Hydrophobically Modified Celluloses as Novel Cholesterol-lowering Polymers

Jan Tůma,[a] Zdeněk Volek,[b] Andriy Synytsya,[a] Dagmar Dušková,[b] and Milan Marounek [b,*]

Hydrophobically modified celluloses were prepared by the esterification of monocarboxycellulose (MCC) and carboxymethylcellulose (CMC) with methanol, followed by the amino-de-alkoxylation of the methyl esters with *n*-octadecylamine. These cellulose derivatives were fed at 30 and 60 g/kg for 4 weeks to female rats. The diets were supplemented with palm fat (60 or 50 g/kg) and cholesterol (0 or 10 g/kg). There was no significant effect of amidated celluloses on the feed intake or body weight of rats. Both MCC and CMC derivatives significantly decreased the concentrations of cholesterol present in the serum and liver. The supplementation of diets with hydrophobically modified celluloses tended to increase fecal concentrations of cholesterol and coprostanol and significantly increased fecal concentrations of total neutral sterols. In rats fed diets containing cholesterol, the total serum cholesterol correlated negatively with the fecal concentrations of neutral sterols. It can be concluded that MCC-C18 and CMC-C18 are effective cholesterol-lowering agents.

Keywords: Modified celluloses; Dietary sorbents; Cholesterol; Neutral sterols; Rats

Contact information: a: Institute of Chemical Technology, Technická 3, 166 28, Prague, Czech Republic; b: Institute of Animal Science, Přátelství 815, Prague 22, CZ-104 00, Czech Republic;
** Corresponding author: marounek.milan@vuzv.cz*

INTRODUCTION

Obesity, diabetes mellitus, and hypercholesterolemia are the main risk factors of coronary heart disease, which is the leading cause of death worldwide. Many methods and agents have been used to treat hypercholesterolemia, including dietary fat restriction, 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins), and bile acid sequestrants. The latter are synthetic resins (cholestyramine, colestipol, and colesevelam), which are able to bind bile acids in the intestine, thereby interrupting their enterohepatic circulation. Such sequestration enhances the conversion of cholesterol to bile acids in the liver, resulting in reduced cholesterolemia (Farmer and Gotto 1995). An alternative to bile acid sequestrants consists of the class of hydrophobic sorbents of neutral sterols. Hydrophobically modified polysaccharides have a potential value as drug carriers and sorbents for non-polar compounds. Amino-de-alkoxylation of highly methylated (HM) citrus pectin with *n*-alkylamines leads to *N*-alkylpectinamides (Sinitsya *et al*. 2000). Among these derivatives, water insoluble *N*-octadecylpectinamide, which carries long alkyl chains, is interesting due to its amphiphilic properties (Synytsya *et al*. 2004). The hypocholesterolemic activity of this modified pectin has been examined *in vivo* in the experiment on rats (Marounek *et al*. 2010). This derivative of HM citrus pectin, at a level of 50 g/kg, significantly decreased the concentrations of cholesterol in the serum and

liver and significantly increased the fecal excretion of sterols. A similar cholesterol lowering effect was observed in a subsequent experiment when *N*-octadecylpectinamide was supplied at 20, 40, and 60 g/kg, or supplied at 20 g/kg for three months (Marounek *et al*. 2013). This suggests that amide bonds are resistant to the enzymes of the intestinal microbiota. Butyl and hexyl amides of pectate were prepared by Hromádková *et al*. (2008). The results of the *in vivo* experiment on rats suggest that these pectin derivatives may serve as nutraceuticals for obesity treatment.

Several cellulose derivatives are prospective cholesterol-lowering agents. The quaternisation of diethylaminoethylcellulose increased its binding capacity for bile acid salts in an *in vitro* experiment (Clas 1991). Similarly, modified celluloses containing tertiary amino and/or quaternary ammonium groups were synthesised by Nichifor *et al*. (1998). Other modified celluloses include soluble fibres, which are not absorbed by the body. In the intestinal lumen, these fibres can increase viscosity and decrease the absorption of cholesterol and fat. Maki *et al*. (1999) reported that high-viscosity hydroxypropylmethylcellulose significantly lowered the total, LDL, and non-HDL cholesterols in patients with mild to moderate hypercholesterolemia. Hydroxypropyl-methylcellulose increased the fecal fat, sterol, and bile acid excretions in hamsters (Bartley *et al*. 2010). Cationic hydroxyethylcellulose significantly decreased the weight gain, adipose weight, total, VLDL and LDL cholesterol, and hepatic lipids in hamsters fed a high-fat diet (Hung *et al*. 2012).

The tasks of the present study were to prepare hydrophobic derivatives (*N*-octadecylamides) of oxidised monocarboxycellulose (MCC) and carboxymethylcellulose (CMC) and to compare their effects on feed intake and serum, liver, and fecal cholesterol in rats. *In vivo* experiments using amidated celluloses have not been reported in the available literature.

EXPERIMENTAL

Chemicals

Oxidised monocarboxycellulose (MCC) in its acidic form with carboxylic groups of 18% m/m was obtained from VUOS Plc., Pardubice, Czech Republic. Sodium salts of carboxymethylcellulose (CMC) containing 0.65 to 0.85 carboxymethyl groups per anhydroglucose unit, *N*-octadecylamin, and cholesterol (≥ 99.0%) were purchased from Sigma Aldrich Chemie GmbH, Steinheim, Germany. Cholesterol, coprostanol, β-sitosterol, epicoprostanol, stigmasterol, and β-sitostanol, were purchased from Sigma-Aldrich (Prague, Czech Republic). Other chemicals were purchased from Lach-Ner, Neratovice, Czech Republic.

Preparation and Characterisation of Modified Celluloses

N-octadecylamide derivatives of MCC and CMC (MCC-C18 and CMC-C18, respectively) were prepared in two steps: (a) esterification with methanol, and (b) amino-de-alkoxylation of methyl ester with *N*-octadecylamine. The methodology of preparation of hydrophobic cellulose derivatives was based on the modified methods of Charpentier *et al*. (1997) and Taubner *et al*. (2013). MCC, CMC, and their derivatives were analysed for their carbon, hydrogen, and nitrogen contents using the EL III Universal CHNOS Elemental Analyser (Elementar Analysensysteme GmbH, Germany). The degrees of

amidation (*DA*) were calculated based on the nitrogen and carbon contents and used for calculation of average molecular weights of MCC-C18 and CMC-C18 monomeric units.

Animals and Diets

Thirty female Wistar rats, approximately 3 months of age and weighing 250 g, were housed in a temperature and humidity controlled room. The animal facility was maintained on a 12-h light/dark daily photoperiod cycle at a temperature of 21 to 23 °C and a relative humidity of 60 ± 5%. The rats were fed the commercial ST-1 rat diet (Velaz Ltd., Lysolaje, Czech Republic), consisting of soybean meal, meat and bone meal, fish meal, wheat, maize, oats, wheat bran, limestone, dicalcium phosphate, salt, and supplements of vitamins, trace elements, and amino acids. The ST-1 diet contained crude protein, fibre, fat, ash, and cholesterol at 208, 54, 41, 19, and 0.28 g/kg, respectively. The diet was supplemented with microcrystalline cellulose and the palm fat AkoFeed Gigant 60 (AarhusKarlshamn Sweden AB, Karlshamn, Sweden) at 60 and 50 g/kg, respectively.

The study was approved by the Ethics Committee of the Institute of Animal Production and Central Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic.

Table 1. Composition of Control and Experimental Diets

Ingredient	Diet (g/kg)					
	1	2	3	4	5	6
Cholesterol	0	10	10	10	10	10
Palm fat[a]	60	50	50	50	50	50
Amidated oxidised MCC[b]	0	0	30	60	0	0
Amidated CMC[c]	0	0	0	0	30	60
Cellulose	60	60	30	0	30	0
Diet ST-1	880	880	880	880	880	880

[a] Palm fat contained lauric, myristic, palmitic, stearic, oleic, and linoleic acid at 1.7, 2.0, 45.6, 30.5, 15.1, and 3.3 g per 100 g of fatty acids determined, respectively.
[b] MCC, oxidised monocarboxycellulose
[c] CMC, carboxymethylcellulose

The palm fat AkoFeed Gigant 60 contains 85% free fatty acids and 99% total fat. After 4 weeks, the rats were randomly divided into 6 groups of 5 animals each. Diets 2, 3, 4, 5, and 6 were supplemented with cholesterol at 10 g/kg at the expense of palm fat. Diets 3 and 4 were supplemented with amidated oxidised MCC at 30 and 60 g/kg, respectively, and diets 5 and 6 included amidated CMC at 30 and 60 g/kg, respectively. Amidated celluloses were added at the expense of microcrystalline cellulose (Table 1). Microcrystalline cellulose was supplied by Sigma-Aldrich (Prague, Czech Republic). Food and water were available *ad libitum.*

Sampling

Feces were collected during the last 5 days of the experiment, pooled, and stored at -40 °C until analysis. The experiment's duration was 4 weeks. At the end of the experiment, the rats received 4 g of feed 4 h before they were killed (Spielman *et al*. 2008). Rats were sacrificed *via* decapitation after anaesthesia by isofluorane inhalation (Nicholas Piramal India Ltd., London). Mixed blood samples were drawn at the time of

euthanasia to obtain the serum. After laparotomy, the livers were excised, weighed, and stored at -40 °C until analysis.

Chemical Analyses

Analyses of the feed were performed as described previously (Marounek *et al*. 2012). The serum levels of total cholesterol, LDL cholesterol, triacylglycerols, and aminotransferases were determined using commercial kits supplied by Erba-Lachema, Ltd. (Brno, Czech Republic). The total hepatic and fecal lipids were extracted with a 2:1 chloroform-methanol solution and then determined gravimetrically (Folch *et al*. 1957). Hepatic lipids were saponified with 2 M ethanolic KOH and extracted with diethyl ether. Cholesterol was silylated using TMCS and HMDS silylation reagents (Sigma-Aldrich, Prague, Czech Republic). The silyl derivatives were quantified on a gas chromatograph equipped with a SAC-5 capillary column (Supelco, Bellefonte, U.S.A.) operating at 285 °C. The fecal neutral sterols were analysed in freeze-dried samples by gas chromatography on a 25-m fused silica capillary column (CP-SIL 5CB, 0.25-mm *i.d.*, and 0.25-µm film thickness; Varian, U.S.A.). The samples were then subjected to *n*-butylation and trimethylsilylation (Sylon HTP was purchased from Supelco), according to Batta *et al*. (2002), except that the reaction mixture was centrifuged prior to evaporation at 1400 g for 10 min. The residue was dissolved in hexane and centrifuged, and then a 1-µL sample was injected. The oven was initially kept at 200 °C for 2 min and then programmed to increase at 8 °C per min to a final temperature of 272 °C. Cholesterol and other neutral sterols were identified based on their retention times and compared to standards.

Statistics

The data were statistically analysed using two-way analysis of variance (ANOVA) with the General Linear Models (GLM) Procedure of SAS (Statistical Analysis System, SAS Institute, Cary, NC, USA, version 9.2, 2003). The main variables were the cellulose type, cellulose dosing, and interaction between these two factors. Each response variable $Y_{i,j,k}$ was modelled as follows (Eq. 1),

$$Y_{i,j,k} = \text{Intercept} + \alpha_i + \beta_j + \gamma_{ij} + \varepsilon_{ijk} \quad (1)$$

where α_i is the effect of cellulose type (i), β_j is the effect of cellulose dosing (j), γ_{ij} is the effect of interaction between cellulose type (i) and cellulose dosing (j), and ε_{ijk} represents the Gaussian (random) error in the population.

All differences were considered significant at $P < 0.05$. The results in the preceding tables are presented as the mean and the root mean square error (RMSE). The relationships among serum cholesterol, hepatic cholesterol, and fecal neutral sterols were evaluated by estimating Pearson's correlation coefficients.

RESULTS AND DISCUSSION

Preparation and Characterisation of Cellulose Derivatives

As described in our previous work (Taubner *et al*. 2013; Sinitsya *et al*. 2000), in the present study a methyl esterification and subsequent amino-de-alkoxylation of the methylesters were used to obtain MCC-C18 and CMC-C18 because the esters are known

to be more reactive than free carboxyls. According to elemental analysis, the degrees of amidation (*DA*) were 29.6 and 63.1 mol% for MCC-C18 and CMC-C18, respectively. Average molecular weights of MCC-C18 and CMC-C18 monomeric units were 224.8 Da and 411.8 Da, respectively.

Alternative means of MCC or CMC amidation have also been described in the literature. Follain *et al*. (2008) described the amidation of MCC by the reaction of carboxyls with alkylamines (*N*-methoxyethylamine, *N*-butylamine and *N*-octylamine) in the presence of carbodiimide. The *DA* values of the prepared derivatives varied from 13 to 59 mol%. CMC derivatives obtained by the direct amidation of the carboxyl group with *N*-hexadecylamine were described by Charpentier *et al*. (1997), but the *DA* values of these derivatives were significantly lower (from 0.9 to 6% per monomeric unit).

In vivo Animal Experiment

Neither hydrophobically modified celluloses nor cholesterol supplementation significantly influenced the body weight. The feed intake in rats fed MCC-C18 at a level of 30 g/kg was significantly higher than the levels in other rats fed cellulose derivatives (Table 2).

Table 2. Effect of Cholesterol and Amidated Celluloses at 30 and 60 g/kg on Weight and Feed Intake in Female Rats

Cholesterol (g/kg)	0	10	10	10	10	10	RMSE
Amidated oxidised MCC (g/kg)	0	0	30	60	0	0	
Amidated CMC (g/kg)	0	0	0	0	30	60	
Initial weight (g)	261	255	262	254	255	263	12.3
Final weight (g)	265	261	263	257	259	252	13.9
Feed intake (g/day)	17.1^{b}	18.8^{ab}	19.3^{a}	17.0^{b}	17.3^{b}	17.0^{b}	1.3

RMSE, Root mean square error
Data are means for 5 rats per diet. Values in the same row with different superscripts are significantly different ($P < 0.05$).

The serum total and LDL cholesterol concentrations in rats fed the control diet were 2.12 and 0.19 μmol/mL, respectively. Cholesterol supplementation significantly increased the total and LDL cholesterol concentrations to 3.31 and 0.79 μmol/mL, respectively, while significantly increasing the hepatic cholesterol concentration from 5.16 to 19.38 μmol/g (Table 3). Hydrophobically modified celluloses significantly decreased both the serum and hepatic cholesterol concentrations. Serum triacylglycerol concentrations were variable, but tended to be lower in rats fed cellulose derivatives. There was no significant treatment effect on the activity of alanine aminotransferase (E.C. 2.6.1.2). The activity of aspartate aminotransferase (E.C. 2.6.1.1) was significantly increased in rats fed CMC-C18 at 30 g/kg. MCC-C18 supplied at 60 g/kg significantly reduced the concentration of hepatic fat. The hepatic cholesterol correlated significantly with total serum cholesterol ($r = 0.740$; $P < 0.001$). Thus, amidated celluloses demonstrated similar effect on serum and hepatic cholesterol, as shown in previous experiments with amidated pectin (Marounek *et al*. 2010; 2013) and chitosan (Zhang *et al*. 2008).

The concentrations of cholesterol, coprostanol, and total neutral sterols in the feces significantly increased in all rats that were fed diets supplemented with cholesterol (Table 3). The supplementation of diets with hydrophobically modified celluloses

increased fecal concentrations of cholesterol and coprostanol and significantly increased fecal concentrations of total neutral sterols. In rats fed diets containing cholesterol, the total serum cholesterol correlated negatively with the fecal concentrations of neutral sterols ($r = -0.723$; $P < 0.001$). This indicates that the hypocholesterolemic effect of amidated celluloses may be based on the sequestration of sterols, which decreases their enterohepatic circulation.

Table 3. Effect of Cholesterol and Amidated Celluloses on Serum Parameters, Hepatic Cholesterol and Fat, and Fecal Neutral Sterols in Female Rats

Cholesterol (g/kg)	0	10	10	10	10	10	RMSE
Amidated oxidised MCC (g/kg)	0	0	30	60	0	0	
Amidated CMC (g/kg)	0	0	0	0	30	60	
Serum concentrations							
Total cholesterol (µmol/mL)	2.12^{bc}	3.31^{a}	2.46^{b}	1.74^{c}	1.82^{c}	1.62^{c}	0.43
LDL cholesterol (µmol/mL)	0.19^{b}	0.79^{a}	0.43^{b}	0.27^{b}	0.22^{b}	0.20^{b}	0.21
Triacylglycerols (µmol/mL)	1.28^{ab}	2.25^{ab}	1.39^{ab}	1.36^{a}	1.14^{ab}	0.77^{b}	0.69
AST (nkat/mL)	3.08^{ab}	2.64^{a}	2.71^{ab}	2.99^{ab}	3.26^{b}	3.22^{ab}	0.46
ALT (nkat/mL)	1.26	1.16	1.24	1.35	1.36	1.43	0.25
Hepatic concentrations							
Cholesterol (µmol/g)	5.16^{c}	19.38^{a}	8.15^{b}	6.78^{b}	7.44^{b}	6.15^{bc}	1.21
Coprostanol (µmol/g)	0.02^{b}	0.04^{ab}	0.03^{ab}	0.04^{ab}	0.03^{ab}	0.04^{a}	0.016
Fat (mg/g)	98.8^{a}	107.8^{a}	84.4^{ab}	57.8^{b}	72.3^{ab}	92.7^{ab}	28.3
Fecal concentrations							
Cholesterol (µmol/g DM)	5.7^{c}	53.0^{b}	60.8^{a}	59.4^{a}	61.9^{ab}	55.4^{ab}	5.4
Coprostanol (µmol/g DM)	2.5^{d}	10.5^{c}	11.1^{bc}	20.2^{b}	15.5^{b}	28.2^{a}	3.7
Neutral sterols (µmol/g DM)*	12.5^{d}	69.0^{c}	77.9^{b}	84.8^{ab}	82.7^{ab}	88.9^{a}	6.1

RMSE, Root mean square error; DM, Dry matter
Data are means for 5 rats per diet. Values in the same row with different superscripts are significantly different ($P < 0.05$).
*Including epicoprostanol and plant sterols.

CONCLUSIONS

1. Hydrophobically modified celluloses are effective cholesterol-lowering agents. In rats receiving amidated celluloses, the serum and hepatic cholesterol concentrations were decreased and fecal concentrations of neutral sterols were increased.
2. Serum cholesterol correlated positively with hepatic cholesterol and correlated negatively with the fecal concentration of neutral sterols.

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