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Preparation of heparin-immobilized PVA and its adsorption for low-density lipoprotein from hyperlipemia plasma

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Abstract In this study, heparin was covalently coupled by glutaraldehyde to Poly(vinyl alcohol) [PVA] in solid-liquid two-phase reaction system by two-step synthesis method to prepare a LDL-selective adsorbent. The parameters (the material ratio, reaction time and dosage of catalyzer) were investigated to evaluate their effect upon the immobilized amount of heparin onto the surface of PVA, IR was used to verify the covalent immobilization result and the heparin-modified PVA was also undergone the evaluation of its adsorption capability for low-density lipoprotein from hyperlipemia plasma, and its hemocompatibility was preliminarily evaluated by platelet adhesion test. Results showed: (1) under optimized reaction conditions the highest immobilization amount of heparin onto PVA surface within the experiments of this study has been obtained; (2) the optimized reaction conditions were: (i) at the refluxing temperature 78°C; (ii) the material ratio of "PVA(g): 50% glutaraldehyde (ml)" was about "1:3"; (iii) the reaction time was about 5 h; and (iv) the amount of catalyzer (concentrated HCL) was about 1% of the 50% glutaraldehyde; (3) within the experiments of this study the highest immobilization amount would be up to 25 μg heparin on the surface of per g PVA granules; (4) the heparin-modified PVA granules showed significant adsorption for LDL under faintly alkaline environment (pH = 7.2–9.5) ; (5) The result of platelet adhesion test showed no platelet adhered to its surface. Therefore, immobilization of heparin onto the surface of a support is one approach to prepare a kind of LDL adsorbent for blood purification.

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1 Introduction

Epidemiologic studies found cardiovascular-cerebrovascular diseases are still the main disease threatening human lives because of its high lethality, and also confirmed the nosogenesis of cardiovascular-cerebrovascular diseases is mainly caused by artherosclerosis [1]. Clinically, the abnormal elevated level of low-density lipoprotein (LDL) in human blood has been confirmed to be a main independent risk factor for the process of artherosclerosis [2, 3], and studies also found the incidence of cardiovascular-cerebrovascular diseases would soar up if the LDL level increases over the normal one [4]. Fortunately, studies also found reducing LDL level in blood would result in the reduction of the incidence [5–7].

At present, the reduction of elevated LDL level has been clinically implemented by drug (e.g. statins [8–13]), alimentary control and LDL-apheresis [14]. However, the effect to reduce LDL by drug was still unsatisfactory because of its disadvantages such as drug fast, inefficacy or side effect [15, 16]. Meanwhile, the effectiveness of

alimentary control was also very limited, which only acts as an adjunctive therapeutic measure. Unfortunately, drug and alimentary control would take no effect if the patient's LDL level was over 300 mg/dl [17] (especially for homozygous and severe heterozygous familial hypercholesterolemia patients).

LDL-apheresis therapy [14, 17–19] is an unconventional therapy, which uses apheresis system to directly remove LDL from plasma or blood by LDL-adsorbent. It is characterized of effectiveness, safety and instant to the clearance of LDL from hyperlipemia (note: hyperlipemia is a group of diseases whose feature is the abnormal elevation of cholesterol and/or triglyceride level in human plasma.) patient's plasma, and clinically accepted by doctors and patients. With respect to LDL-apheresis therapy, the LDL adsorbent is the critical. Nevertheless, something unsatisfactory still exists within the current LDL adsorbent, such as unbearable side effect in use, or poor adsorption selectivity for LDL, or high cost [20–26]. It is, therefore, still indeed valuable to further the therapy and develop new LDL adsorbent that lead to reduce plasma cholesterol in humans.

On the basis of the facts mentioned above, in this study we tried to develop a new LDL adsorbent by covalently immobilizing heparin onto the surface of PVA granule, and the immobilization technology and the adsorption of heparin-modified PVA for LDL from hyperlipemia plasma were also investigated.

2 Materials and methods

2.1 Covalent immobilization of heparin onto PVA surface

Heparin was covalently immobilized onto the surface of PVA-1799 granule (degree of polymerization $= 1700 \pm 50$, degree of alcoholysis $\geq 99\%$, $M \approx 74{,}800$ g/mol) with the aid of glutaraldehyde couplant. The preparation process was briefly described as follow:

At first, 15 g of PVA-1799 granules were added into a 500-ml three-necked flask, and then 45 ml of 50% glutaraldehyde solution followed; Second, 80 ml of cyclohexane was added as solvent and refluxing agent, and then 50 μl of concentrated HCl was followed as catalyzer; Finally the reaction system was allowed to react for 5 h at the refluxing temperature (78°C)with agitation.

When the reaction time was up, the pre-treated PVA granules were filtered out, and then washes with water was followed to elute redundant couplant; Second, the eluted pre-treated PVA granules were again added into a flask, and then 10 ml of water, 5 mg heparin and 2 drop of glacial acetic acid as catalyzer were followed. Finally the reaction system was allowed to reacted overnight at 37–40°C.

When the reaction time was over, the treated PVA granules were filtered out, following washes with water to elute redundant heparin and then dried at 37–60°C, thus the expected adsorbent was made.

2.2 Analysis of the heparin-modified PVA

2.2.1 IR analysis of the heparin-immobilized PVA

Heparin, PVA-1799 and the heparin-modified PVA-1799 were analyzed with conventional IR (Spectrum GX, Perkin Elmer, USA) to obtain their IR information about functional group respectively. This IR analysis was aimed to verify whether heparin was covalently immobilized onto the surface of PVA-1799.

2.3 Assay of the immobilized amount of heparin on the modified PVA

The immobilized amount of heparin was assayed by the toluidine blue colorimetric method according to the literature [27]. The assay principle was based on the fact that the color of metachromatic dye (e.g. toluidine blue) will irreversibly change and its absorption band will shift towards lower wavelength (≈ 630 nm) when it binds to polyanion substrate (e.g. heparin), thus the amount of immobilized heparin can be directly assayed by toluidine blue colorimetric method [27, 28].

2.4 The effect of reaction parameters upon the immobilized amount of heparin

The procedure and reaction parameters were similar to those described in Sect. 2.1. Briefly, the ratio of PVA-1799 granules to 50% glutaraldehyde solution has been varied first with other reaction parameter kept invariable, then followed by variation of the reaction time with the best ratio and finally the catalyzer dosage.

2.5 The adsorption of the heparin-modified PVA for LDL from hyperlipemia patient's plasma

The static adsorption efficiency of the heparin-modified PVA for LDL from hyperlipemia plasma was assayed with LDL-Kits (purchased from Diasys, Germany) by automatic biochemistry analyzer (Dimension RXL, USA).

The procedure was designed as follow. Briefly, first, 1 ml of fresh plasma obtained from hyperlipemia patients mixed with 1 ml of diluent, of which the diluent was buffer solution with different pH or saline, to prepare the diluted plasma sample respectively. Second, the initial LDL-value

and HDL-value of the diluted plasma sample was assayed by Dimension RXL with LDL-Kit and HDL-Kit respectively. (Note: HDL = high-density lipoprotein). Third, 0.5 g of the heparin-modified PVA granules was statically immersed in the 2-ml diluted plasma sample at 37°C for 30 min respectively. Fourth, the end LDL-values and HDL-values of the treated sample were assayed respectively when the static contact time was up. Finally, the adsorption efficiency of the heparin-modified PVA for LDL under different conditions was calculated out respectively, and then the optimal diluent condition within the experiment of this study would be obtained. The unmodified PVA was taken as the control.

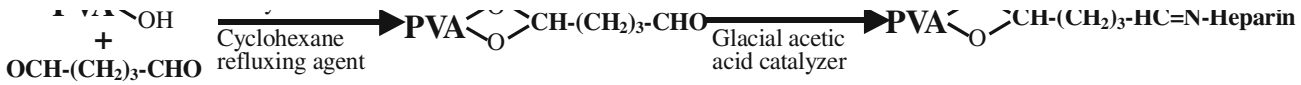
2.6 Platelet adhesion test

This test was aimed to preliminarily evaluate the hemocompatibility of the adsorbent prepared in this study. The evaluation was carried out according to the Chinese National Biological Evaluation Standard for Medical Devices (GB/T 16886.4-2003). Briefly, fresh platelet-rich plasma was first prepared and then allowed to contact the adsorbent at 37°C for 1 h, then the adsorbent was taken out and washed with PBS, following the fixation with 2.5% glutaraldehyde solution. Finally, the adsorbent was prepared as SEM test sample and then examined with scanning electron microscope (KYKY-1000B, China).

3 Results

3.1 Results of IR analysis

The reaction principle in this study was as follow:



According to this reaction formula, if heparin has been covalently immobilized there must form a new chemical bond (viz.–C=N) and the IR characteristic absorption peak of heparin must appear in the heparin-modified PVA substrate. It is well known that IR analysis technology can be used to obtain the information about molecule structure and functional group type, therefore, it can be used to detect whether the chemical bond (–C=N) has newly formed in the modified PVA.

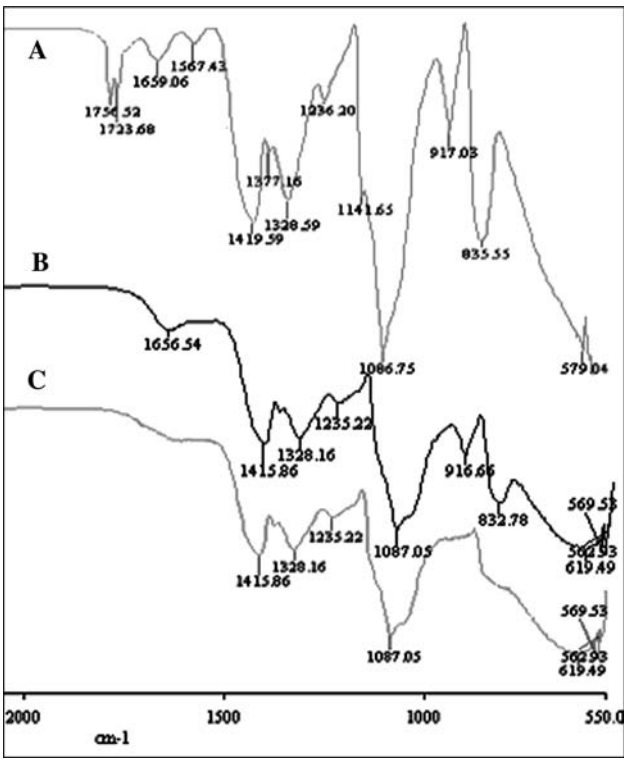


Fig. 1 IR analysis results, scanned by Spectrum GX, PerkinElmer, USA. (**A**): heparin-modified PVA-1799; (**B**) heparin; (**C**) PVA-1799

The IR analysis results of raw material PVA-1799, raw material heparin and the heparin-modified PVA-1799 were shown in Fig. 1 respectively. As for the IR spectra of raw material PVA-1799 (see Fig. 1C), there was no adsorption peak at 830–920 cm^{-1} and 1560–1760 cm^{-1}; As for the IR spectra of heparin (see Fig. 1B), that one adsorption peak at 832 cm^{-1} and the other at 916 cm^{-1} appeared at the same time is the IR characteristic absorption peak [29] of heparin. According to Fig. 1A, the IR characteristic absorption peak of heparin at 832 cm^{-1} and 916 cm^{-1} appeared at the same time in the heparin-modified PVA substrate but not in the raw material PVA-1799, which suggested that heparin has been immobilized; further, a newly-formed chemical bond at 1659 cm^{-1} in heparin-modified PVA substrate but not in raw material heparin and PVA-1799 can be assigned to the stretching vibration peak of –C=N bond on the basis of the reaction formula; from

the two points, it could be concluded that heparin has been covalently immobilized onto the surface of PVA-1799 successfully.

3.2 The effect of materials ratio of PVA to 50% glutaraldehyde on the immobilization amount of heparin

The procedure and reaction parameters were similar to those described in Sect. 2.1. Briefly, the ratio of PVA-1799 granules to 50% glutaraldehyde solution was varied, and other reaction parameters were kept invariable, such as PVA-1799 granules ($D \approx 1.5$ mm, 15 g), reaction time (=5 h), concentrated HCL catalyzer (=50 μl), refluxing agent cyclohexane (=80 ml), refluxing reaction temperature (=78°C) and heparin (=5 mg). Three duplicate tests were done to obtain their mean value.

The effect result was shown in Fig. 2. According to the result, when the best material ratio of PVA-1799 to 50% glutaraldehyde was about 1:3, the highest immobilization amount within the experiments of this study has been obtained.

3.3 The effect of reaction time on the immobilization amount of heparin

The procedure and reaction parameters were similar to those described in Sect. 2.1. Briefly, the reaction time was varied, and other reaction parameters were kept invariable, such as the best ratio of PVA-1799 granules to 50% glutaraldehyde solution (=1:3), PVA-1799 granules ($D \approx 1.5$ mm, 15 g), concentrated HCl catalyzer (=50 μl), refluxing agent cyclohexane (=80 ml), refluxing reaction temperature (=78°C) and heparin (=5 mg). Three duplicate tests were done to obtain their mean value.

The result was given in Fig. 3. According to the result, when the reaction time was about 5 h under the best material ratio of PVA-1799 to 50% glutaraldehyde, the highest immobilization amount within the experiments of this study has been obtained.

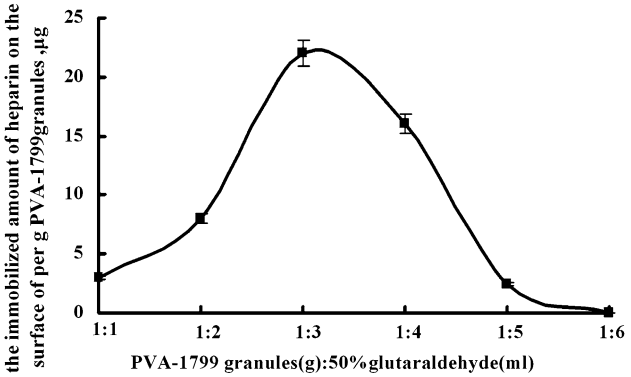


Fig. 2 Effect of the material ratio of PVA to 50% glutaraldehyde on the immobilization amount of heparin

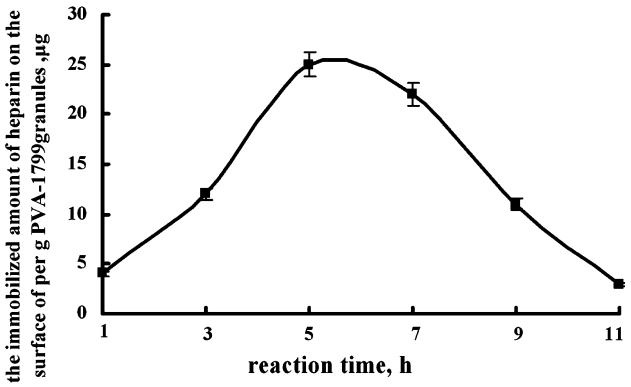


Fig. 3 Effect of reaction time on the immobilization amount of heparin

3.4 The effect of catalyzer dosage on the immobilization amount of heparin

The procedure and reaction parameters were similar to those described in Sect. 2.1. Briefly, the catalyzer concentrated HCl was varied, and other reaction parameters were kept invariable, such as the best ratio of PVA-1799 granules to 50% glutaraldehyde solution (=1:3), PVA-1799 granules ($D \approx 1.5$ mm, 15 g), the optimal reaction time (5 h), refluxing agent cyclohexane (=80 ml), refluxing reaction temperature (=78°C) and heparin (=5 mg). Three duplicate tests were done to obtain their mean value.

The result showed the highest immobilization amount within the experiments of this study has been obtained when the catalyzer dosage was about 50 μl (equal to 1% of 50% glutaraldehyde consumed) under the best material ratio of PVA-1799 to 50% glutaraldehyde and optimal reaction time. In facts, the result also suggested the catalyzer dosage between 20 and 70 μl contributed no remarkable influence on the immobilization amount within the experiments of this study.

3.5 Adsorption efficiency of heparin-modified PVA-1799 for LDL from hyperlipemia plasma

Figure 4 showed the effect of different diluent on the adsorption of heparin-modified PVA-1799 granules for LDL from hyperlipemia plasma.

According to the result, the heparin-modified PVA-1799 granules showed great adsorption for LDL when the hyperlipemia plasma was diluted with faintly alkaline buffer (pH = 7.2–9.5), and under this condition, its adsorption efficiency for LDL was about 60%. Obviously,

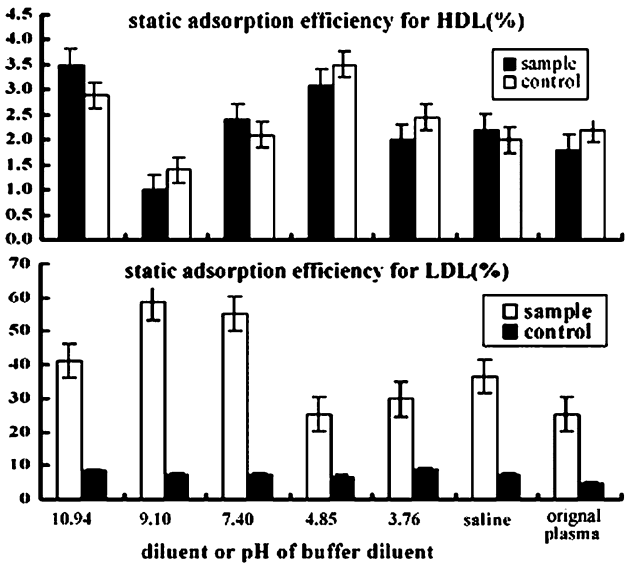


Fig. 4 Static effect of the diluent or the PH of buffer diluent upon the adsorption efficiency for LDL. Sample: heparin-modified PVA; control: unmodified PVA

the adsorption of the heparin-modified PVA for LDL was significantly greater than that of the unmodified. Meanwhile, the modified and unmodified PVA also showed adsorption for HDL, but their adsorption efficiency for HDL was poor (<5%) so that it could be clinically negligible and acceptable.

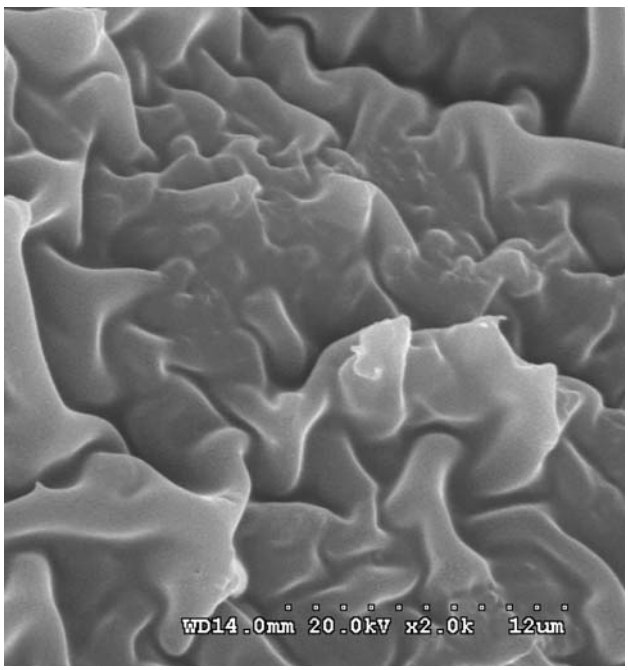


Fig. 5 SEM picture of platelet adhesion test for the adsorbent, scanned with KYKY-1000B

3.6 The result of platelet adhesion test

The result was shown in Fig. 5. It could be seen there was no platelet adhering to the surface of the adsorbent, which suggested the possibility of its good hemocompatibility according to GB/T 16886.4-2003.

4 Discussions

Nowadays, cardiovascular-cerebrovascular disease is still the main disease threatening the human lives; atherosclerosis, a kind of cardiovascular-cerebrovascular disease, has been the main cause of death for the global population [1]. Clinic practices have confirmed the fact that the abnormal elevated LDL in blood is a main independent factor for the process of artherosclerosis [2, 3], and to lower the LDL level would result in the reduction of incidence of the disease [5–7].

Currently, there are three main therapies to lower the elevated LDL level, such as medicine [8–13], alimentary control and LDL-apheresis [14]. With regard to the patient whose LDL level is mildly elevated, medicine therapy in combination with alimentary control has obtained satisfactory clinic effectiveness; however, they would take no effect if the patient's LDL level was elevated over 300 mg/dl [17] (especially for homozygous and severe heterozygous familial hypercholesterolemia patients). As for familial hypercholesterolemia patients, the LDL-apheresis [18, 19] can produce excellent effectiveness, because this therapy can instantly clear LDL from hyperlipemia plasma with the aid of LDL-adsorbent, and this therapy has clinically been accepted by patients and doctors. There are several kind of LDL-apheresis adsorbents used, such as dextran sulfate cellulose [30, 31], heparin buffer solution [32], LDL immunoadsorbent [33, 34], LDL anionic adsorbent [35], and LDL nonionic adsorbent [36]. Among those adsorbents, the general performance of heparin buffer solution has been clinically considered excellent. Although those LDL-apheresis adsorbents have had clinical recognition, they also have shortcomings [18] such as uneasiness to prepare, or high cost, or unsatisfactory efficiency/hemocompatibility, or else, therefore, it is indeed valuable to further the therapy and develop new LDL purification materials that lead to reduce plasma cholesterol in human blood, namely improvements will be also required for them.

Although heparin-mediating LDL precipitation therapy has been clinically considered excellent, there also exist unsatisfactory aspects, such as high cost for patient, sophisticated operation for doctor, and hemorrhagic tendency for the subject. In order to improve this therapy, on the basis of this technology we put forward a different

strategy, namely immobilizing heparin onto a supporter to prepare a new LDL adsorbent.

Study has found the structural domain B100 of LDL molecule has basic amino acids (Lysine and Arginine) with positive charge [37, 38]. Heparin is a polyanion polysaccharide (see Fig. 6), of which there are many functional groups with negative charge, such as ($-COO^-$ and $-OSO_3^-$). Theoretically, LDL could specifically bond with heparin by electrostatic interaction, though heparin is able to displace LDL bound to apoB(LDL) receptors [39]. Based on this, the heparin-mediating LDL precipitation therapy [32] has been successfully developed and clinically used, which suggested the usability of the theory for LDL-apheresis. According to this hypothesis, if heparin were to be modified onto a supporter, the heparin-modifying supporter should acquire the ability to selectively adsorb LDL. Therefore, we took PVA-1799 granules as supporter and immobilized heparin onto its surface to prepare a new adsorbent for LDL.

According to Fig. 2, if the amount of 50% glutaraldehyde continually increased, the amount of immobilized heparin would drop down instead. The reason maybe that the more the 50% glutaraldehyde added the more the free water in the reaction system was, when the amount of water in the reaction system exceeded the critical amount, the PVA granules will dissolve in excess water at high temperature (if not excess, the PVA granules only swells), which would lead to the shape destruction of PVA granules, consequently, the solid surface area of PVA granules for heparin immobilization would reduce, which resulted in the dropdown of the immobilized amount.

From Fig. 3, if the reaction time was prolonged to long, the immobilized amount of heparin would not keep steady but decrease instead, this maybe that the other free functional group (aldehyde group) of the couplant glutaraldehyde took part in the reaction or dismutation reaction happened between free aldehyde groups in the late stage, which directly led to the reduction of amount of free aldehyde group that would be made use of to covalently bond to heparin in the second reaction step, consequently, the immobilized amount of heparin would decrease due to the reduction of amount of free aldehyde groups.

With respect to the effect of catalyzer dosage on the immobilization amount of heparin, the result showed the highest immobilization amount could be obtained when the catalyzer dosage was not more than 2% of the 50% couplant glutaraldehyde. If continually increasing the dosage of catalyzer, the immobilized amount of heparin would drop down instead, this maybe that the more the dosage of catalyzer added, the higher the degree of acidity of the reaction system was. Under highly acid environment, the dismutation reaction of aldehyde groups are prone to take place, which will result in the reduction of free aldehyde group that would be made use of to covalently bond to heparin in the second reaction step, consequently, the immobilized amount of heparin would drop down due to the reduction of free aldehyde groups.

According to Fig. 4, it could be seen the heparin-modified PVA had higher adsorption for LDL than that of its control, which could be attributed to the surface of PVA-1799 modified with heparin. We also found that the heparin-modified PVA had an optimal adsorption for LDL was when the plasma was diluted with the pH = 9.10 buffer diluent, and the adsorption efficiency for LDL under this condition could be at 60%, this maybe the fact that the electronegativity of heparin molecule was enhanced under alkaline environment so that the electrostatic interaction between heparin and LDL was enhanced, thus results in higher adsorption efficiency. In fact, its adsorption for LDL under faintly alkaline environment was better than under other PH environment. And we also found its adsorption for LDL under dynamic condition was similar to that under static condition. From Fig. 4, the modified and unmodified PVA has little adsorption effect upon HDL and the side effect could be clinically acceptable. Additionally, the platelet adhesion test preliminarily illustrated its good hemocompatibility, this is indeed far from proving its good hemocompatibility but it is a first very good hint, and other evaluations are ongoing, and we will report them late when completed.

From the results, the adsorption of the adsorbent developed in this study for LDL was lower than that of the heparin-mediated LDL precipitation technology whose adsorption efficiency is about 100%; however, it was a modified version of heparin-mediated LDL precipitation technology [32] and works in a different strategy, and its adsorption for LDL is also clinically accepted. If it come into clinic use, its cost for patient and hemorrhagic tendency for the subject would be reduced greatly, as well as easy preparation, and the LDL-apheresis therapy system based on this adsorbent would be also simplified. For this reason, it still has practical worth though its adsorption for LDL was not the best.

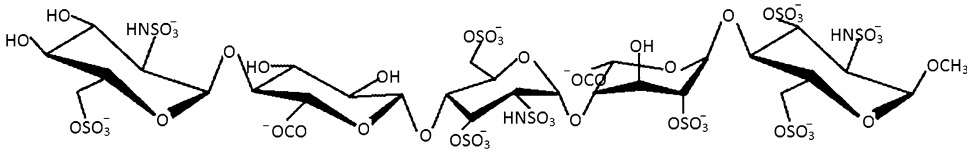


Fig. 6 Molecule structure of heparin

5 Conclusions

Heparin could be covalently immobilized onto the surface of a supporter with the aid of couplant by two-step synthesis method. If the surface of a supporter is modified with heparin, it will acquire a new ability to selectively adsorb LDL from plasma. Based on this finding, new LDL adsorbent for blood purification could be developed by this method.

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