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A Polymeric Antibacterial Agent with Sustained Anti-bacterial Activity: Cellulose Xanthate-metal-neomycin Complexes

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ABSTRACT – Neomycin coupled to a polymer matrix via a metal linker was prepared and evaluated for prolonging antibacterial activity. Microcrystallized cellulose was chemically modified to cellulose xanthate (MCX) to afford metal binding sites. MCX was treated with Cu (II), Fe (III) or Zn (II) followed by reaction with neomycin (Ne). The release of Ne from MCX-Zn (II)-Ne was investigated and its activity duration was measured by ditch plate method. The amount of metal bound to MCX was 0.36 mmol/g matrix, 0.26 mmol/g matrix and 0.56 mmol/g matrix for Cu (II), Zn (II) and Fe (III), respectively. Ne bound to MCX-metal chelates was 0.006 mmol, 0.07 mmol and 0.01 mmol per g MCX for Cu (II), Zn (II) and Fe (III), respectively. The Ne release from MCX-Zn (II)-Ne was sustained even after seven washes, whereas Ne from MC/Zn (II)/Ne mixture was almost completely released in two washes. Antibacterial activity was prolonged with MCX-Zn (II)-Ne and MCX-Fe (III)-Ne, but not with MCX-Cu (II)-Ne when compared with that of free Ne. Taken together, these results suggest that neomycin coupled to MCX via a proper metal linker has a potential as a polymeric antibacterial agent with sustained activity.

Key words – Controlled release, Coordinate bond, Polymeric drug, Microcrystallized cellulose, Sustained antibacterial activity, Neomycin

A great deal of attention has been paid to the development of pharmacologically active macromolecular compounds during recent years. Interest in the development of polymeric drugs is based on their postulated advantages over simple drugs, such as the prolongation of action, the variation in reactivity and toxicity and a possible change in the normal distribution of the drugs.[1-3]

It has been investigated in such diverse areas including medication, weed control and insect control to develop polymer matrices as a controlled release system. A major approach for the release control of small molecular bioactive agents leading to prolonging the action duration involves chemical conjugation with synthetic or naturally occurring macromolecules.[4] The formation of a hydrolysable covalent bond is one of the methods which have been adopted most frequently. However, there is no absolute requirement that this highest energy type of bond be the linkage used. Conceptually at least, it should be possible to develop controlled release system based on other types of chemical bonds with lower energy. These could comprise the van der Waals forces (ΔH 1 Kcal/mol), ionic bonds (ΔH 10-15 Kcal/mol) and coordinate or chelate bonds (ΔH 50 Kcal/mol). Unlike covalent bonds, coordinate covalent bonds of chelate type vary in binding stability over several orders of magnitude depending on the atomic characteristics of metals and ligands that are involved in the coordinate bond. Therefore, using vast body of background chemical information defining the stability constants of such entities, it should be possible to create organometallic structures acting as a controlled release system.[5]

In previous reports, we showed that chemical modification, xanthation, of cotton cellulose markedly increases the metal binding capacity of cotton leading to attachment of a large amount of bioactive agents via chelation between the metal-bound polymer and the bioactive agents. Further, the duration of action of the bioactive agents is prolonged by sustained release from the polymer matrix.[6,7] It was thought that applicability of a polymeric drug varies depending on physical form of a polymeric matrix. Therefore, in the present study, we adopted microcrystallized cellulose as a polymer matrix to carry an anti-microbial agent, neomycin and prepared polymeric antibacterial agents using various metals as a linker between the polymer matrix and drug, thus probably showing controlled release profiles of the active agent from the particle matrix. Our data demonstrate that the final product, cellulose xanthate-metal-neomycin complexes (MCX-metal-Ne) maintained the physical integrity of MC and xanthation of cellulose

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increased the metal binding capacity leading to effective coupling of neomycin to the matrix via metal chelation. The release of Ne from MCX–Zn (II)-Ne was sustained compared with cellulose/Zn (II)/Ne where the metal does not act as a chelating linker between cellulose and Ne. Moreover, the antibacterial activity of MCX-metal-Ne complexes except for MCX-Cu (II)-Ne lasted longer than that of free neomycin.

Materials and Methods

Microcrystallized cellulose (Sigmacell, particle size 50 μm) and neomycin were purchased from Sigma Chemical Co. (St. Louis, MO. U.S.A.). Cupric and zinc nitrate and ferric chloride were obtained from Wako Chemical Co. (Tokyo, Japan). Carbon disulfide was purchased from Junsei Chemical Co. (Tokyo, Japan). The ingredients of the medium used for antibacterial test were received from DIFCO Laboratories, (Detroit. MI. USA). The sources of bacteria employed were as follows: *Staphylococcus aureus* (ATCC 12228), *Enterobacter aerogenes* (IFO 3320). Quantitative analysis of neomycin was done using a fluorescence spectrophotometer (Shimadzu RF-5301PC, Tokyo, Japan). Concentration of metal ions was analysed using Inductively Coupled Plasma Quantorecorder (Shimadzu ICPQ-1000, Tokyo, Japan).

Preparation of cellulose xanthate

Microcrystallized cellulose (MC, 7.5 g) was suspended in a reaction flask containing 1 M-NaOH (100 mL) and mixed with carbon disulfide (5 mL) and the mixture was stirred at room temperature. As the reaction proceeded, the color of reaction mixture turned into orange. After reaction for 4 h, the pale-yellow product was centrifuged, washed thoroughly with water, ethanol and acetone in turn, and air-dried. The resultant dry material, cellulose xanthate (MCX), was stored at −20°C. The aforementioned reaction condition was optimal in physical integrity of the matrix and degree of xanthation.

Preparation of cellulose xanthate-metal chelates and determination of metals bound to cellulose xanthate

MCX (1 g) was added into 100 mL of Zn (II) solution (0.01 M in pH 4.0, 0.05 M sodium phosphate buffer) and stirred at room temperature for 30 min. The white product, MCX-Zn (II), was isolated by centrifugation, washed thoroughly with distilled water and air-dried. The supernatant was subjected to ICPQ to determine the concentration of metal ions. The amount of metal bound to the matrix was deduced by the difference between the amount in the final solution and the amount added for the reaction. MCX-Cu (II) (dark olive green) and MCX-Fe (III) (dark brown) were obtained using the same procedure as described above.

Preparation of cellulose xanthate-metal-neomycin and determination of neomycin bound to cellulose xanthate-metal chelates

MCX-Zn (II) chelate was placed in 100 mL of neomycin (Ne) solution (100 mg in pH 4.5, 0.05 M sodium phosphate buffer) and stirred at 10°C for 1 h. After centrifugation, the precipitated product, MCX-Zn (II)-Ne, was washed thoroughly with water, 0.5 M NaCl solution, ethanol and acetone in turn and dried in air. The supernatant was subjected to fluorescence spectrophotometer to determine the concentration of Ne.[8] The amount of Ne bound to the matrix was deduced by the difference between the amount in the final solution and the amount added for the reaction. MCX-Zn (II)-Ne and MCX-Fe (III)-Ne were obtained using the same procedure as described above.

Release of neomycin from cellulose xanthate-Zn(II)-neomycin

MCX-Zn (II)-Ne complex (0.5 g) was placed in 50 mL of 0.05 M sodium phosphate buffer solution (pH 4.5) and stirred for 5 min at 20°C. After centrifugation, Ne in the supernatant was analysed. This process was repeated with the residue. The same experiment was done with a mixture of cellulose/Zn (II)/Ne (500 mg/8.5 mg/25 mg).

Antibacterial activity of cellulose xanthate-metal-tetracycline

Antibacterial activity was measured indirectly by the agar diffusion method.[7,9] To a culture plate (diameter: 90 mm, height: 15 mm), was added 30 mL of nutrient agar and were placed two rings (o.d.: 10 mm, height: 10 mm). After solidifying, the rings and the contents were removed to form two cylindrical holes. The sample at an appropriate amount as indicated in the table legend was planted in each hole and added a few drops of molten agar medium upon it. The plates were inoculated with cultures of *E. aerogenes*, and *S. aureus*, incubated at 37°C for 24 h and the diameter of inhibition zone was measured. The contents of the cylindrical hole, after 24 h of incubation, were removed and transferred to a hole of a freshly prepared plate as described above, incubated again for 24 h and measured the diameter of the inhibition. To investigate the duration of the antibacterial activity of each sample, this procedure was continuously repeated until the inhibition of growth was no longer noticed. The same procedures were followed using free 0.22 mg Ne (equivalent to 30 mg of MCX-Fe

(III)-Ne) to compare the duration of antibacterial activities of the free and matrix-bound drug.

Results

Formation of cellulose xanthate-metal chelates

In a previous report, xanthation of cotton cellulose increases metal binding capacity by affording xanthate group, a chelating moiety, to cellulose.[6] We examined whether this applied to microcrystallized cellulose (MC). MC was treated with carbon disulfide in 1 M-NaOH solution and pale-yellow MC xanthate (MCX) was obtained. Under this synthesis condition, the matrix maintained the physical integrity of the polymer matrix, MC. MCX was treated with 0.01 M metal ion solutions for 30 min, at which the metal uptake by the matrix reached equilibrium (data not shown), and the metal uptake was determined. The same experiment was done with MC to compare metal binding capacity between MC and MCX. While the amount of metal bound to MCX was 0.36 mmol Cu (II)/g matrix, 0.26 mmol Zn (II)/g matrix and 0.56 mmol Fe (III)/g matrix, the metal binding to MC was negligible.

Coupling of neomycin to cellulose xanthate-metal chelates

It was speculated that the metals bound to MCX-metal chelates might be available for coordinate bond with a ligand that contains electron-donating groups. To prepare a polymeric antibacterial agent, we treated MCX-metal chelates with neomycin (Ne) which possesses electron-donating primary and secondary hydroxyl and primary amino groups and binding of Ne to the matrix was measured. The same experiment was done with MCX and MC. The binding amount of Ne per gram of the matrices was 4.5 mg (0.006 mmol), 49.1 mg (0.07 mmol) and 7.5 mg (0.01 mmol) for MCX-Cu (II), MCX-Zn (II) and MCX-Fe (III), respectively. However, MCX and MC that do not have the metals showed no Ne uptake. The binding of Ne to MCX-Zn (II) chelate reached maximum at 1 hr (data not shown).

Release of neomycin from cellulose xanthate-Zn (II)-neomycin

To examine whether neomycin bound to cellulose-xanthate-metal chelates was released in a controlled manner, the amount of Ne released for each washing-cycle was measured.

As shown in Figure 1, about 90% of Ne was removed from cellulose/Zn (II)/Ne mixture at the first washing and the rest was almost completely washed off by three cycles of the process. On the contrary, the Ne release from MCT-Zn (II)-Ne was sustained maintaining relatively constant level up to at least six-cycles of washing after the first one.

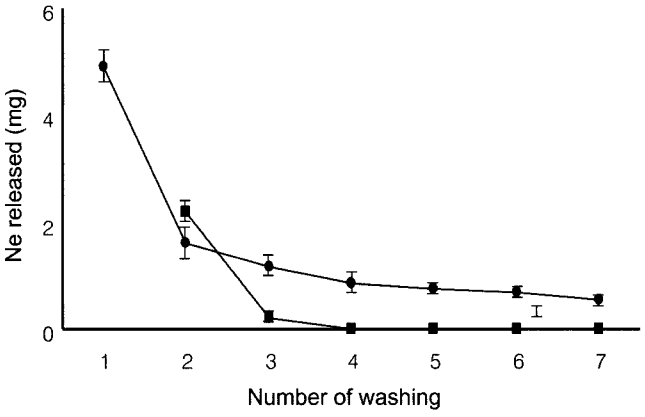

Figure 1–Release of neomycin from cellulose xanthate-Zn (II)-neomycin and a mixture of cellulose/Zn (II)/neomycin. MCX-Zn (II)-Ne (0.5 g) or cellulose/Zn (II)/Ne (500 mg/8.5 mg/25 mg) mixture was immersed in 50 mL of 0.05 M sodium phosphate buffer (pH 4.5) and stirred and centrifuged. This process was repeated 7 times with the residue. Ne released from the first washing of the mixture was 22.8 mg (data not shown). Data represent the mean of three independent experiments. ●, MCX-Zn (II)-Ne; ■, Cellulose/Zn (II)/Ne mixture.

Antibacterial activity of cellulose xanthate-metal-neomycin complexes

Although Ne was released from MCX-Zn (II)-Ne in a controlled manner, it still remained unclear whether MCX-metal-Ne complexes may exhibit sustained antibacterial activity. To examine this, the antibacterial activity of MCX-metal-Ne was evaluated by the agar diffusion method where a zone of inhibition produced by the diffusion of the antibacterial agent from the matrix into the agar was measured. The results are summarized in Table I. Whereas MCX, MCX-Fe (III), MCX-Cu (II) and MCX-Zn (II) did not show antibacterial activity (data not shown), free Ne and MCX-metal-Ne complexes showed activity at varied duration of time. The inhibition zone lasted for 6, 24, 12 and 4 days for free Ne, MCX-Zn (II)-Ne complex, MCX-Fe (III)-Ne complex and MCX-Cu (II)-Ne complex, respectively, for *S. aureus* and *E. aerogenes*, indicating that MCX-metal-Ne complexes exhibited prolonged antimicrobial activity except MCX-Cu (II)-Ne complex.

Discussion

In this report, we demonstrated that xanthation of microcrystallized cellulose (MC) increased the metal binding capacity of the matrix and neomycin (Ne) was coupled to microcrystalline cellulose xanthate-metal chelates, probably

Table I–*Antimicrobial Activity of Cellulose Xanthate-metal-neomycin Complexes*

Strains	Sample[a]	Inhibition zone[b] : diameter[c] (mm)												
S. aureus	Ne	35	20	18	17	17	17	0						
	A	21	21	21	21	21	21	21	20	19	18	18	17	17
		18	18	18	17	17	17	17	17	17	17	17	0	
	B	20	19	18	18	17	18	17	16	17	17	17	17	0
	C	19	17	16	15	0								
E. aerogenes	Ne	40	20	18	18	17	17	0						
	A	24	22	23	23	23	21	21	21	20	18	18	19	18
		18	17	17	17	16	17	16	17	15	16	0		
	B	21	20	20	19	18	17	17	17	17	17	16	17	0
	C	18	17	17	16	0								

[a] : 0.22 mg of free neomycin and 30 mg of MCX-metal-Ne complexes were used.
[b] : The inhibition zone was measured once a day.
[c]: Data represent the mean of three separate experiments.
Ne: Free neomycin, A: MCX-Zn (II)-Ne, B: MCX-Fe (III)-Ne and C: MCX-Cu (II)-Ne.

via coordinate bond. We also showed that MCX-Zn (II)-Ne released Ne in a controlled manner and furthermore, MCX-Zn (II)-Ne and MCX-Fe (III)-Ne presented sustained anti-bacterial activity.

In agreement with our previous report, where cotton was used as a polymer matrix,[6] the metal binding capacity of MC was increased markedly by the chemical modification, xanthation, indicating that xanthation is an effective way to increase metal binding capacity of the cellulosic matrix regardless of physical shape of matrices. Tenacious metal binding did not take place with cellulose itself, which suggests that xanthation provides available binding sites for covalent bonding of metal ions to the matrix as a xanthate group. Although binding of metal ions to the xanthate group has been described as 1:1 and 1:2 complex,[10] formation of 1:1 complex might be favored. This is based on the facts that there should be a steric hindrance imposed by the rigid polymeric structure of MCX and preparation of MCX-metal chelates was conducted with excess metal ions, thus probably limiting formation of 1:2 complexes. Thus, it is very likely that the transition metals bound to the xanthate group in MCX are still available for another coordinated bond with neomycin. Moreover, since the final product was washed thoroughly by a buffer including 0.5 M NaCl and organic solvents, neomycin non-specifically adsorbed on the matrix should be removed. These suggest that the dissociation of Ne from the coordinate bond leads to the sustained releases of Ne from MCX-Zn (II)-Ne. This was illustrated by the data showing that whereas the Ne release from cellulose matrix without a chelating linker, cellulose/Zn (II)/Ne mixture, did not show controlled release pattern. MCX-Zn (II)-Ne, indeed, sustained the release of Ne from the matrix. Considering that the binding of Ne to MCX-metal chelates takes place primarily via the coordinate bond, the binding efficiency of neomycin to each metal bound to MCX might be dependent on the affinity of electron donating groups in neomycin to each metal on MCX. This implies that neomycin binds to MCX-Zn (II) with greater affinity than MCX-Cu (II) and MCX-Fe (III).

Ne released from MCX-metal-Ne complexes was shown to be available to inhibit bacterial growth in the agar diffusion experiment (Table I). MCX-metal-Ne complexes as well as free Ne showed antibacterial activity represented by inhibition zone. However, the duration of the antibacterial activity of MCX-metal-Ne complexes was dependent on amount of neomycin bound, i.e., that the antibacterial activities of MCX-Zn (II)-Ne, MCX-Fe (III)-Ne and MCX-Cu (II)-Ne lasted for approximately 24, 12, and 4 days, respectively. Consistent with a previous paper,[6] it was noticed that the degree of the decrease in antibacterial activity was not significant on each successive test. Since the phenomena were observed even for free drug, the results suggest that, along with dissociation of neomycin from the complexes, the diffusion of neomycin across the agar media plays a role in prolonging the activity of the complexes. The contribution of diffusion to sustained action may be relevant to the observation that the activity of free Ne lasted longer than MCX-Cu (II)-Ne. However, our data showing that the activity of MCX-Fe (III)-Ne lasted longer than 0.22 mg Ne (equivalent to MCX-Fe (III)-Ne used) strongly suggest that the dissociation of the active agent from the polymer matrix is the predominant factor that sustained the activity of the complexes.

As demonstrated in our data, the polymeric matrix (MCX-metal chelates) is likely to be a polymeric matrix to control the release of many bioactive agents with chelatable groups in them. Considering the physical shape of MCX-metal-bioactive

agent complexes and biodegradability of cellulose, the polymeric bioactive agents may be applicable to aqua- and agriculture, where they can be used to release antibiotics, pesticides or herbicides in a controlled manner, thus reducing the frequency of administration and the fluctuation of chemical concentration, improving environmental side effects and bioactivity.[11,12]

Abbreviations

MC: Microcrystallized cellulose, MCX: Microcrystallized cellulose xanthate, MCX-metal-Ne: Microcrystallized cellulose xanthate-metal-neomycin complex, Ne: Neomycin.

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