Reduction of Free Radicals and Endotoxin by Conjugated Linoleic Acid Loaded in an *In Situ*-Synthesized Poly(*N*-isopropyl acrylamide) Thin Layer

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ABSTRACT: 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. Then, conjugated linoleic acid (CLA) was loaded into the pNIPAAm layer. The effects of CLA on the blood coagulation and oxidative stress were evaluated with the use of human blood. The level of reactive oxygen species (ROS) was measured by the chemiluminescence (CL) method to evaluate the oxidative stress. Furthermore, the removal of bacterial endotoxin (lipopolysaccharide) by CLA-loaded PAN-NIPAAm was measured with enzyme-linked immunosorbent assay. The results show that the LCST swelling curve was at 37°C. PNIPAAm chains form expanded structures under 37°C. At temperatures greater than 37°C, however, the chains form compact structures by dehydration. This makes PNIPAAM membrane due to rapid hydration and dehydration changes of the polymer chain. 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 lipopolysaccharide for clinical applications. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 113: 3222–3227, 2009

Key words: *in situ* synthesis; poly(*N*-isopropyl acrylamide); conjugated linoleic acid; oxidative stress; endotoxin

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 have attract more and more interest 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 to temperature less than LCST, and they collapse when heated at temperatures greater than 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 endotoxins, 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 also can be removed by CLA. Endotoxin, also called lipopolysaccharide (LPS), is

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commonly found in our environment and is the most significant pyrogen in parenteral drugs and medical devices. Their presence in the bloodstream may cause septic reactions with a variety of symptoms, such as fever, hypotension, nausea, shivering, and shock.[10]

In this study, 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 this membrane was used for hemodialysis, it released CLA during the process. The results of this work should be helpful in developing a heparin-less hemodialyzing technique that can simultaneously reduce both oxidative stress and the presence of LPS.

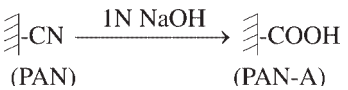
EXPERIMENTAL

Materials

Polyacrylonitrile powders were purchased from Sigma-Aldrich (St. Louis, MO). *N*,*N*-Dimethyl formamide (DMF) was purchased from Merck (Whitehouse Station, NJ). Lucigenin, luminal; 1,3-propanediol; 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC); and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. Isopropylamine (IPA) was purchased from Acros Organics (Geel, Belgium). 4-Morpholineethanesulfonic acid monohydrate (MES) buffer was purchased from Sigma-Aldrich. Reagents for activated partial thrombin time (APTT), prothrombin time (PT), fibrinogen time (FT), and thrombin time (TT) were purchased from Dade Behring (Now Siemens Healthcare Diagnostics, Deerfield, MA). Endotoxin and limulus amebocyte lysate (LAL) kit was purchased from Associates of Cape Cod (Cape Cod, MA).

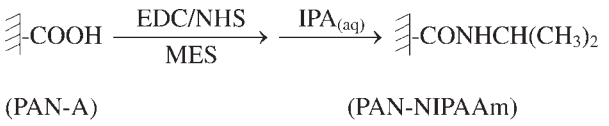
Preparation of polyacrylonitrile membranes

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 0.25 mg/mL IPA solution at 4°C for 24 h. The resulting samples were denoted as PAN-NIPAAm.



Characterization analysis

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

For the study of temperature-dependent equilibrium swelling ratios, the membranes were equilibrated in DI 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 equilibrated 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 retrieved and rinsed three times at 4°C with ethanol thoroughly and followed by DI water.

Determination of LPS level

The reduction of LPS level was measured by enzyme-linked immunosorbent assay. A piece of

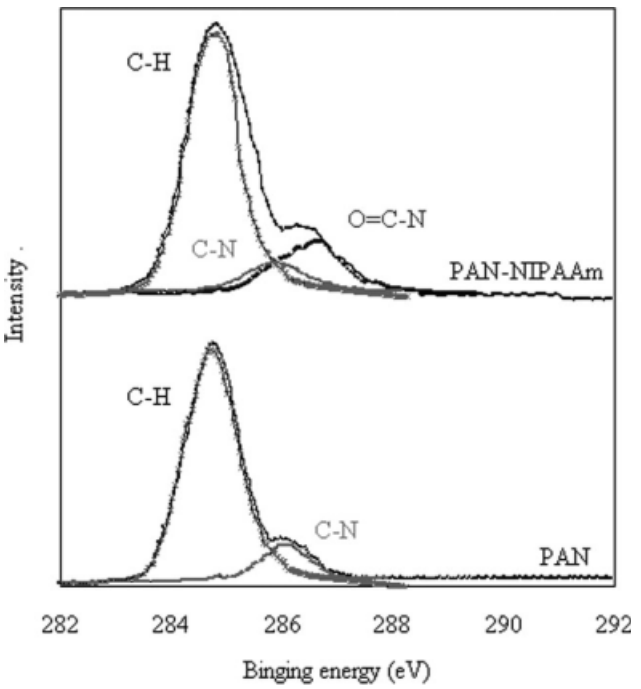


Figure 1 XPS spectra of unmodified PAN and modified PAN membranes.

membrane (1 × 1 cm^2) was placed in 500 μL of LPS (2 EU/mL) at 25°C for 30 min. Afterwards, 100 μL of residual LPS were added to each well in a 96-well TCPS plate at 37°C for 10 min. Then, 50 μL 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 enzyme-linked immunosorbent assay reader. The test was repeated three times for each sample.

Blood coagulation time

The *in vitro* coagulation times, including APTT, PT, TT, and FT were determined by the use of 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 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 reactive oxygen species (ROS) in the samples, a chemiluminescence (CL) method was adopted using lucigenin (1 mg/mL) as an amplifier for measuring superoxide (O_2^-), and luminal (1 mg/mL) as an amplifier for measuring hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). In brief, 200 μL 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). 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]

The hydrolytic reaction occurred at the interface of the solid and the solution[12]; thus, those nitrile groups contacting the solution can be converted into carboxylic groups. Table I shows that the surface density of carboxyl groups for PAN-PAA was 0.13 μmol/cm^2, which was equivalent to a thickness of 60 nm.[12] As shown in Figure 2(a), the swelling ratios of PAN-PAA and PAN-NIPAAm increased with the pH. When pH was greater 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 Figure 2(b), 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

TABLE I
Surface Characterization of Unmodified and Modified PAN Membranes (Mean ± SD) (n = 3)

Membrane	Surface density of carboxyl groups (μmol/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

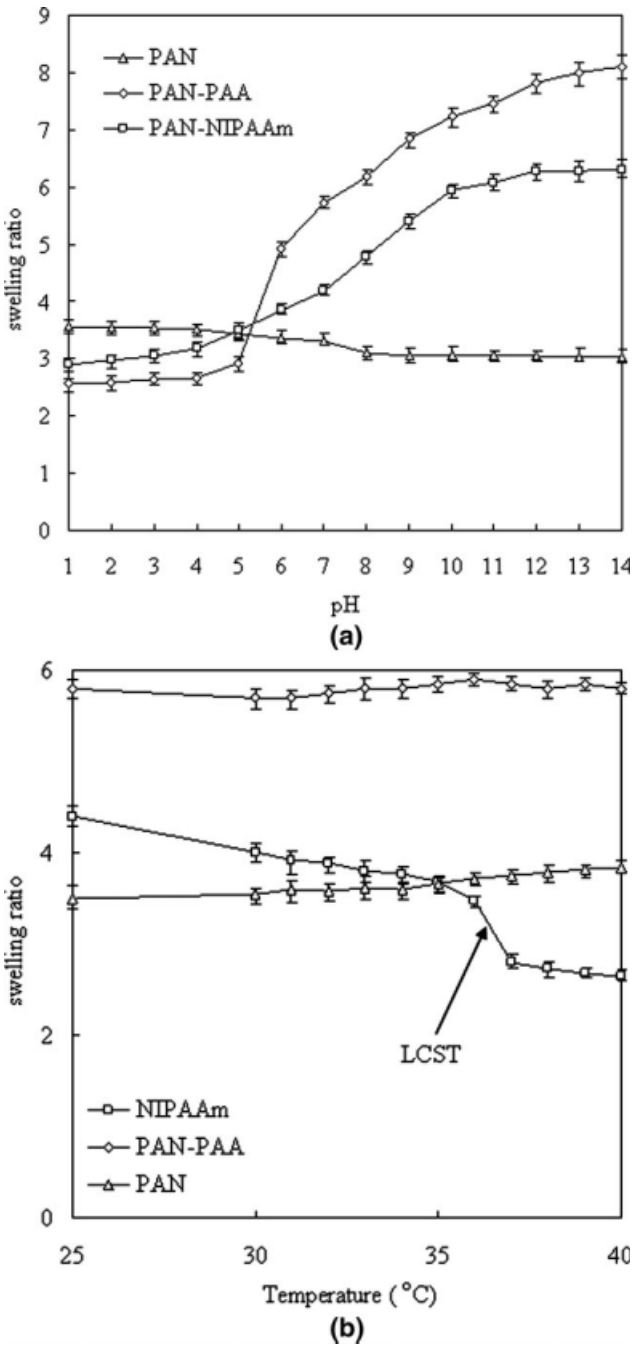


Figure 2 (a) pH-dependent swelling ratio of PAN-NIPAAm at room temperature ($n = 3$). (b) Temperature-dependent swelling ratio of PAN-NIPAAm in aqueous media of pH 7 ($n = 3$).

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 I show that LPS can be removed greatly by PAN-NIPAAm entrapped CLA. As a result, 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,[17] the kinin system, leukocytes, platelets, and endothelial cells. Banni et al. reported that CLA can decrease prostaglandin E2 (PGE2) production via the inhibition of LPS.[18] 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.[19] 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 3 shows that the coagulation times of PAN membrane are nearly the same as the control, and those of PAN-NIPAAm are slightly greater 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

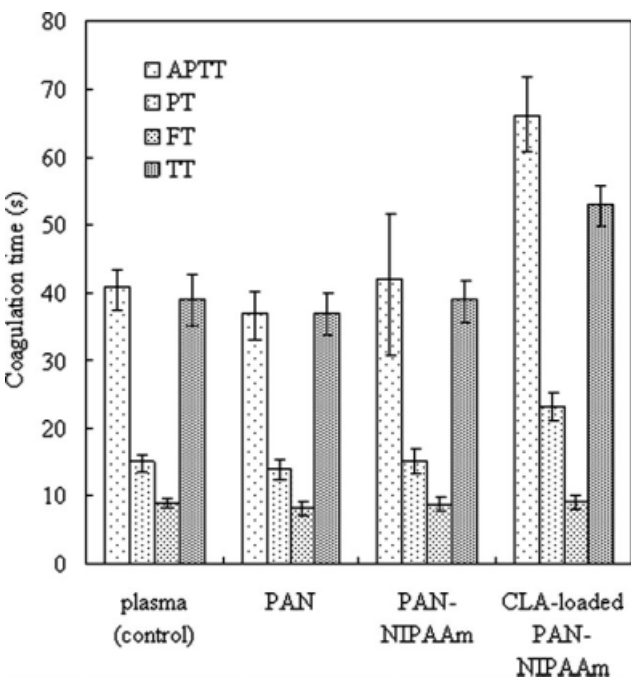


Figure 3 Comparison of coagulation times of PAN membranes ($n = 3$).

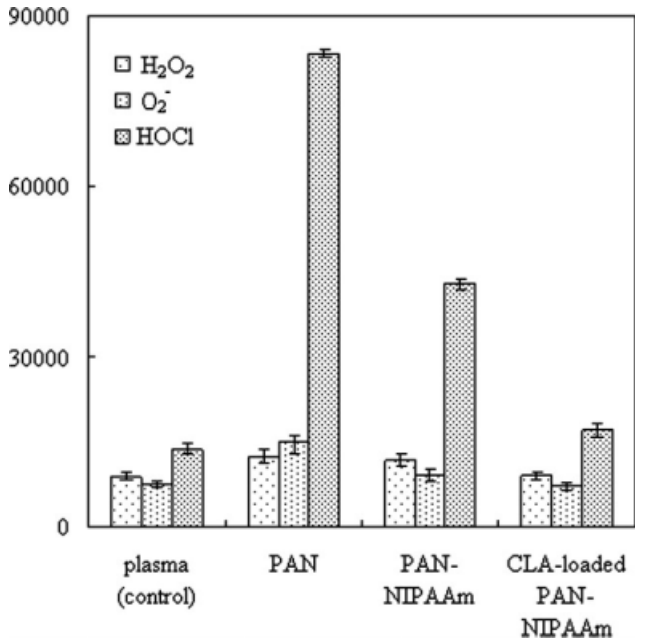


Figure 4 The variation of CL counts measurement for PAN and PAN-NIPAAm membranes ($n = 3$).

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.[20]

Figure 4 show that the ROS level for CLA-loaded PAN-NIPAAm is similar to that of the control, whereas the ROS level for the PAN and PAN-NIPAAm membranes is greater. 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. An 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) whereas 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.[21,22] 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 undergoing chronic hemodialysis therapy.

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 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 come in contact with blood, the coagulation time can be prolonged greatly. This finding 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 composed of CLA loading PAN-NIPAAm membrane can significantly reduce the need for injection of anticoagulant in clinical applications Although 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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