Polymer Bulletin 57, 261–267 (2006)
DOI 10.1007/s00289-006-0557-8

Polymer Bulletin

Synthesis and characterization of heparin immobilized PAN-based resin

Feng Li [1] (✉), Guangxu Cheng[1], Shipiao Liang[2]

[1] School of Material Science & Engineering, Xi'an Jiaotong University, Xi'an 710049, China
[2] Department of Material Science, Xi'an University of Science and Technology, Xi'an, 710054, China
E-mail: lifeng@mail.xjtu.edu.cn

Received: 23 November 2005 / Revised version: 15 February 2006 / Accepted: 22 March 2006
Published online: 7 April 2006 –

Summary

The role of low-density lipoprotein cholesterol (LDL-C) as a major risk factor for coronary heart disease (CHD) is well established [1-5]. Hemoperfusion treatment for familial hyperlipidemia (FH) and serious cardiovascular diseases is currently employed when the reduction of low-density lipoprotein (LDL) in patient's plasma concentration appears impossible to be achieved by diet or drugs administration [6.7]. Since late 1970s, many scientists have been engaged in developing different kinds of adsorbents which included non-specific, selective adsorbents and immunoadsorbents [8]. Up to date, Polyacrylate coated polyacrylamide (DALI) extracorporeal LDL apheresis [9.10], and heparin-induced extracorporeal LDL apheresis Precipitation [11] had been developed. Most of the LDL adsorbents were used in plasma for the removal of LDL-C and a primarycell/plasma separation by a centrifuge or a membrane separator was required [12].So far no corresponding observation was reported for the heparin immobilized PAN-based resin. In this paper, the resin was synthesized and the properties or factors affecting its adsorption were investigated.

Keywords:

PAN-based resin, heparin, selective adsorption, LDL

Experimental

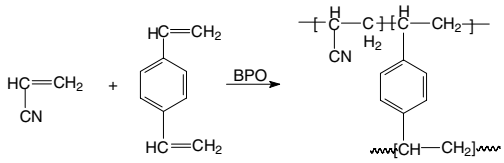
Materials

Divinylbenzene (56%, g/mL), gelatine, benzoyl peroxide (BPO), toluene, furanidine, acrylonitrile (A.R), glutaraldehyde (A.R), hexadecyltrimethylammonium bromide (CTMAB, A.R) glutaraldehyde (GA, A.R) and low molecular weight heparin(LMWH) were purchased from Shanghai Chemical Reagent Co (Shanghai, PRC). Plasma was drawn from HALP patients aged between 45 and 65 years old in The First Hospital of Xi'an Jiaotong University, who were not taking drugs for lipid-lowering or metabolism altering for at least 2 months. In order to prevent blood coagulation, EDTA disodium salt was added to the plasma.

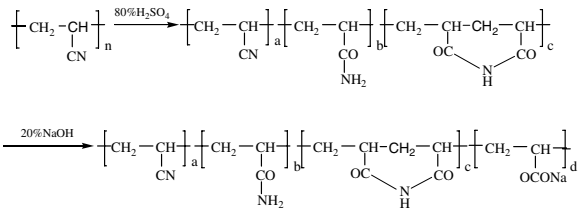
Synthesis of PAN-based resin

Scheme 1-2 shows the route of the preparation of macroporous cross-linked PAN-based resin bearing cyano, amido and carboxyl as functional group. The resin was synthesized by suspension polymerization using BPO as the initiator, divinylbenzene as the cross-linker, and toluene as the porogen.

Scheme 1



Scheme 2

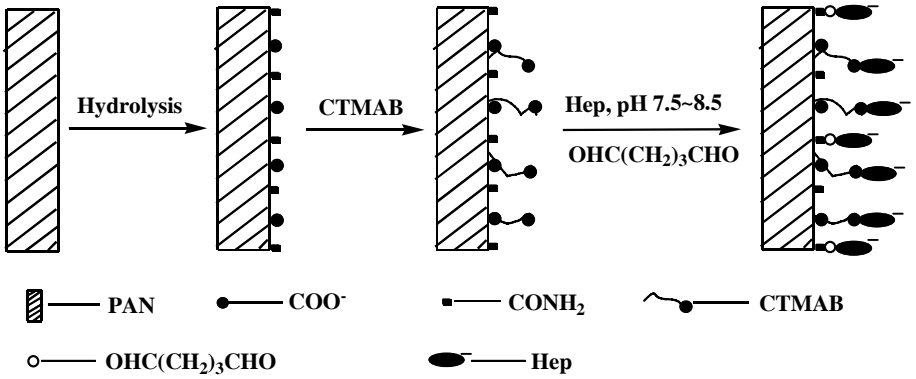


The preparation was carried out in a 500 mL four-neck, round-bottom flask kept at 60°C. First, a mixture of 40.0 g NaCl and 1.0 g gelatine in 200 mL deionised water (DDW) was prepared. Then, the redistilled acrylonitrile 50 g, divinylbenzene 13.9 g (56% v/v) and toluene 60 mL were added to the mixture with moderate stirring. Nitrogen was introduced into the reaction vessel and the solution stirred under nitrogen atmosphere. After 4 h reaction of the mixture at 60°C, white-colored microsphere resin appeared, and the temperature was raised to 80°C to react for 4 h. With a color change of the microsphere resin from white to yellow, the temperature of the reaction was finally raised to 90°C and kept for 1 h before the polymerization was terminated. The microsphere resin was then filtered and washed with hot DDW (60°C), dried at room temperature, and subsequently extracted in furanidine for 8 h to eliminate the unreacted acrylonitrile monomer and the excessive toluene. The macroporous cross-linked PAN resin 20g was then added into 50 mL 80% of H_2SO_4 solution at 90°C for 5 h, subsequently, the resin was filtered and washed with hot DDW (60°C). The resin 20g was base-hydrolyzed in 50mL 20% of NaOH solution at 90°C for a specific time. The extent of the base hydrolysis was varied with the hydrolysis time from 1 h to 5 h [13].

Synthesis of heparin immobilized PAN-based resin

Typically, heparin immobilization onto the surface of the crosslinking, functionalized resin was performed using methodologies already described[14-16] in the literature. Scheme 3 shows the synthesis of heparin immobilized PAN-based resin.

Scheme 3



Firstly, 10g of the PAN resin derived from the above procedures was mixed with 20 mL 1.0% CTMAB solution that premixed with definite ratio of thetrahydrofuran and alcohol (3/2,v/v). Then the reaction mixture was heated to 55°C and sustained at this temperature for 10 h while stirring. The resultant beads were filtrated and washed with DDW, the resin beads were dried at 45°C under reduced pressure. Subsequently, the resin beads were added to 20 mL 1.0% GA/0.05M boric acid buffer solution (pH 8.5) at 35°C for 2 h with stirring. The product was separated by filtration and then washed with a 0.1M phosphate buffer solution (pH 7.5). The product was mixed with 20 ml of a 0.1M phosphate buffer solution (pH 7.5) containing1.5% LMWH at 35°C for 10 h while stirring. Finally, the resin beads were washed with DDW for 48 h and dried at 25°C in vacuum.

Measurement of SEM

The surface of the prepared resins was observed with an S-570 SEM (Hitachi, Japan).

Measurement of ATR-FTIR

The IR spectra of the prepared resins were measured on 300E FTIR spectrometer (Jasco).

Determination of adsorption capacity

Adsorption percentage and adsorption capacity were calculated according to the following equations:

$$AP = \frac{[C]_B - [C]_A}{[C]_B} 100\% \qquad (1)$$
$$AC = ([C]_B - [C]_A)V_p$$

Where AP and AC stand for adsorption percentage and adsorption capacity respectively, $[C]_B$ is the concentration before adsorption, $[C]_A$ is the concentration after adsorption, V_P is the volume of plasma used during adsorption. Exactly 0.5 ml of

heparin immobilized PAN-based resin was incubated with 1.5, 2.0 ml hyperlipidemia plasma, respectively, and stirred for 3 h at 37°C. Total cholesterol (TC) LDL-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C) and plasma total proteins were determined by using the corresponding commercial test kits purchased from Zhongsheng High Tech Bioengineering Company Beijing, PRC.

Results and Discussion

FTIR Analysis

The FTIR spectra of the resin before and after different hydrolysis treatments are shown in Fig 1 a-c. Without any hydrolysis treatments, a stretching band for C-H at 2925 cm^{-1}, and a characteristic band for cyano group at 2243 cm^{-1} are presented. Bands at 1670 cm^{-1} and 1607 cm^{-1} are the characteristic bands for amido groups resulting from a small quantity of cyano groups hydrolyzed. The band at 1453cm^{-1} is the characteristic band for $–CH_2–$ and the band at 711cm^{-1} is due to the bending of C-C in benzene. The FT-IR spectra of the resin after acid hydrolysis showed that there is an increase in the characteristic band of amido groups at 1671 cm^{-1} and 1607 cm^{-1}, though there is a slight decrease in the characteristic band of cyano groups at 2243 cm^{-1}, indicating clearly the formation of amido groups due to the hydrolysis of cyanogens groups.

After the subsequent base hydrolysis, FTIR spectra of the sample in Fig 1c indicated that the characteristic band of the cyano groups at 2243 cm^{-1} decreased further. It is because cyano groups were converted into sodium carboxylic groups when the hydrolysis of the resin was catalyzed in 20% NaOH solution. The intensity of the characteristic band of amido groups around 1670 cm^{-1} kept constant or decreased, indicating there is a partial conversion of amido groups into $–COO^-$ groups in the base-hydrolysis.

Based on the FTIR spectra analysis, it is concluded that three kinds of functional groups, cyano, amido and carboxyl groups, are presented at the surface of the

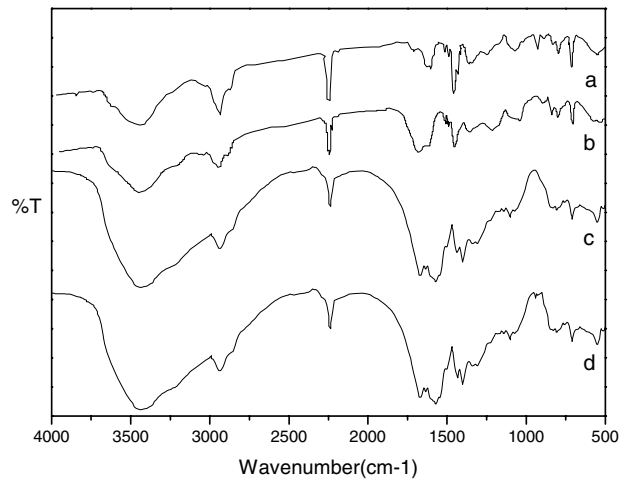


Fig 1. FTIR of the resins: (a) Initial sample; (b) after acid hydrolysis; (c) after base hydrolysis; (d) after heparin immobilized

particles. Resins of such nature have good mechanical properties derived from polyacrylonitrile framework [17]. Its excellent bio-compatibility and anionic adsorption capability result from amido and carboxyl groups, respectively [18].
Fig 1d show the FTIR spectra of heparin immobilized PAN-based resin. The strengthen of band at 1430^{-1} may be contributed by the group of $N(CH_3)_4^+$ in the CTMAB. The characteristic band of the heparin at 940 cm^{-1} indicated the heparin is immobilized to the surface of PAN-based resin [19].

Optical Microscope Analysis

During the polymerization, when the porogen dose M (the volume ratio of porogen to monomer and cross-linker) was less than 3/4, the resin granule appeared to be spherical with smooth surface under optical microscope, and the diameter of the spheres was about 1mm (Figure 2, a). When M increased to 3/4, a few resin granules became elliptical (Figure 2, b) and cracks emerged after dried (Figure 2, c).

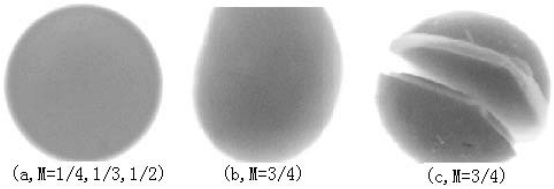


Fig 2. Correlation between figure of resin granule and porogen dose

As resin with spherical granules has the best adsorption capability and mechanical properties [20], the maximum volume of porogen should not exceed 3/4 times of the volume of the monomer and the cross-linker.
During the base hydrolysis, the sodium carboxylic groups are increased with the hydrolysis time, which were determined by acid-base titration. When in excess of 3 hours, the cracks also could be found after dried. As LDL was known to bind negatively charged substances, the sodium carboxylic groups were favoured for selective adsorption for LDL in human whole blood, therefore the base hydrolysis time was determined to 3 hours, and the amount of the sodium carboxylic groups is 1.55mmol/g.

SEM Analysis

The SEM micrographs of the resins shown in Figure 3 and data (obtained by calculating the statistical average value of one hundred pores) in Table 1, which indicated that as the porogen dose increases, the average size of the pore increases and the structure becomes more homogeneous.

Table 1. Correlation between porogen dose and average pore size (D=6%)

Sample (M)	1/4	1/3	1/2	3/4
Average pore size (nm)	112	175	213	280

(D = Concentration of the cross-linker. M = the ratio of volume of the porogen to that of monomer and cross-linker.)

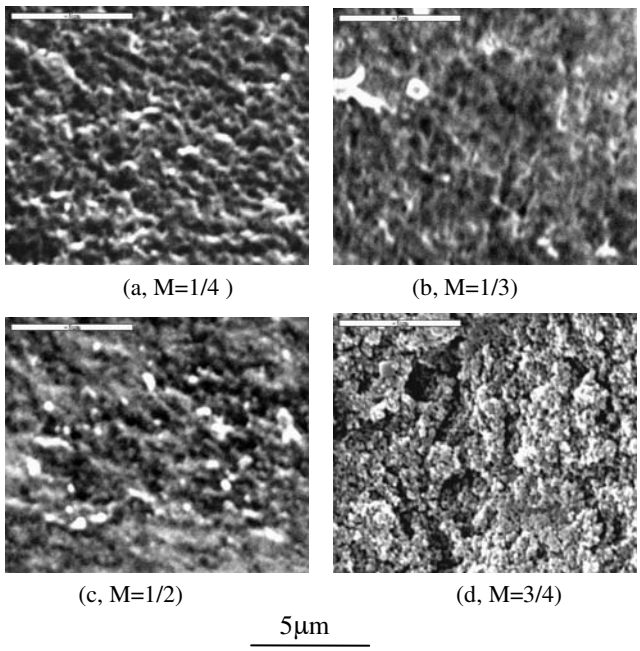


Fig 3. SEM of resins (D=6%)
(M is the ratio of volume of the porogen to that of monomer and cross-linker)

Effect of the volume ratio of adsorbent to plasma on adsorption capacity

According to literature [21-23], the volume ratio of adsorbent to plasma was 1:3 for the adsorbent to reach the highest adsorption capacity. In this paper, 0.5 ml heparin immobilized PAN-based resin was mixed with 1.5 and 2.0 ml HALP plasma, respectively and stirred for 3 h at 37°C. TC, TG, LDL-C and HDL-C levels were determined and the results were shown in Table 2-3. It is evident that optimal volume ratio of heparin immobilized PAN-based resin to plasma is 1:3. In contrast, the adsorption percentage was reduced with the increment of the volume of plasma used.

The mechanism of the action is assumed to be the electrostatic interaction between the negatively charged groups at the surface of the resin beads and the positively charged apolipoprotein B moiety of the LDL particles.

Table 2. The volume ratio of PAN-based resin to plasma vs. the adsorption results

Adsorbent:plasma V:V	LDL		TC		TG		HDL	
	AC (mg/ml)	AP (%)	AC (mg/ml)	AP (%)	AC (mg/ml)	AP (%)	AC (mg/ml)	AP (%)
1:3	0.633	36	0.145	8	0.127	9	0.004	5
1:4	0.684	21	0.210	7	0.157	8	0.005	4

Note: the results were gained from single-test runs. AC and AP stand for Adsorption capacity and Adsorption percentage

Table 3. The volume ratio of heparin immobilized PAN-based resin to plasma vs. the adsorption results

Adsorbent:plasma V:V	LDL		TC		TG		HDL	
	AC (mg/ml)	AP (%)	AC (mg/ml)	AP (%)	AC (mg/ml)	AP (%)	AC (mg/ml)	AP (%)
1:3	2.137	91	0.658	27	0.324	23	0.031	11.9
1:4	2.312	76	0.661	22	0.392	20	0.034	9.8

Note: the results were gained from single-test runs. AC and AP stand for Adsorption capacity and Adsorption percentage

Conclusions

The heparin immobilized PAN-based resin has many advantages that make it an ideal adsorbent for the removal of LDL in plasma with high efficacy and good selectivity. Meanwhile the concentrations of TC, TG in plasma also have obvious reduction.

Acknowledgements. The financial support of the National Natural Science Fundation of China (Grant 19972052).

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