih PET vzorcev, in sicer: kemijsko predobdelan PET

### Abstract

**Purpose:** Internationally-accepted standards have been developed for a range of tests and parameters for characterising the in-vitro interactions of biomaterials with blood. However, there are, as yet, no standards concerning the size, design and type of such in-vitro testing systems (1). Since the development of hemocompatible biomaterials provides a very important challenge in material science, there is a need for further progress in finding reliable and standardized methods for hemocompatibility testing (2).

The aim of this research was to introduce a method for analyzing the hemocompatibility of different chemically-modified surfaces. Polyethylene terephthalate (PET), with surface modifications using different polysaccharides and their derivatives, were chosen because of their

ter PET obdelan s hitosanom, fucoidanom, sulfatiranim chitosanom in heparinom. Steklo je bilo uporabljeno kot standardna trombogena površina.

**Rezultati:** Rezultati pokažejo, da mešanje raztopine hemoglobina in pri 40 tresljajih na minuto zmanjša standardno deviacijo za 68 % v primerjavi s 120 tresljaji na minuto.

Dodatek pufru in s tem zvišanje pH iz 5.2 na 7.4 je še dodatno zmanjšalo standardno deviacijo (56 %). Merilno napako smo še nekoliko zmanjšali s hlajenjem krvi po odvzemu in naknadnim segrevanjem na 37 °C.

**Zaključki:** Znižanje hitrosti mešanja raztopine, dodatek pufru in ohlajanje krvi pred eksperimentom, značilno zniža standardno deviacijo merilnih rezultatov, in sicer za 89 %.

Optimirana metoda in njihovimi derivati niso bile značilne.

*promising biocompatible properties and numerous potential biomedical applications.*

**Methods:** A modified hemoglobin-free method was used to determine the antithrombogenicity of the modified PET surfaces (4). The method was optimized for shaking rate, the addition of buffer, and blood temperature to decrease measuring errors. Five differently-modified PET surfaces were analyzed: chemically pre-treated PET and PET treated with chitosan, fucoidan, sulphated chitosan and heparin. Glass was used as a standard thrombogenic surface.

**Results:** The results showed that a lower shaking rate, the addition of buffer, and blood cooling prior to measurement significantly decreased the standard deviation of the measurement results by a total of about 89 %.

**Conclusions:** We believe this optimized hemoglobin-free method is suitable for distinguishing between chemically and structurally-different surfaces, such as glass and PET. The differences between PET surfaces coated with different polysaccharides were, however, less pronounced.

## INTRODUCTION

The increasing life expectancy of the general population is adding to the number of people worldwide in need of cardiovascular care; global demand for cardiovascular devices will, therefore, continue to rise (5). Although there has been more than 50 years of synthetic cardiovascular implant development and therapy adjustments, the same problems persist: hemolysis, thrombosis, thromboembolic complications, anticoagulation-related hemorrhage, infection, and pannus formation (tissue overgrowth).

Numerous methods have been proposed in order to reduce the thrombogenicity of synthetic implants, such as improvement of physico-chemical properties, pre-treatment with proteins, incorporation of negative charges, application of anticoagulant and anti-platelet agents, and lining the prosthetic implant with cultured endothelial cells that could prevent thrombus formation (6). However, no biomaterial has yet produced a satisfactory performance when in contact with blood for long time periods (3).

Polyethylene terephthalate (PET) is one of the most frequently used biomaterials in cardiovascular surgery. It is well-known that uncoated PET possesses moderate biocompatibility, which is insufficient for cardiovascular replacements. By modifying PET surface properties, better biocompatibility should be achievable – in particular, better antithrombotic properties – but so far no surface modification has produced satisfactory results (7)

Since the development of hemocompatible biomaterials is a very important challenge in material science, there is a need for further progress in finding reliable and standardized methods for hemocompatibility testing (2). A wide variety of different testing systems and techniques are currently used in the development of new materials (8)(12). The majority of these methods differ in their designs and the types of in-vitro systems used, such as the incubation systems and procedures. Major changes can take place in blood as a result of the complex processes that occur in in-vitro systems. A thorough study of all the influences that could alter the results of whole blood tests of the hemocompatibility of biomaterials is therefore of fundamental importance.

We previously developed a haemoglobin-free method (4) for the in-vitro evaluation of hemocompatibility. In the past, clotting-time was used for evaluating the hemocompatibility of a range of chemically-different materials, such as Teflon, glass and collagen (9). This method, however, has not yet been used to distinguish chemically similar surfaces, such as differently-modified PET surfaces. In the present research, we developed a suitable method for evaluating the clotting-times of same-base materials, such as PET surfaces modified by using different biopolymers. In order to increase the method's sensitivity and to decrease measuring errors and deviations, the method was optimized for the shaking-rate of the incubated samples, pH or buffer addition, and blood temperature. Five different chemically-modified PET surfaces, modified using amino- and sulpho-polysac-

charides, were analysed and the results compared to those of glass, which was used as a standard thrombogenic surface.

## **MATERIALS AND METHODS**

Mylar® (PET) foil with a thickness of 175 µm was used for the experiments. All chemicals used were of analytical grade and used without further purification. Chitosan from crab shells with low molecular weight (Aldrich, 448869) and a deacetylation degree of 75–85 % was applied. Sulphated chitosan with a 15.8 % sulphur content was synthesized from the chitosan sample. Fucoidan from *Fucus vesiculosus* (Fluka, 47865) and heparin sodium salt from porcine intestinal mucosa (Fluka 51551) were used.

### **PET foil pre-treatment**

175 µm PET foil was immersed in 98 % ethanol and cleaned in an ultrasonic bath for 10 minutes, then washed thoroughly with demineralized water and air dried. The PET foil was hydrolyzed using 4 M NaOH solution to activate the surface for later chitosan adsorption. Hydrolysis was stopped and the foils neutralized using 1 M HCl. The foils were air dried after thorough rinsing with demineralized water.

### **PET surface modification**

PET surfaces were chemically modified using chitosan and sulphated polysaccharides to improve their blood-contact properties. During the first step chitosan was adsorbed at 60°C, with a solid/liquid ratio of 1:50. The samples were then thoroughly washed until constant conductivity of the rinsing water was reached, then the samples were vacuum dried. Individually selected sulphated polysaccharides (fucoidan, sulfochitosan and/or heparin) were adsorbed onto the chitosan-modified PET surface. Fucoidan and heparin adsorption was carried out using 0.6 w/v % aqueous solution at 40°C with a

solid/liquid ratio of 1:50. The adsorption of sulfo-chitosan was carried out using 0.3 % aqueous solution at pH 7.4.

The samples were labelled as follows:

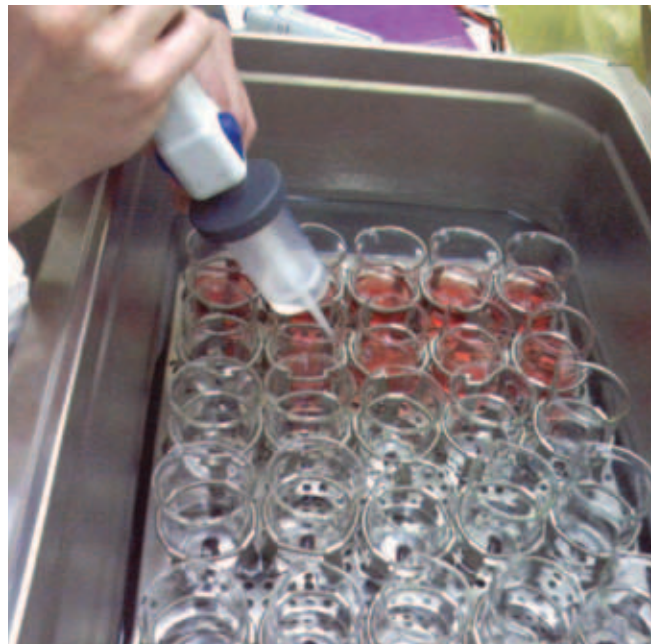
- Glass - reference thrombogenic surface
- PET-H - pre-treated PET foil
- PET-HC - chitosan treated PET foil
- PET-HCF - chitosan and fucoidan treated PET foil
- PET-HCSH - chitosan and sulfochitosan treated PET foil
- PET-HCHEP - chitosan and heparin treated PET foil

### In-vitro blood compatibility determination

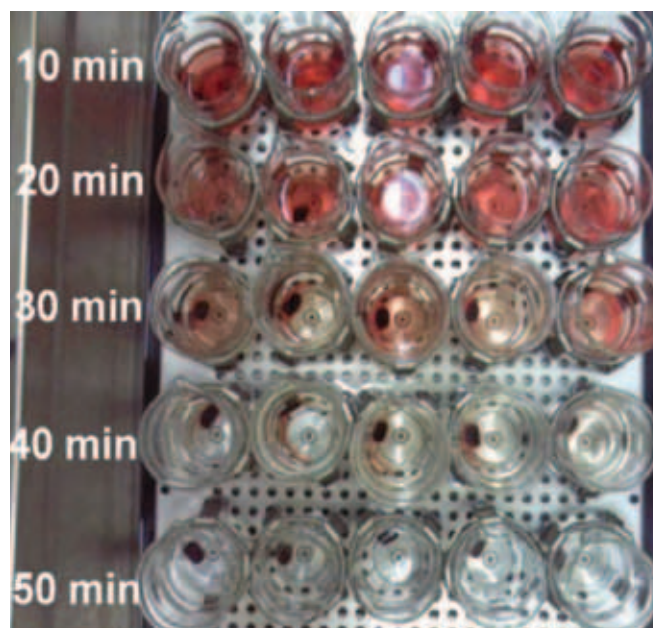
The thromboresistant properties of differently coated PET samples were evaluated using the hemoglobin-free method (7). PET foil samples were cut into 8 x 20 mm rectangular pieces, rinsed with ethanol, and dried in a vacuum at 37°C for 24 hours. The cleaned and dried samples were then placed into 25 mL beakers in a water bath at 37°C and the temperature monitored with a thermostat. Blood was donated by healthy young male adult volunteers and used fresh, without cooling or the addition of an antithrombogenic agent. 0.1 mL fresh blood was placed on each sample piece. After set times (10, 20, 30, 40 and 50 minutes) the clotting procedure was terminated by adding a defined amount of distilled water or buffer solution (Fig. 1). This stopped any clotting processes. Those red blood cells not entrapped within the blood clot were hemolyzed and the freed hemoglobin was dispersed into the liquid (Fig. 2). The concentration of free hemoglobin was determined colorimetrically by measuring the absorbance values at a wavelength of 540 nm. The results from 5 parallel sample pieces were used for the statistical evaluation of the measurement results.

The influences of the shaking rate of the beakers after liquid addition, the amount of buffer added (pH), and the temperature of the added blood

were investigated to optimize the procedure. Shaking rates from 0–120 shakes/minute were investigated.



**Figure 1:** Termination of the clotting procedure with the addition of water and/or buffer



**Figure 2:** Samples after buffer addition: time-dependency of hemoglobin release

## RESULTS

Figure 3 shows the results of the optimization for the shaking rate of the fluid.

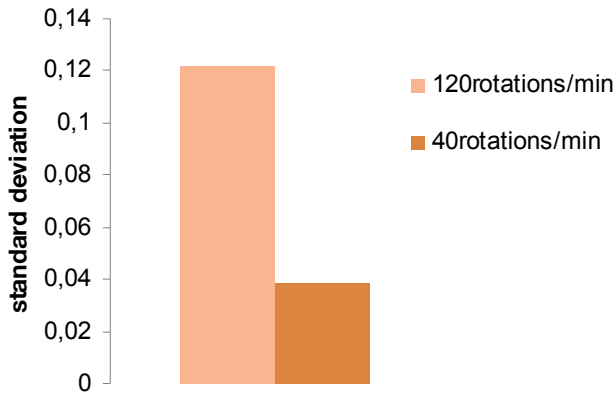


Figure 3: Influence of the shaking rate on the standard deviations of the measurements

Figure 4 shows the results of the optimization for the phosphate buffer addition.

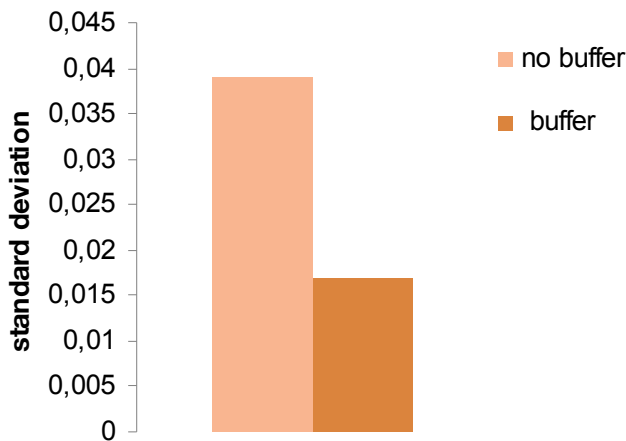


Figure 4: Influence of buffer addition on the standard deviations of the measurements

Figure 5 show the results of blood cooling.

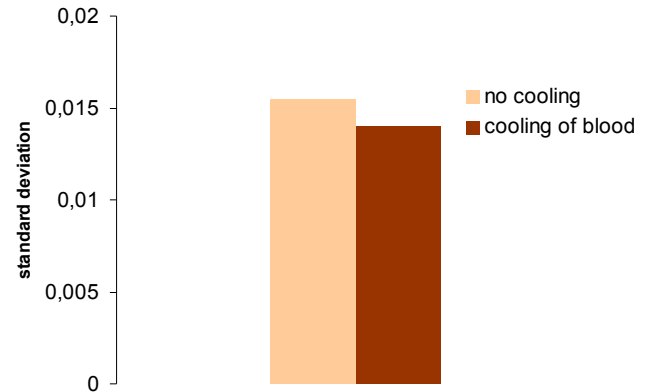


Figure 5: Influence of blood cooling on the standard deviations of the measurements

Figure 6 shows the results of the applied optimized free haemoglobin method, for determination of antithrombogenic properties of different PET samples.

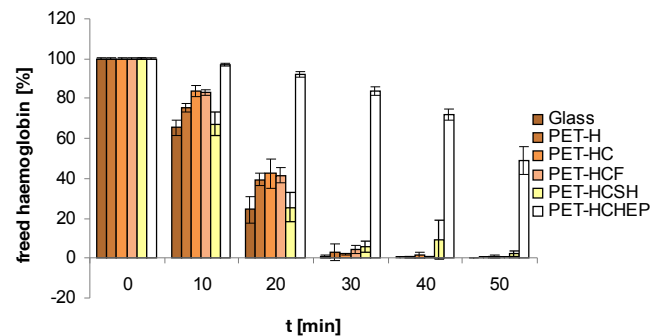


Figure 6: Proportion of hemoglobin released as a function of contact time between a single blood drop and the surface of the samples being studied

## DISCUSSION

The shaking rate of the sample holder was examined during the first optimization phase. During incubation and after the addition of the liquid (water and/or buffer), a certain degree of movement was needed to disperse all the freed hemoglobin from the blood drop into the liquid. This motion has to be precisely tuned. When the motion of the liquid

was too rough, the complete blood clot that had formed on the PET or glass surface was destroyed, and hemoglobin from the red blood cells entrapped within the clot was also freed. This, to a great extent, influenced the results.

The influence of different shaking rates on the standard deviations of the results are presented in Figure 3. A rate of 40 shakes/minute was optimal, as it was high enough to disperse blood in the solution yet gentle enough not to destroy the clots that had already formed. The most sensitive blood clots were those that had not yet completely formed, and these could be destroyed by moderately intense movement of the liquid.

Basic measurements of detection of free hemoglobin were performed according to the method of Boccafoschi et al. (3) and Huang et. al. (4) using distilled water at pH 5.2. Hemoglobin unfolded at low pH to a 'molten globular' state with different levels of structure depending on how low the pH was. At low pH, heme loses contact with its protein but it is not released until the protein is fully dissociated (1). Buffer addition enabled solid stabilization of the hemoglobin molecules and prevented protein degradation.

With addition of phosphate buffer in the testing procedure, mean standard deviations decreased from 0.04 to 0.015 (Fig. 4).

The influence of blood temperature on the coagulation rate was also investigated. With cooling of the fresh blood, the starting point of the coagulation cascade was delayed until the blood temperature rose to 37°C, with delays ranging from a few seconds to a minute. Cooling to app. 4 °C facilitated execution of the experiments and slightly reduced measurement error (Fig. 5).

The optimized conditions defined during the introductory investigations were then used to determine the in-vitro blood compatibility of different chemically-modified PET surfaces. Glass was used as a standard, control thrombogenic surface; and,

as highly antithrombogenic surface, heparin coated PET was applied..

The percentage of hemoglobin released from single blood drops placed onto the samples' surfaces is presented in Figure 6 as a function of contact time with the surface. The glass surface was the most thrombogenic surface, and after 20 minutes only about 20 % of the hemoglobin had been released from the blood drop placed on it, while after 30 minutes the blood clot that formed was so solid that practically no more hemoglobin release could be detected. The opposite results occurred for the PET surface treated with chitosan and heparin (PET-HCHEP sample). After 20 minutes of blood contact, about 90 % of the hemoglobin was being released to the buffer solution, and even after long period of blood contact (50 minutes) about 50 % of hemoglobin was being released. All the other PET surfaces, modified with different polysaccharides, had much higher thrombogenicity. Blood drops placed on PET surfaces modified by natural polysaccharides than other heparin released only about 40 % of hemoglobin after 20 minutes and after 40 minutes no more hemoglobin was released.

## CONCLUSIONS

We optimized the "hemoglobin-free method" for testing the hemocompatibility of polyethylene terephthalate surfaces. The optimal shaking rate after liquid addition was 40 shakes/minute; this decreased the standard deviation of the measurements by about 68 % compared to the results obtained from a shaking rate of 120 shakes/minute. Adding buffer and increasing the pH of the added solution from 5.2 to 7.4 decreased the standard deviations of the measurements by a further 56 %. A further slight decrease in standard deviation of 13 % was achieved when the blood was initially cooled to app. 4°C, as this delayed activation of the coagulation cascade until the blood temperature returned to 37°C. Overall, these steps decreased the standard error to 5–10 %.

The optimized haemoglobin-free-method described here readily distinguished chemically and structurally different surfaces, such as glass and PET, but was less useful for differentiating between PET surfaces coated with different polysaccharides. Measurements using heparin-coated PET (heparin being an ideal

antithrombogenic substance) revealed significantly different antithrombogenic properties compared to all the other coatings studied. Further research needs to be done to discover why other samples (especially samples coated with sulphated chitosan and fucoidan) showed such low antithrombogenicity.

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