In vitro blood compatibility of polyacrylamide grafted polypropylene membrane*☆

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Abstract

BACKGROUND: Contacting with blood, most of polymer materials lead to different extents of blood coagulation, which limits their clinical application. Therefore, developing polymer materials with excellent anticoagulant property has become a key to clinical study of bioartificial liver materials.

OBJECTIVE: To *in vitro* detect the blood compatibility of polyacrylamide grafted polypropylene (PP) membrane (PP-g-AAm), a novel artificial liver reactor material.

METHODS: Prior to and after modification, hemolytic test, prothrombin time and activated partial thromboplastin time tests of PP membrane were performed; blood platelet CD62P and CD63 expression rates were determined by flow cytometry, and platelet adhesion on PP and PP-g-AAm membranes by scanning electron microscopy.

RESULTS AND CONCLUSION: The hemolysis ratio of PP and PP-g-AAm membranes was 1.32% and 1.46%, respectively. Compared with PP-g-AAm membrane, prothrombin time and activated partial thromboplastin time of PP membrane were markedly shorter ($P < 0.05$). CD62P and CD63 expression rates in the PP-g-AAm membrane were significantly lower than PP membrane ($P < 0.05$). Scanning electron microscopy results revealed that there were obvious changes of platelets adhering to these two membranes, but platelets adhering to PP-g-AAm membrane were fewer than PP membrane. These results indicate that PP-g-AAm membrane exhibits good blood compatibility.

INTRODUCTION

A bioreactor is a key region where material exchange between liver cells from *in vitro* bioartificial liver support system and blood/plasma of acute hepatic failure patients takes place and supports the metabolism of bioartificial liver[1-3]. Recently, some novel non-hollow fiber bioreactors adopt specific polymer membrane materials, such as cuprophane membrane, polysulfone membrane, polyurethane, and polypropylene (PP), which break through conventional hollow fiber semipermeable membrane. However, contacting with blood, most of polymer materials would lead to different extents of blood coagulation, which limits their clinical application. Therefore, it is critical to develop polymer materials with excellent anticoagulant property to study bioartificial liver materials for clinical use. But there is no experiment able to solely evaluate the blood compatibility of materials due to complicated blood coagulation mechanism and *in vivo* environment. Up to date, there have been no overall assessment criteria established[4]. The Second Affiliated Hospital of Zhejiang University collaborated with Institute of Polymer Materials, Zhejiang University on developing polyacrylamide grafted PP membrane (PP-g-AAm), a novel bioartificial liver bioreactor material, and investigated its blood compatibility through detecting hemolysis, blood coagulation, platelet adhesion and activation, taking non-modified PP membrane as control.

MATERIALS AND METHODS

Design

In vitro material blood compatibility detection, controlled observation experiment.

Time and setting

This study was performed at the Institute of Polymer Materials, Zhejiang University and Central Laboratory, Second Affiliated Hospital, Zhejiang University between January 2005 and December 2007.

Materials

Material and equipment	Source
PP membrane	Shanghai Medical Materials Factory, China
Full automatic blood coagulation analyzer	Coulter, USA
Stereoscan 260 scanning electron microscope	CAMBRIDGE, England
FACScan flow cytometer, anti-human CD62P-FITC, anti-human CD63-FITC	B&D Company, USA

Methods

Material preparation

Polypropylene membrane was cut into small pieces (2 cm × 5 cm) and then soaked into Quartz tube containing 30% H_2O_2 solution and oxidized for 4 hours under ultraviolet light. Following removal of H_2O_2 using much deionized water, PP membrane was soaked in tube containing 10 mL 5% acrylamide(AAm) solution, followed by oxygen removal and nitrogen addition. Through the use of syringe, 1 mL 0.015 mol/L ferrous sulfate solution was added to evoke the graft polymerization of AAm on PP membrane surface. Following 30-minute graft polymerization at 30 ℃, PP-g-AAm membrane was obtained and washed with much deionized water to remove the unreacted monomers on PP membrane surface.

Determination of contact angle

The contact angle was determined through the use of JJC-1-type contact angle

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measuring instrument. At room temperature, deionized water droplets with a diameter of 2.0–3.0 mm were dropped onto material surface using microsyringe. Three minutes later, contact angle was rapidly determined. The mean value was obtained by averaging five determination results.

Hemolysis experiment

PP and PP-g-AAm membranes (6 cm^2/mL) were respectively soaked in physiological saline at 37 ℃ for 72 hours. 10 mL material leaching liquor, as well as equal amount of sterilized distilled water (positive control), physiological saline (negative control), was placed into 15 mL centrifuge tube individually for 30-minute water bath at 37 ℃. After 0.2 mL anticoagulative diluted rabbit blood was added, each tube was placed in 37 ℃ water for 60 minutes, followed by 5-minute centrifugation at 1 000 r/min. Supernatant was collected for determination of absorbance at 545 nm through the use of 722 spectrophotometer. Ten parallel samples were chosen from each group.

Preparation of platelet-rich plasma (PRP)

15 mL venous blood was collected from 10 healthy young blood donors, anti-coagulated using 2% EDTA-Na (1: 9), and centrifuged at 800 r/min for 5 minutes to prepare PRP. 3 mL PRP from each blood donor was collected and aliquoted into three Quartz tubes, 1 mL per tube.

PP and PP-g-AAm membranes (1 cm^2) were placed into two tubes separately, and PRP in the remaining tube served as blank control. All three tubes were incubated at 300 r/min and 37℃ on constant-temperature vibrator.

Prothrombin time (PT) and activated partial thromboplastin time (APTT) tests

Following 30-minute incubation on vibrator, 500 μL PRP from each tube was centrifuged at 3 000 r/min for 15 minutes. Supernatant was taken to detect PT and APTT by turbidimetry through the use of full automatic blood coagulation analyzer and equipped reagents. Ten parallel samples were used in each group.

Detection of platelet CD62P and CD63 expression by flow cytometry

Following 30-minute incubation on vibrator, 100 μL PRP supernatant from each group was fixed with 100 μL 2% formalin for 10 minutes and washed with PBS, and platelet concentration was adjusted to 1×10^{10}/L. 100 μL platelet suspension from each group was incubated for 20 minutes with monoclonal antibody CD42b-FITC, DC62P-PE, CD63-PE (each 10 μL) at room temperature. CD62P and CD63 expression rates were detected through the use of FACScan flow cytometer, and experimental data were analyzed using CellQuest 3.1 software. Ten parallel samples from each group were used. PRP sample not contacted with PP or PP-g-AAm membrane served as control.

Observation of platelet adhesion by scanning electron microscopy

1 cm^2 of PP or PP-g-AAm membrane was mixed with 1 mL PRP and incubated for 1 hour at 300 r/min and 37 ℃ on vibrator. Following two PBS washes, PP and PP-g-AAm membranes were fixed with 2.5% glutaraldehyde for 2 hours, then gently washed with PBS for 10 minutes, dehydrated with gradient ethanol, dried at critical point, gold-spread, and finally observed by scanning electron microscopy.

Main outcome measures

In vitro hemolysis ratio of PP and PP-g-AAm membrane materials; PT and APTT after contacting with these two materials; effects of these two materials on CD62P and CD63 expression; scanning electron microscopy observation of morphology of platelets activated.

Design, enforcement and evaluation

The second author designed this study, the first and third authors collected data and performed experimental procedures, the first and fourth authors assessed experimental results. A single blind method was employed.

Statistical analysis

Experimental data were processed using Statistics Software SPSS 10.0 software and expressed as Mean±SD. One-factor analysis of variance was adopted for comparison between groups. A level of $P < 0.05$ was considered statistically significant.

RESULTS

Contact angle

The water contact angle was (72.3±0.1)° for the PP membrane and (29.6±0.1)° for the PP-g-AAm membrane.

Hemolysis results of PP and PP-g-AAm membranes

Hemolysis ratio was calculated according to the following formula: Hemolysis ratio (%) = (Absorbance $_{sample}$ −Absorbance $_{negative\ control}$)/ (Absorbance $_{positive\ control}$−Absorbance $_{negative\ control}$) × 100%. A less than 5% hemolysis ratio indicated that biomaterials corresponded to experimental requirement of hemolysis. An equal to and higher than 5% hemolysis ratio indicated that biomaterials exhibited hemolytic reaction. Results demonstrated that prior to and after modification, hemolysis ratio was less than 5%, indicating that two membrane materials could not lead to hymolytic reaction (Table 1).

Table 1 Hemolysis of PP and PP-g-AAm membranes ($\bar{x}\pm s$, n =10)

Group	Absorbance	Hemolysis ratio (%)
Negative control	0.018±0.0021	0
Positive control	0.773±0.0338	100
PP	0.028±0.0027	1.32
PP-g-AAm	0.029±0.0031	1.46

PP: polypropylene membrane; PP-g-AAm: polyacrylamide grafted PP membrane

PT and APTT results

PT was shorter in the PP group than in the blank control group (P = 0.01), and in the PP group than in the PP-g-AAm group (P = 0.027), but there was no significant difference between PP-g-AAm and blank control groups (P = 0.179). Significant difference in APTT was observed between PP, PP-g-AAm and blank control groups (P = 0.000, P = 0.032), as well as between PP and PP-g-AAm groups (P = 0.039) (Table 2).

Table 2 Prothrombin time (PT) and activated partial thromboplastin time (APTT) detection results ($\bar{x}\pm s$, n =10, s)

Group	PT	APTT
Blank control	14.87±3.81	41.38±6.92
PP	9.68±2.42[a]	30.20±4.64[a]
PP-g-AAm	12.94±3.01[c]	35.67±5.09[bc]

[a]P < 0.01, [b]P < 0.05, *vs*. blank control group; [c]P < 0.05, *vs*. PP group

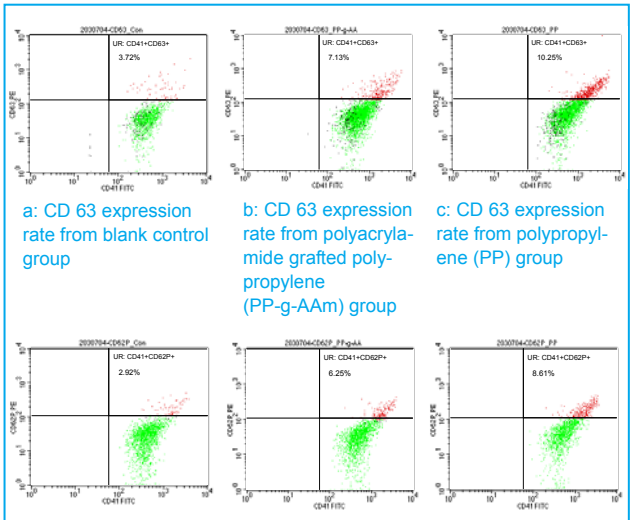
CD62P and CD63 expression rates in platelets activated by PP and PP-g-AAm membranes

Compared with the blank control group, CD62P and CD63 expression rates were significantly higher in the PP and PP-g-AAm groups (P < 0.05), and CD62P and CD63 expression rates were significantly lower in the PP-g-AAm group than in the PP group (P < 0.05). (Table 3, Figure 1).

Table 3 CD62P and CD63 expression rates in platelets ($\bar{x}\pm s$, n =10, %)

Group	CD62P	CD63
Blank control	2.29±0.84	3.68±1.43
PP-g-AAm	5.27±1.52[a]	6.07±1.97[a]
PP	7.60±2.21[ab]	8.98±2.53[ac]

PP: polypropylene membrane; PP-g-AAm: polyacrylamide grafted PP membrane; [a]P < 0.01, *vs*. blank control group; [b]P < 0.05, [c]P < 0.01, *vs*. PP-g-AAm group

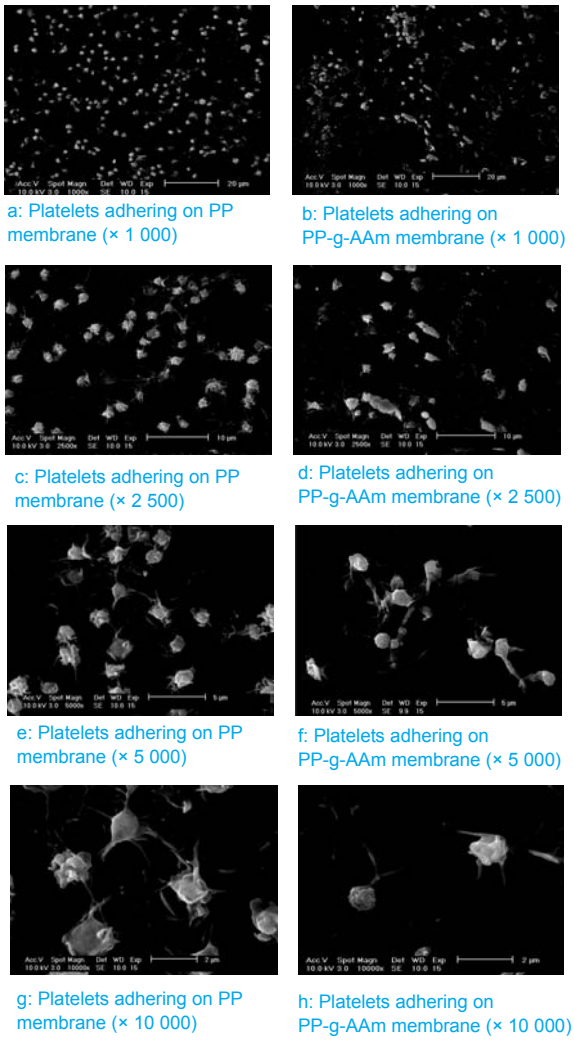


a: CD 63 expression rate from blank control group
b: CD 63 expression rate from polyacrylamide grafted polypropylene (PP-g-AAm) group
c: CD 63 expression rate from polypropylene (PP) group
d: CD62P expression rate from blank control group
e: CD62P expression rate from PP-g-AAm group
f: CD62P expression rate from PP group

Figure 1 Flow cytometry results of CD63 and CD62P expression rates in platelets from different groups

Scanning electron microscopy results of platelet adhesion

Following 1-hour incubation at 37 ℃, platelets adhesion was observed on both PP and PP-g-AAm membranes. Under 1 000-fold magnification, platelets were more on the PP membrane than on the PP-g-AAm membrane. Under 2 500-, 5 000-, and 10 000-fold magnification, obvious platelet pseudopodia could be observed. Under 10 000-fold magnification, PP membranes presented obviously deformed platelets with many long pseudopodia (Figure 2).



a: Platelets adhering on PP membrane (× 1 000)
b: Platelets adhering on PP-g-AAm membrane (× 1 000)
c: Platelets adhering on PP membrane (× 2 500)
d: Platelets adhering on PP-g-AAm membrane (× 2 500)
e: Platelets adhering on PP membrane (× 5 000)
f: Platelets adhering on PP-g-AAm membrane (× 5 000)
g: Platelets adhering on PP membrane (× 10 000)
h: Platelets adhering on PP-g-AAm membrane (× 10 000)

Figure 2 Scanning electron microscopy results of platelets adhering on polypropylene (PP) and polyacrylamide grafted PP (PP-g-AAm) membranes

DISCUSSION

Enhancing surface hydrophilicity has become a widely accepted method to improve the histocompatibility of materials[5-6]. Previous findings demonstrated that following grafting modification, PP surface presents with enhanced hydrophilicity and markedly increased immune compatibility[7]. Contact angle determination results showed that following grafting modification, PP membrane surface exhibits small tension and strong hydrophilicity.

Generally, the hemolytic reaction caused by materials with toxic substance is greater than the toxic reaction in cell culture. Hemolytic crisis from hemolysis test indicates material toxicity. Using the methods recommended by ISO, the present study tested the in vitro hemolytic activity of materials. Results showed that the hemolysis ratio of PP and PP-g-AAm membranes was 1.32% and 1.46% respectively, which was 5% lower than critical value. So these two membranes were considered no hemolytic activity. These results suggest that prior to and after grafting modification, PP and Pp-g-AAm membranes produce little destruction to erythrolysis, and

correspond to the hemolysis requirements of biomaterials. Evidence exists that once materials contact with blood, plasma protein will adhere to material surface. The adhering fibrinogen degrades into fibrin monomer and fibrinopeptide (platelet factor) by electric charge and activity changes. The adhering fibrinogen and γ-globulin would lead to platelet adhesion and activation at the same time, resulting in thrombosis[8]. The adhering albumin would reduce platelet adhesion. The absorption characteristics of fibrinogen on interface correlate to the hydrophilicity and charges of material surface[9]. The plasma fibrinogen of material surface can promote platelet adhesion, because fibrinogen can specifically bind with glycoprotein GP Ⅱb-Ⅲa on material surface, and micro amounts of fibrinogen (not less than 0.02 μg/cm^2) is enough to lead to platelet adhesion and activation [10]. The binding of fibrinogen and platelet further activates signal transduction system, which contributes to platelet activation. The completely activated platelets would express and release some biochemical active substances (such as β-TG, P selectin, and IV factors), resulting in the activation and aggregation of other platelets[11].

When platelets are activated, their appearance turns into globular shape, and some presented with formed pseudopodia. Scanning electron microscopy results showed that platelets with pseudopodia could be observed on PP and PP-g-AAm membranes, but compared with PP membrane, low number of platelets was observed on the PP-g-AAM membrane. Under normal condition, CD62P and CD63 are stored in α granule and lysosome of resting platelets, respectively. But when platelets are activated, α granule and lysosome fuse with plasma membrane and become the markers of activated platelets. Most studies report the whole level change following platelet activation by materials, *e.g.*, detection of β-TG and CD62P by radioimmunity assay or double antibody sandwich method. The present study determined the existence of platelet activated subsets by detecting CD62P and CD63 expression using flow cytometry, and meanwhile investigated platelet morphology and immunity changes based on single cell level. Studying platelet changes using flow cytometry is a novel method in the field of histocompatibility.

Taken together, polyacrylamide grafting modification can enhance the hydrophilicity of PP membrane and thereby obviously improve the blood compatibility of membrane materials. The novel bioartificial liver membrane material exhibits good blood compatibility, which can reduce the adverse events when contacting with blood in clinical use.

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聚丙烯酰胺接枝改性聚丙烯膜的血液相容性*☆

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摘要

背景：绝大多数高分子材料在与血液接触时都导致不同程度凝血，使其应用受到限制。因此研制有优良抗凝血性能的高分子材料成为生物人工肝材料临床研究中的关键问题。

目的：体外检测新型人工肝反应器材料——聚丙烯酰胺接枝改性聚丙烯膜(PP-g-AAm)的血液相容性。

方法：对改性前、后的聚丙烯膜行溶血试验、凝血酶原时间和活化部分凝血激酶时间试验，用流式细胞术检测血小板 CD62P 和 CD63 的表达率，扫描电镜观察两种膜上血小板的黏附情况。

结果与结论：聚丙烯膜和 PP-g-AAm 膜的溶血率分别为 1.32%和 1.46%；聚丙烯膜的凝血酶原时间和活化部分凝血激酶时间较 PP-g-AAm 膜明显缩短 ($P < 0.05$)；PP-g-AAm 膜激活血小板表达 CD62P、CD63 的百分率都明显少于聚丙烯膜($P < 0.05$)；扫描电镜观察两种材料表面黏附的血小板都有明显变形，但 PP-g-AAm 膜表面黏附的血小板明显少于聚丙烯膜。提示 PP-g-AAm 膜具有良好的血液相容性。

关键词：人工肝；血液相容性；生物相容性；聚丙烯；膜生物材料

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