Carbohydrate Polymers 78 (2009) 828–832

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Adsorption of lipoproteins with the aid of carboxymethylchitosan mircrospheres crosslinked with poly(ethylene glycol) bisglycidyl ether

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ARTICLE INFO

Article history:
Received 10 September 2008
Received in revised form 26 June 2009
Accepted 6 July 2009
Available online 12 July 2009

Keywords:
Carboxymethylchitosan
PEG
Low-density lipoprotein apheresis

ABSTRACT

Carboxymethylchitosan microspheres crosslinked with poly(ethylene glycol) bisglycidyl ether were prepared and then tested as an adsorbent for selective removal of low density lipoprotein (LDL) in human plasma. The microspheres were formed by a method of electrostatic instillation and crosslinked with poly(ethylene glycol) bisglycidyl ether. FTIR spectral analyses and X-ray photoelectron spectroscopy revealed that carboxymethylchitosan was crosslinked through amino groups to poly(ethylene glycol) bisglycidyl ether. The plasma lipoprotein sorption tests showed that the adsorption properties of the crosslinked microspheres for LDL were dependent on the concentrations of carboxymethylchitosan and poly(ethylene glycol) bisglycidyl ether. When the concentrations of carboxymethylchitosan and poly(ethylene glycol) bisglycidyl ether were 3.5% and 6%, respectively, 40% LDL and lower than 10% high density lipoprotein in plasma could be removed and the adsorption could be reach an equilibrium in 30 min.

1. Introduction

It is well know that the high level of low-density lipoprotein (LDL) in the organism leads to anterosclerosis development, coronary diseases, etc. The use of LDL apheresis in the treatment of patients with homozygous familial hypercholesterolemia (FH) and severe heterozygous FH provides an effective means of reducing LDL in plasma and has gained broad clinical acceptance during recent years (Bosch et al., 2006; Dräger et al., 1998; Knisel et al., 1994; Moriarty, Gibson, Shih, & Matias, 2001; Richter et al., 1993). But there are limitations to extending the application of the techniques. One of the main limitations is that the cost of the treatment remains very high. So developing economical and effective adsorbent for LDL apheresis has been attracting much attention (Li, Zhang, & Chen, 2004; Wang, Yu, Cui, & Cheng, 2003).

Chitosan is an amino-polysaccharide formed from deacetylated chitin, which is the second most abundant polysaccharide in nature. Because of its excellent properties such as biocompatibility, biodegradability and antimicrobiality, chitosan has been used as food packing materials (Arvanitoyannis, 1999), biomedical engineering scaffolds (Hsieh, Chang, & Lin, 2007; Kim et al., 2008). Chitosan is also used as a dietary fiber (Hossain et al., 2007; Xu, Huang, Qiu, Wu, & Hu, 2007) or a potential medicine against hypercholesterolemia (Lehtimäiki, Wuolijoki, Hirvelä, Lehtinen, & Ylitalo, 1995) because it can either adsorb cholesterolemia in the food or interact by electrostatic force with fatty acid and bile acid which have negative charge, in the digestive tract (Yao, Huang, & Chiang, 2008).

The surface of LDL is rich in basic amino acids, so is positively charged. Therefore most of the affinity adsorbents for LDL were designed based on the electrostatic interaction between the positively charged LDL and the negative charges on the ligands (Fu et al., 2006). Carboxymethylchitosan (CMC) is an amphiprotic derivative of chitosan. The carboxyl (—COOH) groups in the molecule make it possible to interact with LDL by electrostatic interaction. As CMC is water soluble, crosslinking of CMC is required when it is applied to medicine in the non-soluble form, for instance, as a matrix in drug delivery systems (Zhu et al., 2007), and a sheet to prevent tissue adhesion (Pan et al., 2006; Zhou, Elson, & Lee, 2004.). The commonly used crosslinking agent is glutaraldehyde (Chen, Tian, & Du, 2004; Zhao, Wang, & Wang, 2003), which may cause cytotoxicity both in vitro and in vivo (Walter Fürst & Banerjee, 2005). PEG is a highly water soluble amphipathic polymer and frequently used for chemical modification of nature and artificial macromolecules for biomedical applications (Otsuka, Nagasakib, & Kataoka, 2001). Poly(ethylene glycol) bisglycidyl ether, a diepoxy compound of PEG, exhibited lower cytotoxicity than glutaraldehyde (Nishi, Nakajima, & Ikada, 1995) and has been used as an crosslinking agent for biomaterials (Segura, Chung, & Shea, 2005; Tornihata & Ikada, 1997).

In the present study carboxymethylchitosan microspheres were prepared by a method of electrostatic instillation and crosslinked with poly(ethylene glycol) bisglycidyl ether. The resultant

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0144-8617/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2009.07.008

microspheres were characterized by FTIR spectroscopy, X-ray photoelectron spectroscopy (XPS), and environmental scanning electronic microscopy (ESEM) and used as an adsorbent for removal of low-density lipoprotein in human plasmas. The effect of preparation conditions on the adsorption properties of the microspheres was investigated in detail.

2. Experimental

2.1. Materials

Chitosan with a degree of deacetylation 90% and average molecular weight of about 10 KDa was obtained from Yuhuan Biochemical Co. Ltd. (Zhejiang, China). Total cholesterol (TC), high density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol kits were purchased from Zhejiang Dongou Bioengineering Co. Ltd. (Wenzhou China). All the other reagents were of analytical grade. Human plasma was kindly supplied by The Second Hospital of Hebei Medical University.

2.2. Preparation of carboxymethylchitosan

Chitosan (2 g) was immersed in 40 ml of isopropanol contained 4 g potassium hydroxide and allowed to stand overnight. Monochloroaceticacid (1 g) was added batched to the mixture at room temperature and stirred for 5 h. The mixture was adjusted to pH 8 by adding hydrochloric acid and then precipitated with 100 ml of ethyl alcohol. The precipitate was rinsed with 70–90% ethyl alcohol, ethyl alcohol absolute, ether absolute and vacuum dried at room temperature. The product was K salt CMC.

2.3. Preparation of poly(ethylene glycol) bisglycidyl ether (PEGB)

PEGB was prepared by the method of reference (Que & Liu, 1996), thus 20 g of PEG-400 was mixed with 6.2 g of potassium hydroxide, 14 g of epichlorohydrin, 0.6 g of tetrabutylammonium bromide and 40 ml of dichloromethane in a flask and stirred for 8.5 h at 60 °C. The reaction mixture was neutralized, filtrated and washed three times by water (20 ml each time), then the resulted mixture was distillated to remove dichloromethane and a light yellow oil-like liquid was obtained.

2.4. Preparation of carboxymethylchitosan–poly(ethylene glycol) bisglycidyl ether microspheres

A solution of 3% CMC was dropped through an electrostatic droplet generator to a solution of 6% zinc nitrate. Gel formation in the shape of microspheres was formed instantaneously. The microspheres were allowed to harden for 1 h and CMC–Zn microspheres were obtained. CMC–Zn (5 ml) microspheres was mixed with PEGB and stirred at 70 °C for 2.5 h. The crosslinked microspheres were rinsed with ethyl alcohol and acetone to remove the organic reagents on the surface of the microspheres and then immersed in a solution of 3 mol/l sodium citrate for 3 h to dissolving out the zinc ions in the microspheres. The CMCPB microspheres were kept in a saline solution after rinsed extensively with distilled water.

2.5. Measurement

Infrared spectra were performed in a ETS-3000 spectrophotometer (USA) with KBr disc technique. The morphological characteristics of microspheres were recorded by using an environmental scanning electron microscope XL130 TMP (USA). The variations in chemical composition on the surface at different crosslinking conditions were examined by using an EDAX-1600 X-ray photoelectron spectrometer (USA).

2.6. The adsorption properties of the microspheres for lipoproteins in human plasma

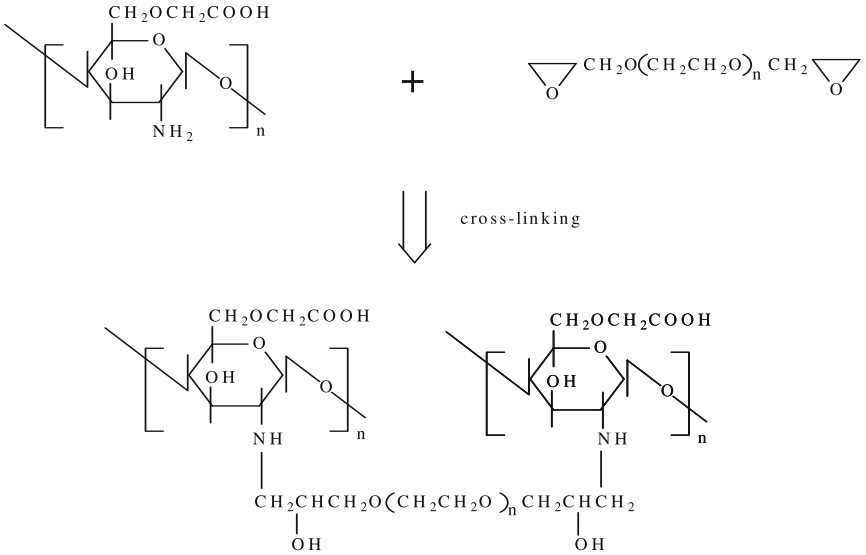
Microspheres (1 ml) was mixed with 1 ml of human plasma and incubated at 37 °C for 40 min. Amount of TC, LDL and HDL were measured with commercial assay kits. For various cholesterol removal ratio was calculated using the relation

$$\text{Removal efficiency } \% = \frac{C_1 - C_2}{C_1} \times 100\%$$

where C_1 and C_2 are the concentrations before and after adsorption, respectively.

3. Results and discussion

The reaction scheme for the crosslinking of CMC with PEGB is shown below (Scheme 1).



Scheme 1. Reaction scheme of CMC with PEGB.

3.1. Environmental scanning electron microscope

The ESEM images of CMC–Zn gel microspheres and CMCPB microspheres were shown in Fig. 1a and b. Comparing these pictures, it could be seen that the morphologies of the microspheres before and after crosslinking were very similar. This fact exhibited that the network of CMC–Zn gel microspheres was remained after crosslinked with PEGB. After adsorption of lipoproteins the surface of wet CMCPB microspheres became crinkled (Fig. 2) compared with the smooth surface before adsorption (ESEM image not given).

3.2. X-ray photoelectron spectroscopy

The effect of PEGB concentration on the chemical composition of CMCPB surface was studied by determining carbon/nitrogen (C/N) ratios. It is apparent from Fig. 3 that the C/N ratio increases with increasing PEGB concentration as expected from reaction of CMC with PEGB. This trend clearly indicated that CMC was crosslinked to PEGB chains.

3.3. IR spectroscopy

The IR spectra for CMC and representative crosslinked (1%, 5% PEGB) CMCPB microspheres were shown in Fig. 4. The CMC spectrum showed the characteristic absorption bands at 1599 cm^{-1} (superposed by $-NH_2$ bending and C=O bending in $-COO^-$), 1321 cm^{-1} (amide III) and 1078 cm^{-1} (skeletal vibrations of C—O stretching). The intensity at 1599 and 1324 cm^{-1} in CMCPB de-

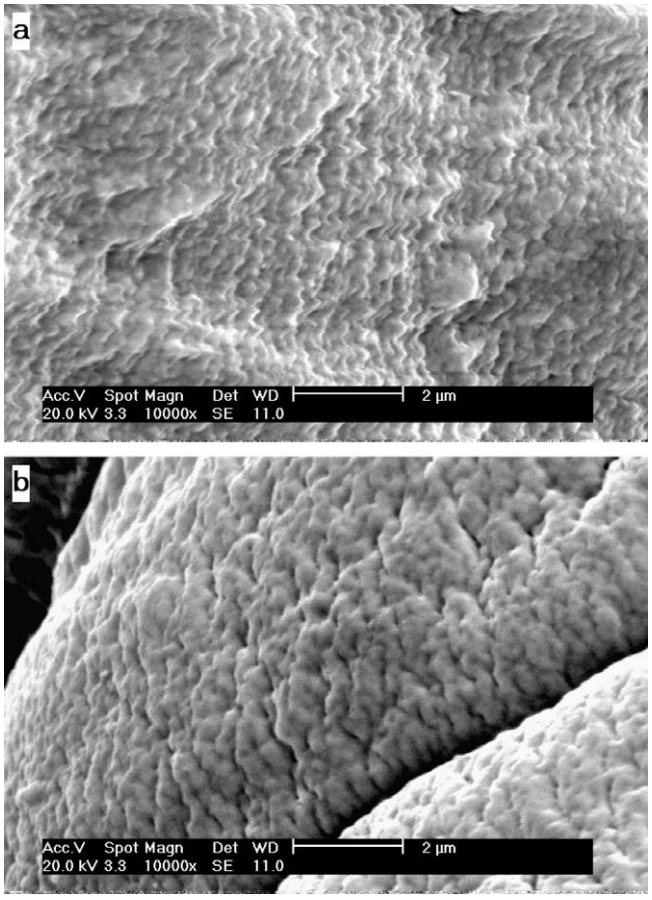


Fig. 1. Environmental scanning electronic microscopy of dry microspheres surface. (a) Carboxymethylchitosan–Zn, (b) crosslinked microspheres.

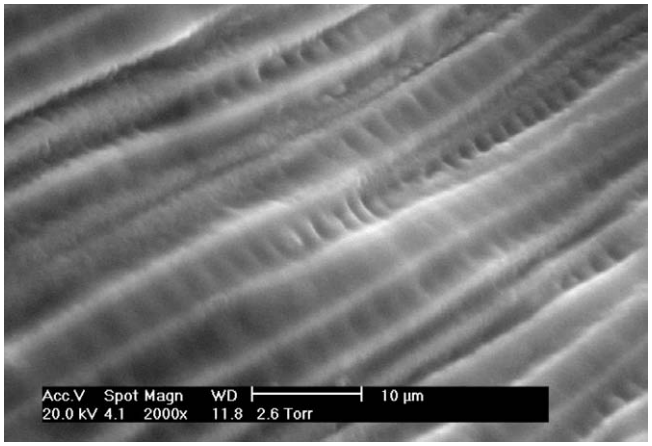


Fig. 2. Environmental scanning electronic microscopy of wet crosslinked microspheres surface after adsorption of lipoproteins.

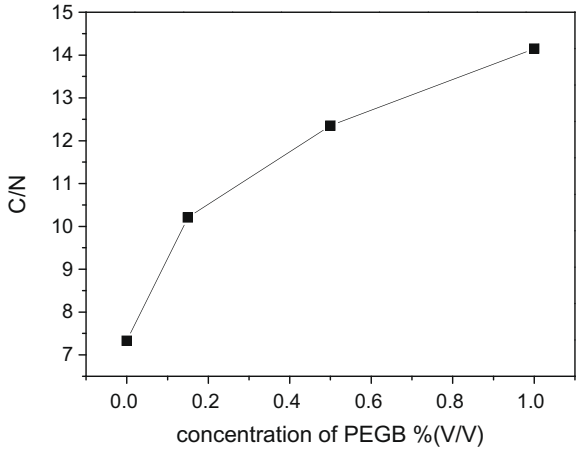


Fig. 3. Effect of the concentration of poly(ethylene glycol) bisglycidyl ether on the C/N ratio of crosslinked microspheres. Concentration of lipoproteins in human plasma before adsorption: TC, 5.08 mmol/l; HDL, 1.11 mmol/l; LDL, 3.97 mmol/l.

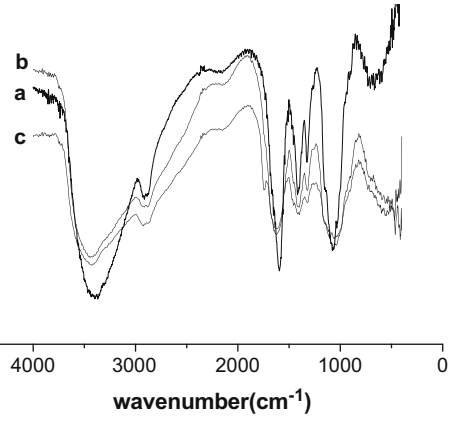


Fig. 4. IR spectra of (a) carboxymethylchitosan, (b) crosslinked microspheres (1% poly(ethylene glycol) bisglycidyl ether), (c) crosslinked microspheres (5% poly(ethylene glycol) bisglycidyl ether).

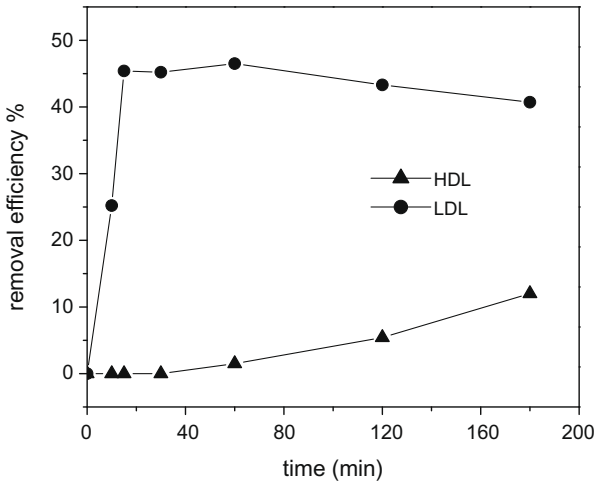


Fig. 5. Kinetic curves of crosslinked microsphere adsorption for lipoproteins. Concentration of lipoproteins in human plasma before adsorption: TC, 4.99 mmol/l; HDL, 1.13 mmol/l; LDL, 3.86 mmol/l.

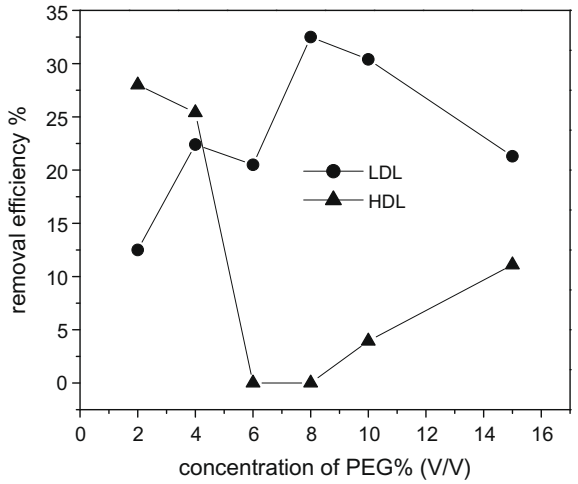


Fig. 6. Effects of concentration of poly(ethylene glycol) bisglycidyl ether on adsorption selectivity for LDL. Concentration of lipoproteins in human plasma before adsorption: TC, 4.95 mmol/l; HDL, 1.08 mmol/l; LDL, 3.87 mmol/l.

creased with increasing the PEGB concentration. As —C═O was not participate in the reaction of PEGB with CMC, we supposed that the crosslinking was formed between PEGB and CMC chains through —NH_2 groups.

3.4. The adsorption properties of CMCPB microspheres for LDL

The adsorption isotherms of lipoproteins onto CMCPB as a function of time were demonstrated in Fig. 5. The removal ratio for LDL increased rapidly within 30 min and changed little during 30–60 min, and then decreased. HDL was not adsorbed in the first 30 min, then the removal ratio of HDL increased. The short adsorption equilibrium time for LDL was related to the preparation process of the microspheres. As the solution of CMC was dropped into $ZnNO_3$ solution at a high speed, the CMC–Zn gel was formed instantly in the surface of the microspheres, the diffusion of Zn^{2+} from solution to the center of CMC microspheres slowed down. It means the gel was formed mostly on the surface of the microspheres. Crosslinked with PEGB the structure of the microspheres was remained. So LDL can be absorbed on the surface of the microspheres without diffusing into the center.

The influence of PEGB concentration on the adsorption properties for lipoproteins was shown in Fig. 6. It could be seen form this figure that the adsorption capacity for LDL increased with an increasing of PEGB concentration from 1% to 8%, and then decreased. As a contrast the adsorption capacity for HDL decreased with PEGB concentration from 1% to 6% and then increased with the increasing of PEGB concentration. These facts were due to the structure of the microspheres. At lower PEGB concentration the network of the microspheres was not sufficient, most CMC was in flowing state that was unfavorable to LDL adsorption. As the concentration of PEGB increased more CMC was fixed in the network and the amount of LDL adsorbed on the microspheres increased. Meanwhile the HDL adsorbed on the microsphere was edged out by LDL, so the adsorption capacity for HDL decreased. When the concentration of PEGB exceed the optimum value for LDL absorption, the adsorption amount of LDL decreased with the decreasing pore diameter of the microspheres. The diffusion of HDL was not affected by the pore diameter due to its smaller molecule diameter so the adsorption capacity for HDL increased again.

The adsorption selectivity of CMCPB microspheres for LDL was also depended on the concentration of CMC as shown in Table 1.

Higher concentration of CMC led to higher selective adsorption capacity for LDL. Considering LDL is a kind of negative charged particle, this result reflects the electronic interaction between the functional group of the adsorbent and LDL particle plays a main part in the adsorption.

4. Conclusion

This study concluded that CMCPB microspheres could adsorb LDL in human plasma rapidly and selectively.

The increase in C/N ratio on the CMCPB microspheres with increasing PEGB concentration, observed by XPS spectroscopy, indicated an increase in crosslinking. IR spectra showed characteristic peaks of CMC, the intensity at 1599 and 1324 cm^{-1} in CMCPB decreased with increasing the PEGB concentration. These two observations revealed the crosslinking of CMC with PEGB was through —NH_2 group.

The adsorption properties of CMCPB microspheres for lipoproteins in human plasma were related to the concentrations of CMC and PEGB. When the concentrations of CMC and PEGB were

Table 1
Effects of concentration of carboxymethylchitosan on the adsorption properties of the crosslinked microspheres for lipoproteins in human plasma.

Sample no.	CMC concentration (g/dl)	Concentration of lipoproteins in plasmas after adsorption[a] (mmol/l)			Removal efficiency (%)		
		HDL	LDL	TC	HDL	LDL	TC
1	2.5	0.96(±0.03)	2.95(±0.09)	3.89(±0.14)	19.5	23.8	22.8
2	3	1.01(±0.03)	2.50(±0.09)	3.51(±0.15)	15.3	35.4	30.6
3	3.5	1.06(±0.04)	2.32(±0.08)	3.38(±0.13)	10.7	40.0	33.1

Each value represents mean ± SE. The number of tests was three.
[a] Concentration of lipoproteins in human plasma before adsorption: TC, 5.05 ± 0.21 mmol/l; HDL, 1.18 ± 0.04 mmol/l; LDL, 3.87 ± 0.17 mmol/l.

3.5% and 6%, respectively, 40% LDL and lower than 10% HDL could be removed by CMCPB microspheres and the adsorption could reach an equilibrium in 30 min.

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