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科　　別：化學

作 品 名 稱：Reduction of free radicals and endotoxin by conjugated linoleic acid loaded in-situ synthesized poly(N-isopropyl acrylamide thin layer

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作者簡介

我是吳銘哲，目前就讀開南商工綜高科三年級，從小對於知識的攝取抱持比較積極的態度，在學習態度上，我始終抱持著在能力許可範圍下盡量做到最好的理念，由於這一年中老師曾帶我出去比賽過亞太抗震盃拿到不錯的成績，再加上同學找我去參加科展，使我對科展，或是一些需要實驗的報告有進一步了解和嚮往。

作者簡介

我是于子捷，目前就讀開南商工實驗研究班一年級，對於實驗研究方向有著非常高的興趣，畢竟動手實做與紙上談兵不太一樣。曾參與2007國際海鳥協會研討會所辦的創藝鳥裝比賽得到佳作。很高興加入實驗研究班中學校給予推薦參與這次2007國際科展比賽的資格，感謝學校老師給與這樣的機會，希望也能不辜負老師的期望。

中文摘要

本研究首先利用 NaOH 將 PAN 薄膜改質形成 PAA 水膠膜表面，進一步與異丙胺鍵結形成 NIPAAm 結構，以做爲藥物傳輸的載體。反應所得之 PNIPAAm 薄膜其溫度轉換點爲 34 °C 其 pH 敏感性介於 pH 5 至 10 之間，實驗進行進一步於 34 °C 將 PNIPAAm 薄膜浸入共軛亞麻油酸(CLA)中，取出後置入 4 oC 二次水中，最後測試經改質接枝後 PAN 薄膜於膽固醇、三酸甘油酯的吸收效果，並以化學冷光儀分析改質接枝後 PAN 薄膜其抗氧化效率。結果顯示改質接枝之 PNIPAAm 薄膜於包埋 CLA 其對於血液中膽固醇及三酸甘油酯有明顯的吸收效率，此外對血液中的自由基也有明顯較未改質之 PAN 薄膜穩定的趨勢。

關鍵字：聚異丙基丙烯醯胺、共軛亞油酸、氧化壓力

英文摘要

A thin layer of poly(N-isopropyl acrylamide) (pNIPAAm) was synthesized *in situ* on the surface of hydrolyzed polyacrylonitrile (PAN) membrane. This thin layer exhibited both pH response due to the poly(acrylic acid) moiety and temperature response due to the pNIPAAm moiety. The swelling behavior of the membranes was evaluated under various temperatures and pH. The curve of the swelling ratio for the PAN-NIPAAm showed a lower critical solution temperature (LCST). Then conjugated linoleic acid (CLA) was loaded into the pNIPAAm layer. The effects of CLA on the blood coagulation and oxidative stress were evaluated using human blood. The level of reactive oxygen species (ROS) was measured by chemiluminescence (CL) method to evaluate the oxidative stress. Furthermore, the removal of bacterial endotoxin (lipopolysaccharide, LPS) by CLA-loaded PAN-NIPAAm was measured with ELISA. The results show that the LCST swelling curve was at 37°C. In addition, the swelling ratio increased by 71% when the pH increased from 5 to 10. The concentration of LPS can be reduced by CLA-loaded PAN-NIPAAm 2.1 and 1.2

times of that by unmodified PAN and PAN-NIPAAm membranes, respectively. In addition, the level of ROS against CLA-loaded PAN-NIPAAm was reduced significantly than that against unmodified PAN and PAN-NIPAAm. Therefore CLA-loaded PAN-NIPAAm membrane could offer protection for patients against oxidative stress and could also inhibit LPS for clinical applications.

Keywords: poly(N-isopropyl acrylamide)、conjugated linoleic acid、oxidative stress

目錄

INTRODUCTION

Stimuli-responsive drug delivery systems have been investigated for their applications in pulsatile delivery of certain hormone drugs.[1,2] Temperature- and pH-sensitive hydrogels have been suggested for use in a variety of controlled drug delivery systems. Among these hydrogels, poly(N-isopropylacrylamide) (pNIPAAm) hydrogels attract more and more interests in biomedical applications because they exhibit a well-defined lower critical solution temperature (LCST) in water around 31~34°C which is close to the body temperature. Poly(NIPAAm) hydrogels swell when cooled below LCST, and they collapse when heated above the LCST. In the literature, pNIPAAm thin layer is grafted to membrane surface through free-radical initiated polymerization of NIPAAm monomers.[3,4] However, NIPAAm monomer is a neurotoxin and a carcinogen.[5] On the contrary, in this study, isopropylamine (IPA) was amidated with poly(acrylic acid) (PAA) on the surface of hydrolyzed polyacrylonitrile (PAN) membranes to produce a thin layer of pNIPAAm. By so doing, the membrane is sensitive to both temperature and pH.

Conjugated linoleic acid (CLA) has been shown to decrease carcinogenesis, decrease atherosclerosis, increase body protein, remove endotoxin and decrease body fat.[6] In addition to the aforementioned features, CLA also modulates the immune system by increasing lymphocyte blastogenesis,[7] lymphocyte cytotoxic activity,[6,8] and macrophage killing ability, as well as protecting against end-stage body wasting in autoimmune disease.[9]

Endotoxins can also be removed by CLA. Endotoxin, also called lipopolysaccharide (LPS), are commonly found in our environment and are the most significant pyrogen in parenteral drugs and medical devices. Their presence in the blood stream may cause septic reactions with a variety of symptoms such as fever, hypotension, nausea, shivering and shock.[10]

In this studying, we adopted a new approach to synthesize the pNIPAAm layer onto the PAN membrane. By loading CLA, the biocompatibility of PAN-NIPAAm

membrane was improved. When such membrane was used for hemodialysis, it can release CLA during the process. The results of this work would be helpful for developing a heparin-less hemodialyzing technique which can simultaneously reduce both oxidative stress and LPS.

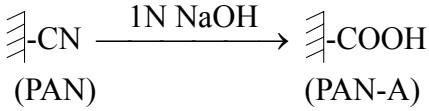
EXPERIMENTAL

Materials

Polyacrylonitrile powders were purchased from Aldrich, USA. N, N-dimethyl formamide (DMF) was purchased from Merck, USA. Lucigenin, luminol, 1,3-propanediol, 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), and N-hydroxysuccinimide (NHS) were purchased from Sigma, USA. Isopropylamine (IPA) was purchased from Acros, USA. 4-morpholineethanesulfonic acid monohydrate (MES) buffer was purchased from Aldrich. Reagents for activated partial thrombin time (APTT), prothrombin time (PT), fibrinogen time (FT), and thrombin time (TT) were purchased from Dade Behring Inc, USA. Endotoxin and limulus amebocyte lysate (LAL) kit was purchased from Associates of Cape Cod, USA.

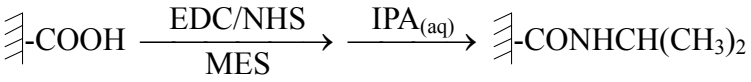
Preparation of polyacrylonitrile membranes

The PAN membranes were prepared by the phase inversion method. The polymer solution (15 wt% PAN in DMF) was cast on a glass plate and immersed in deionized (DI) water to form a flat membrane. The resulting membrane was rinsed with DI water for 12 h, and then was dried under vacuum for 6 h. A piece (5×5 cm^2) of PAN flat membrane was immersed in 100 ml of aqueous 1 N NaOH solution at 50 °C for 10 min. Afterwards, the membrane was removed and washed thoroughly with DI water. Subsequently, the hydrolyzed membranes (designated as PAN-A) were dried in a vacuum oven at 25°C for 4 h. The chemistry reaction was described as follows:



Surface modification

The PAN-A membrane was incubated in 20 mL of 0.01 M EDC/0.01 M NHS of pH 4 MES buffer at 4 °C for 2 h, and then reacted in 0.25mg mL^{-1} IPA solution at 4 °C for 24 h. The resulting samples were denoted as PAN-NIPAAm.



(PAN-A) (PAN-NIPAAm)

Characterization analysis

The characteristic peaks of the functional groups of unmodified and modified PAN membranes were detected using an X-ray photoelectron spectroscopy (XPS) (ESCALAB 250, Thermo VG Scientific, West Sussex, UK) equipped with Mg K_α at 1253.6 eV and 150W power at the anode.

For the study of temperature-dependent equilibrium swelling ratios, the membranes were equilibrated in deionized water (pH 7.0) for at least 24 h at a predetermined temperature between 25 and 40°C. For the pH dependence study, the membranes were equilibrating in the aqueous media at 38°C for at least 24 h at a predetermined pH of 1–14.

The swelling ratios of the samples were measured gravimetrically. After the excess water on the sample surfaces was wiped off with moist filter papers, the samples were weighted (W_w). The dry weight (W_d) of each sample was determined after drying to a constant weight under vacuum overnight at 50°C. The weights from three measurements were averaged, and the swelling ratio was calculated from the following:

$$\text{Swelling ratio} = \frac{W_s}{W_d}$$

where W_s is the weight of water in the swollen sample at a specific temperature and pH, and $W_s = W_w - W_d$.

Loading of CLA

A piece (5×5 cm^2) of PAN-NIPAAm membrane was immersed in 100 ml of DI water at 50 °C for 10 min. Then the membrane was quickly immersed in 10 ml of pure CLA at 4°C for 30 min. Afterwards, this CLA-loaded PAN-NIPAAm membrane was rinsed 3 times with ethanol at 4°C to remove adsorbed ethanol.

Determination of LPS level

The reduction of LPS level was measured by ELISA. A piece of membrane (1×1 cm^2) was place in 500μl of LPS (2 EU/mL) at 25°C for 30 min. Then 100μl of which were added to each well in a 96-well TCPS plate at 37°C for 10 min. Then 50μl of LAL kit were added to each well and incubated at 37°C for 10 min. After adding 10 μl of the chromogenic substrate, the reading was taken from the ELISA reader.

Blood coagulation time

The *in-vitro* coagulation times, including activated partial thrombin time (APTT), prothrombin time (PT), thrombin time (TT), and fibrinogen time (FT) were determined using an automated blood coagulation analyzer (CA-50, Sysmex Corp., Kobe, Japan). In addition, the bioactivity of immobilized CLA was accessed by comparing the APTT with the calibration curve of free CLA in the platelet-poor plasma (PPP) control.

Measurement of the level of reactive oxidants in plasma.

Heparinized blood samples were wrapped with aluminum foil to prevent light exposure until testing for reactive oxidant levels. To measure the production of ROS in the samples, a chemiluminescence (CL) method was adopted using lucigenin (1 mg/mL) as an amplifier for measuring superoxide (O_2^-), and luminol (1 mg/mL) as an amplifier for measuring hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). In brief, 200μL of blood sample were immediately placed in a 96-well dish for the oxidative stress assay using a chemiluminescence analyzer (TopCount System; Packard, Meriden, CT, USA). For each sample, the assay was performed in triplicate, and the reactive oxidant level was expressed as CL counts.

RESULTS AND DISCUSSION

Surface characterization

Figure 1 shows the expanded scale of the C1s XPS spectra for the PAN and the PAN-NIPAAm. The C1s core-level spectra of PAN-NIPAAm were composed of three peak components at about 284.6, 285.7 and 287.4 eV, attributable to the C-H, C-N and N-C=O species, respectively.[11]

According to our previous study,[12] the hydrolytic reaction occurred at the interface of the solid and the solution, thus those nitrile groups contacting the solution can be converted into carboxylic groups. Table 1 shows that the surface density of carboxyl groups fro PAN-PAA was 0.13 μmol/cm^2, which was equivalent to a thickness of 60 nm [12]. As shown in Fig.2a, the swelling ratios of PAN-PAA and PAN-NIPAAm increased with the pH. When pH was higher than 5, the swelling ratio of PAN-PAA increased sharply from 2.9 to less than 4.9. This phenomenon is similar to that reported in the literature [13]. As for PAN-NIPAAm, the swelling curve did not show such a jump, although increased with the pH as well. The swelling ratio changed little at pH above 10. This can be attributed to that part of carboxyl groups of PAA were reacted with IPA to form pNIPAAm, thus the pH-sensitivity was less obvious than PAA.

The LCST of thermo-responsive NIPAAm based materials is the consequence of hydrophobic (associated with the isopropyl groups) and hydrophilic (associated with the amide moiety in the pendant groups) interaction in NIPAAm.[14] As shown in Fig. 2b, the LCST of the PAN-NIPAAm membrane was 37°C, which was higher than the LCST (32°C) of pure pNIPAAm. Incorporation of hydrophilic moieties may increase the LCST of pNIPAAm and vice versa.[15,16] When a hydrophilic component was incorporated into pNIPAAm, the hydrophilic-hydrophobic balance will shift towards a more hydrophilic nature, and the LCST shifts to a higher temperature.

Removal of LPS

Table 1 show that LPS can be removed greatly by PAN-NIPAAm entrapped CLA. As the results, the LPS inhibition of the PAN-NIPAAm entrapped CLA and PAN-NIPAAm was 2.09, 1.73 times of the PAN, respectively. Endotoxin can activate complement, the kinin system, leukocytes, platelets, and endothelial cells. Banni et al. reported that CLA can decrease prostaglandin E2 (PGE2) production via inhibition of LPS.[17] The initial response of the immune system to LPS is the activation of circulating monocytes or tissue-resident macrophages. The anticarcinogenic effect of CLA in endotoxin activated macrophages may be related to its ability to decrease both PGE2 and NO synthesis by suppressing transcription of COX-2 and iNOS.[18] In this study, CLA was loaded into the NIPAAm layer. This suggests that the loaded CLA still possesses the ability to remove LPS.

Blood coagulation

Figure 3 shows the improvement on the coagulation times by the CLA entrapping in PAN-NIPPAAm membranes. The effect of CLA entrapping can be observed by comparing the values of APTT, PT, TT, and FT of PAN and PAN-NIPAAm to those of CLA-loaded PAN-NIPAAm. Figure 4 shows that the coagulation times of PAN membrane are nearly the same as the control, and those of PAN-NIPAAm are slightly higher than those of the control, whereas those of CLA-loaded PAN-NIPAAm membranes are much longer. The values of APTT, PT, TT and FT for CLA-loaded PAN-NIPAAm were 1.78, 1.64, 1.10 and 1.43 times of those of PAN while the PAN-NIPAAm were 1.14, 1.07, 1.06 and 1.05 times of those of PAN, respectively. This indicates that CLA loading can indeed reduce the blood coagulation on the PAN-NIPAAm membrane surface.

Effect on the level of reactive oxidants in plasma.

The chemiluminescence emission spectrum analysis was used to assess the effect of CLA-loaded PAN-NIPAAm on the scavenging activity for plasma ROS (H_2O_2, HOCl and O_2^-). Although hemodialysis can remove dityrosine and creatinine, hemodialysis can also increase oxidative stress, and reduce the plasma ROS

scavenging activity. During hemodialysis, neutrophil contacts the dialysis membrane, activating blood granulocytes, thereby increasing ROS levels.[19]

Figure 4 show that the ROS level for CLA-loaded PAN-NIPAAm is similar to that of the control, while the ROS level for the PAN and PAN-NIPAAm membranes is higher. The CL counts of O_2^-,H_2O_2 and HOCl for PAN increased significantly with the time, indicating that PAN membrane would induce the formation of ROS. Increase in oxidative stress has been closely associated with the untoward effects related to the extracorporeal circulation. The ROS measurements, shown in Figure 4, indicate that CLA-loaded PAN-NIPAAm membrane could directly react with and quench H_2O_2, HOCl and O_2^-. As shown in Figure 4, the HOCl level for CLA-loaded PAN-NIPAAm was 1.24 times of the control (whole blood) while that for PAN and PAN-NIPAAm were 6.07 and 3.13 times of that for the control after 3 min. Similarly, the H_2O_2 level for CLA-loaded PAN-NIPAAm was 0.95 times of the control (whole blood) while that for PAN and PAN-NIPAAm were 2.00 and 1.21 times of that for the control after 5 min. The O_2^- level for CLA-loaded PAN-NIPAAm was 1.01 times of the control (whole blood) while that for PAN and PAN-NIPAAm were 1.37 and 1.31 times of that for the control after 5 min. This suggests that the loading of CLA can reduce hemodialysis-enhanced production of H_2O_2, HOCl and O_2^- and minimize the oxidation caused by a regular PAN membrane.

The antioxidant ability of CLA could function by directly reacting with free radicals to terminate the radical chain reaction or by chelating transition metals to suppress the initiation of radical formation. Recent studies have demonstrated the direct radical scavenging effects of CLA.[20,21] These findings indicate that CLA has radical-scavenging capacity. The chemical mechanism for the CLA-radical reaction cannot be clearly elucidated without identifying the reaction products and/or intermediates. However, it is clear that the conjugated double bonds made contributions to the radical scavenging capacity of CLA.

In summary, our results are in agreement with those reports that CLA can reduce the ROS levels of patients with end-stage renal disease (ESRD) undergoing chronic

hemodialysis therapy.

Table 1. Surface characterization of unmodified and modified PAN membranes. (mean ± std) (n=3)

Membrane	Surface density of carboxyl groups (μmole/cm^2)	LPS Removal (EU/cm^2)
PAN	--	0.11±0.04
PAN-PAA	0.13±0.6	0.13±0.06
PAN-NIPAAm	0.10±0.3	0.19±0.06
CLA-loaded PAN-NIPAAm	--	0.23±0.09

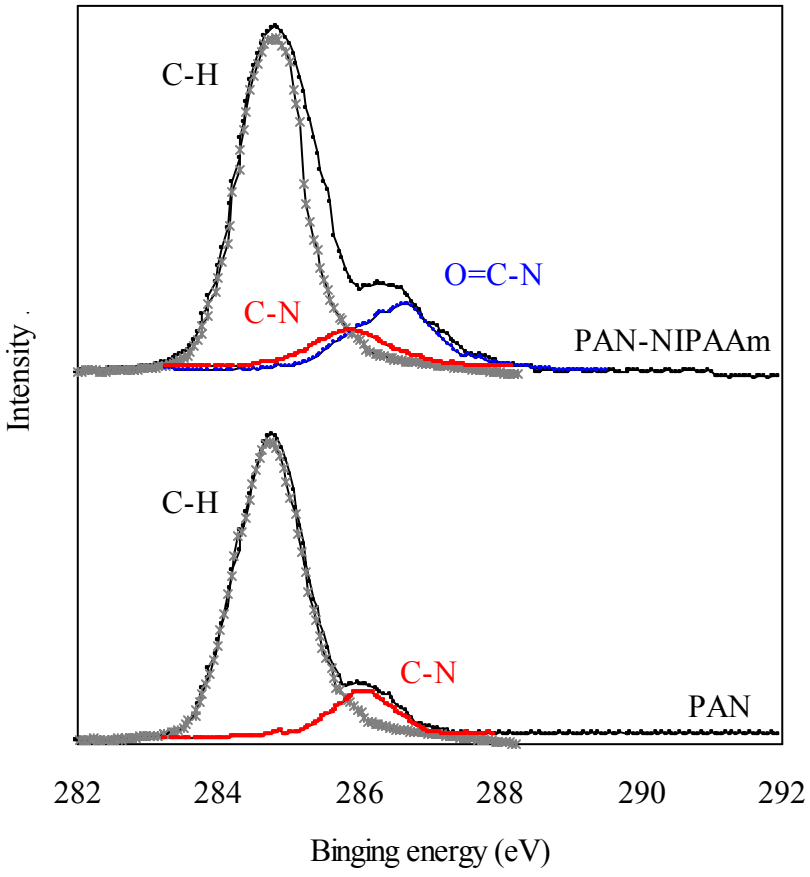


Figure 1.XPS spectra of unmodified PAN and modified PAN membranes.

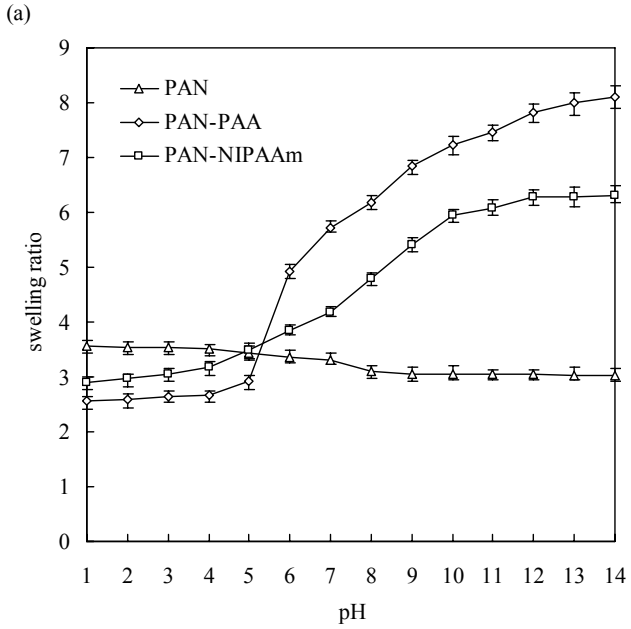


Figure 2a. pH-dependent swelling ratio of PAN-NIPAAm at room temperature. (n=3).

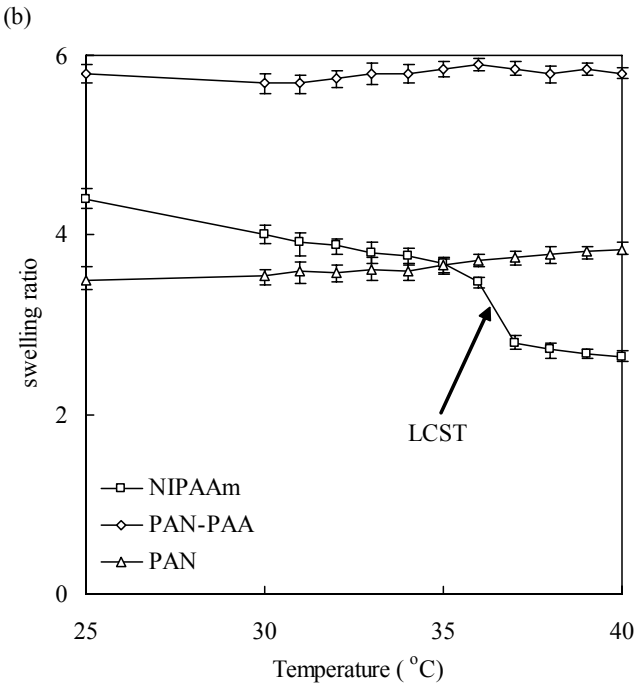


Figure 2b.Temperature-dependent swelling ratio of PAN-NIPAAm in aqueous media of pH 7. (n=3).

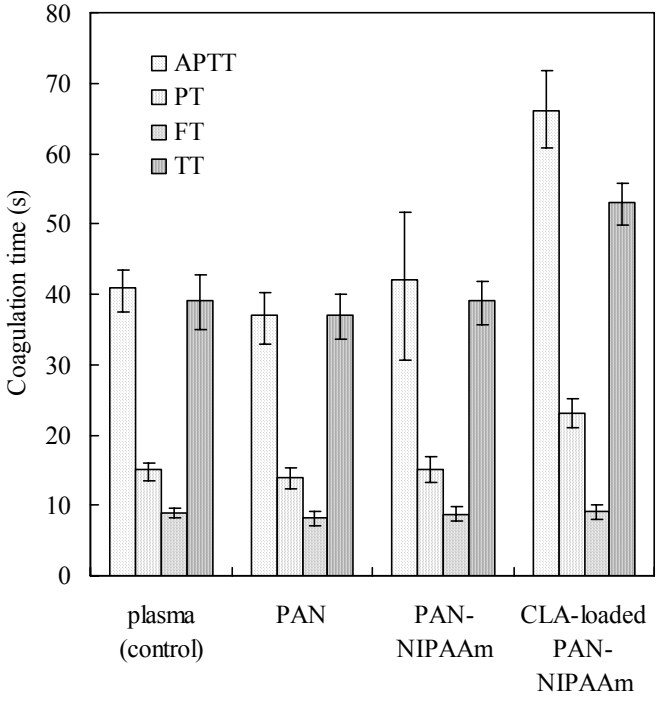


Figure 3. Comparison of coagulation times of PAN membranes. (n=3).

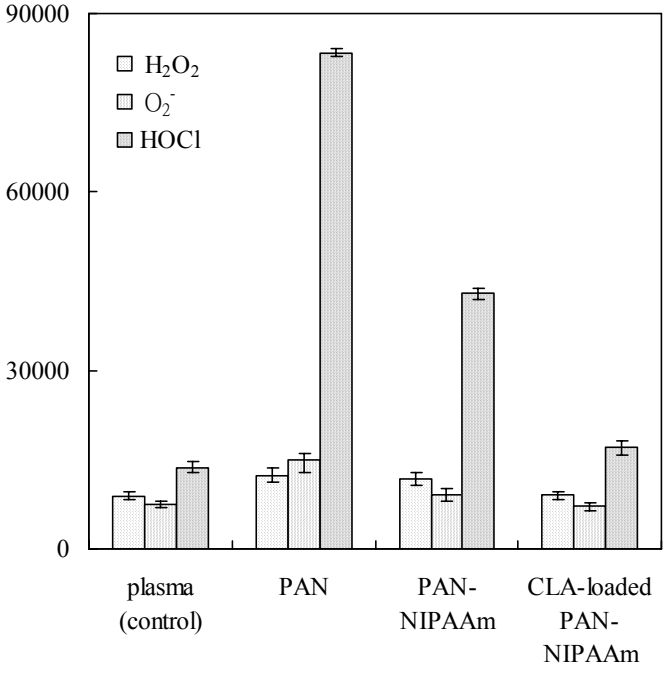


Figure 4. The variation of CL counts measurement for PAN and PAN-NIPAAm membranes. (n=3).

CONCLUSIONS

The surface of the PAN membrane can be hydrolyzed with $NaOH_{(aq)}$ to convert the surface into polyacrylic acid. The surface was then reacted with isopropylamine (IPA) and produced a layer of NIPAAm. Thus the PAN-NIPAAm membranes were responsive to both pH and temperature. The hemocompatibility of PAN membranes can be improved by loading CLA onto the NIPAAm surface. When such CLA-loaded PAN-NIPAAm membranes contact blood, the coagulation time can be prolonged greatly. This indicates that CLA loading can reduce blood coagulation. This hemocompatibility improving treatment via loading CLA can be applicable for hemodialysis. The results of this work suggest that a hemodialyzer made of CLA loading PAN-NIPAAm membrane can significantly reduce the need for injection of anticoagulant in clinical applications While the biocompatibility of PAN membranes has been significantly improved, the problem of ROS production was also reduced. Furthermore, the endotoxin in the blood can also be reduced by CLA-loaded PAN-NIPAAm. Thus CLA-loaded PAN-NIPAAm exhibits multiple advantages over a regular PAN membrane.

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評語

兩位同學在作作品介紹時非常的流暢，但是研究內容中許多數據的處理皆委託他人處理，實際親自執行的實驗並不多。