In vitro RELEASE OF CHLORAMPHENICOL FROM POLY[N-(2--HYDROXYPROPYL)METHACRYLAMIDE] CARRIERS BY CATHEPSIN B

Karel ULBRICH[a], Olga NAZAROVA[a], Eugenii PANARIN[a],
Miroslav BAUDYŠ[c] and Michail V. SOLOVSKII[b]

[a] *Institute of Macromolecular Chemistry,*
Czechoslovak Academy of Sciences, 162 06 Prague, Czechoslovakia,
[b] *Institute of Macromolecular Compounds,*
Academy of Sciences of the U.S.S.R., 199 004 Leningrad, U.S.S.R. and
[c] *Institute of Organic Chemistry and Biochemistry,*
Czechoslovak Academy of Sciences, 166 10 Prague, Czechoslovakia

It has been shown in recent years that copolymers of N-(2-hydroxypropyl)methacrylamide may be used as targeted polymer drug carriers. The wide-spectrum antibiotic chloramphenicol has been bound to these carriers by means of biodegradable oligopeptidic sequences. The rate of drug release from the carrier in aqueous buffer solutions pH 7·4 and 6·0 was measured and the rates were compared with that of the enzymatic drug release from the carrier by means of the enzyme Cathepsin B. It was shown that the active drug may be released from the carrier by simple hydrolysis or by acting with an enzyme and that the rate of drug release depends on the structure of the oligopeptidic sequence which acts as a link between the drug and the polymer. The results obtained may be employed in the synthesis of a polymer compound potentially possessing anti-microbial activity.
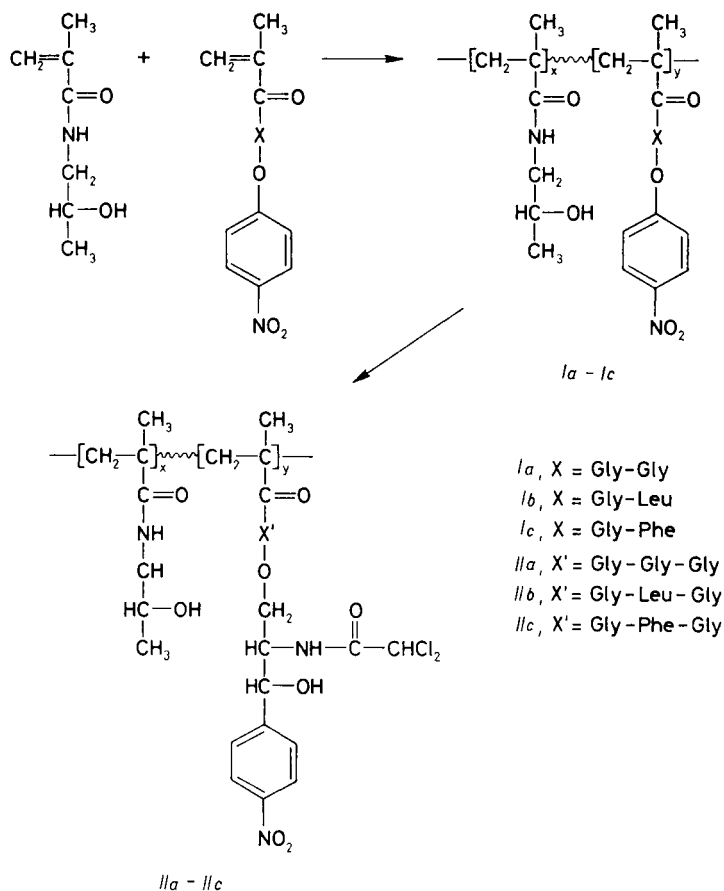
Copolymers of N-(2-hydroxypropyl)methacrylamide are water-soluble polymers which may be used as carriers of biologically active compounds[1]. These compounds — drugs — may be bound to the carriers through "spacers" consisting of oligopeptidic sequences which are relatively stable in the blood plasma and blood serum[2] and which may be degraded by lysosomal enzymes, isolated (Cathepsin B (ref.[3]), Cathepsin H and L (ref.[4]), or present in a mixture (Tritosomes[5,6]).

By binding drugs to a high-molar mass carrier, it is possible to modify their properties to an important extent[7], in the first place, solubility, duration of their stay in the organism, and mechanism of penetration into cells; quite often, too, toxicity is considerably reduced and side effects on the organism are suppressed. Moreover, the use of N-(2-hydroxypropyl)methacrylamide copolymers as the carrier enables the drug to be released in its active form, not during its transport through the organism, but only after it has penetrated into the target cell and interacted with intracellular enzymes.

By using the biodegradable oligopeptidic sequence, a number of drugs have

already been bound to the poly[N-(2-hydroxypropyl)methacrylamide] carriers, such as antibiotics ampicilin and aminopenicilanic acid[8], cancerostatics bis(2-chloro-ethyl)amine[9], sarcolysine[10], daunomycin and puromycin[11,12], adriamycin and the like.

The objective of this study has been to bind a wide-spectrum antibiotic, possessing intracellular activity and inhibiting the protein synthesis, chloramphenicol (ChP), i.e. D(−)-threo-2,2-dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)]-ethylacetamide, to the polymer carriers mentioned above by means of a biodegradable oligopeptidic sequence through an ester bond (Scheme 1) and to investigate the effect of changes in the structure of the bond and sequence used on the rate of drug release from the carrier brought about by simple and enzyme-catalyzed (Cathepsin B)

Ia, X = Gly–Gly
Ib, X = Gly–Leu
Ic, X = Gly–Phe
IIa, X' = Gly–Gly–Gly
IIb, X' = Gly–Leu–Gly
IIc, X' = Gly–Phe–Gly

SCHEME 1

hydrolysis. In the case of promising results (biodegradable bond, sufficient rate of drug release, higher solubility of the samples), the binding procedure should be used in the preparation of a polymer analog of chloramphenicol (chloramphenicol bound to the polymer) in an amount sufficient for biological testing.

EXPERIMENTAL

Chemicals

Amino acids (L-configuration), N-(tert-butoxycarbonyl)glycine (BOC-Gly-OH)*, 2,2'-azobis(iso-butyronitrile) (AIBN), dimethyl sulfoxide (DMSO), methacryloylchloride, 1-amino-2-propanol, dicyclohexylcarbodiimide (DCC) were from Fluka AG, Buchs, Switzerland. AIBN was re-crystallized from ethanol, DMSO was dried on a molecular sieve and rectified under N_2 on a column packed with Halipack at 55°C/533 Pa. The other chemicals were used in the required purity.

Monomer N-(2-hydroxypropyl)methacrylamide (HPMA)[13] and comonomers 4-nitrophenyl N-methacryloylglycyl-glycinate[14], 4-nitrophenyl N-methacryloylglycyl-leucinate[14], and 4-nitro-phenyl N-methacryloylglycyl-phenylalaninate[14] were prepared as described earlier.

Polymer Precursors

Copolymers *Ia—Ic* were prepared by the radical precipitation copolymerization (24 h) of HPMA with 4-nitrophenyl ester comonomers (Scheme 1) initiated with AIBN in acetone at 50°C (12·5 mass % monomers, 0·6 mass % AIBN related to the solution)[14]. The content of respective comonomer units in the copolymer was determined by UV spectroscopy from the absorption of ONp groups[15]: 9·0 mole % in *Ia*, 7·6 mole % in *Ib*, 9·9 mole % in *Ic*.

Molar masses of the copolymers were determined after the aminolysis of precursors with 1-amino-2-propanol by GPC analysis in a 1·6 × 90 cm column packed with Sepharose 4B + 6B (1 : 1), buffer 0·05M-tris(hydroxymethyl)methylamine + 0·5M-NaCl, pH 8·0, flow rate 11 ml/h.

Chloramphenicol Ester of Glycine (Gly-ChP)

The ester was prepared by reacting 2·16 g BOC-Gly-OH (12·3 mmol) with 4·0 g ChP (12·4 mmol) in 40 ml of tetrahydrofuran in the presence of 2·6 g DCC (12·6 mmol) at −5°C. After stirring for 2 h at −5°C the temperature was raised to 20°C and the stirring continued for 8 h. The mixture was cooled to 4°C, dicyclohexylurea that separated from solution was filtered off with suction and the solvent was evaporated to dryness. The product was purified in a column 1·7 × 60 cm, packing Kieselgel 60 (Fluka AG), eluent an acetone/hexane mixture 2 : 1, injected quantity 500 mg/10 ml. After evaporation of the solvent an oily product was obtained, yield 50%. For $C_{18}H_{23}Cl_2N_3O_8$ (480·43) calculated: 45·01% C, 4·83% H, 14·78% Cl, 8·75% N; found 45·88% C, 5·12% H, 13·98% Cl, 8·45% N, R_F (acetone : hexane 2 : 1) 0·80.

The BOC protecting group was deblocked from the aminogroup of glycine by using a 20% HCl solution in dry methanol (25°C, 1 h). After evaporation of the solvent and drying in vacuo over P_2O_5 the oily product HCl.H·Gly-ChP was used directly for binding to the polymer carrier.

---

* The nomenclature and symbols of the amino acids, amino-acid residues and protecting groups obey the IUPAC–IUB recommendation.

Binding of Chloramphenicol to the Carrier

The chosen polymer precursor *Ia—Ic* and HCl.H-Gly-ChP in the molar ratio 1 : 1·1 were dissolved in DMSO to a 15% solution (by mass), a molequivalent of triethyl amine (with respect to HCl.H-Gly-ChP) was added at 25°C with stirring, and the mixture was left to react for 24 h. The unreacted ONp groups were removed by adding 1-amino-2-propanol, and the polymer analog of chloramphenicol was precipitated into acetone. Reprecipitation of polymer drug from methanol into acetone.

The polymer drug was purified by gel filtration in methanol on a column 3×40 cm (Sephadex LH 20). After evaporation of the solvent the product was dried by lyophilization from an aqueous solution. The amount of chloramphenicol bound to the carrier (Table I) was determined by elemental analysis (Cl content) and checked by UV spectrophotometry in DMSO, 278 nm, molar absorption coefficient $8\,900\ 1\,\text{mol}^{-1}\,\text{cm}^{-1}$. The polymer did not contain free chloramphenicol (detection by GPC, column 1·30 cm Sephadex G-15, UV detection at 280 nm, eluent 0·2M-phosphate buffer pH 6·0, flow rate 0·45 ml/min.

Drug Release from the Carrier

The amount of chloramphenicol and its derivatives released hydrolytically from the carrier in various time intervals was determined by GPC on columns packed with Sephadex G-15 (cf. above). The contents of the particular components were calculated from the areas of the respective peaks.

The hydrolytic stability was investigated in 0·2M-phosphate buffers having pH 6·0 or 7·4 with 0·001M-ethylenediaminetetraacetic acid (EDTA) added, 37°C, polymer concentration $1\,.\,10^{-3}$ mol $.\,1^{-1}$ (related to the bound chloramphenicol).

Enzymatic hydrolysis. The hydrolysis took place in an aqueous solution of Cathepsin B (3.4.22.1) isolated from bovine spleen[16]. The molar content of active sites was determined by titration of the latter with ^{14}C iodoacetic acid[16]. The enzyme used contained 37% of active protein. The polymer concentration in solution was $1\,.\,10^{-3}$ mol 1^{-1} (related to ChP), 37°C, 0·2M-phosphate buffer pH 6·0 with 0·001M-EDTA and 0·005M-reduced glutathione (GSH) added. Concentration of Cathepsin B was $1·9\,.\,10^{-7}$ mol 1^{-1}. Enzymolysis was stopped by adding leupeptine as inhibitor. Evaluation by GPC.

TABLE I

Characterization of HPMA copolymers containing bound chloramphenicol

Polymer drug	Spacer unit	M_w	M_n	Drug content	
				mole %	wt. %
IIa	Gly-Gly-Gly	17 000	13 000	1·9	3·9
IIb	Gly-Leu-Gly	19 000	13 000	2·9	5·6
IIc	Gly-Phe-Gly	17 000	13 000	4·4	8·1

RESULTS AND DISCUSSION

Up to now, compounds (drugs or their models) bound to poly[N-(2-hydroxypropyl)-methacrylamide] have been those which contained a primary amino group in their molecule, i.e., they are bound to the carrier through an amide bond. It has been shown that the amide bond is hydrolytically (uncatalyzed hydrolysis) stable in an aqueous medium in the pH range 5·5−8·0 and that it can be degraded depending on the structure of the spacer used (the part of which the amide bond is) only by acting with enzymes possessing peptidase activity, which also involved Cathepsin B.

In this study polymer samples were prepared in which a drug, antibiotic chloramphenicol, was bound to the poly[N-(2-hydroxypropyl)methacrylamide] carriers by means of the oligopeptidic sequence through an ester bond (Scheme 1). The objective of the study was to compare the properties of polymer compounds in which the drug was bound to the carrier through an amide or ester bond, and in the case of degradability of the ester bond to prepare a major amount of polymer bound chloramphenicol for further biological testing.

As can be seen in preceding papers[4,9], carriers containing the spacers Gly-Leu-Gly and Gly-Phe-Gly allow the drug model to be bound to the polymer by a comparatively strong bond in blood serum, which can be readily degraded with Cathepsin B or with a mixture of lysosomal enzymes, Tritosomes. The spacer consisting of glycine units only is degraded very slowly. In all the cases reported above the drug model is bound to the carrier through an amide bond which is stable in the aqueous medium between pH 5·5 and 8·0.

The hydrolytic stability of polymer samples *IIa−IIc* prepared by us was investigated in buffers modelling pH of the medium in a living organism (blood, cells). The results of measurements are summarized in Table II. It is obvious that in a mildly acid medium (pH 6·0) the drug is released from the carrier only very slowly: within 24 h, 8% of the originally bound chloramphenicol is released at most. On the other hand, however, in a mildly alkaline medium (pH 7·4) the rate of noncatalyzed hydrolysis quickly increases, especially in those samples in which chloramphenicol is bound through a spacer containing phenylalanine and leucine. These polymers release approximately one third of the total bound drug within 24 h. The results of GPC measurements showed that in this case chloramphenicol itself is released, not its derivatives, consequently, the ester bond between the last amino acid of the spacer and chloramphenicol is hydrolyzed. The results show that, unlike the hydrolytically stable amide bond, the ester bond is hydrolytically labile, more in buffers modelling the blood stream and less in buffers modelling the medium inside the cells.

If *IIa−IIc* are incubated in buffers pH 6·0 with Cathepsin B (Fig. 1a), the rate of drug release from the carrier is considerable compared with the noncatalyzed hydrolysis. Polymer *IIa* releases the drug more slowly due to the nonadvantage-
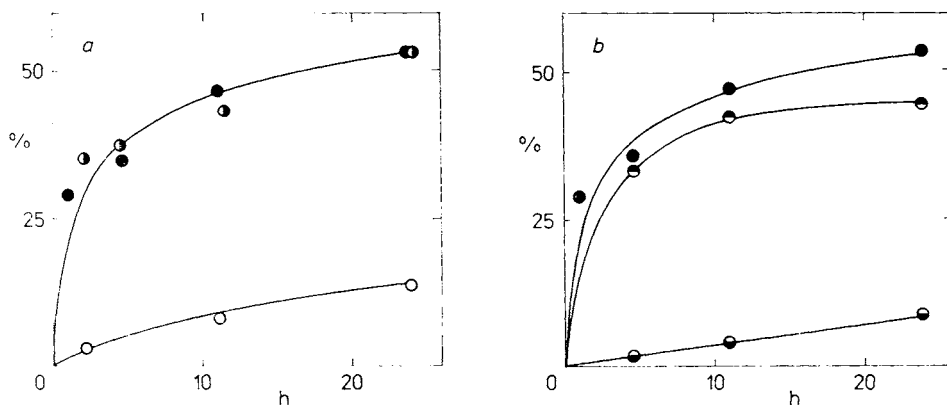
FIG. 1

Enzymatic release of chloramphenicol (ChP in mass % with respect to the total bound amount) from polymer drugs (Cathepsin B concentration $1·9 . 10^{-7}$ mol 1^{-1}, 37°C, 0·2M-phosphate buffer pH 6·0, 0·001M-EDTA, 0·005M-GSH). a Release of ChP from polymers ○ IIa, ◑ IIb, ● IIc. b Release of ChP from IIc: ◓ in the form of glycine derivative, ◔ free ChP, ● total amount
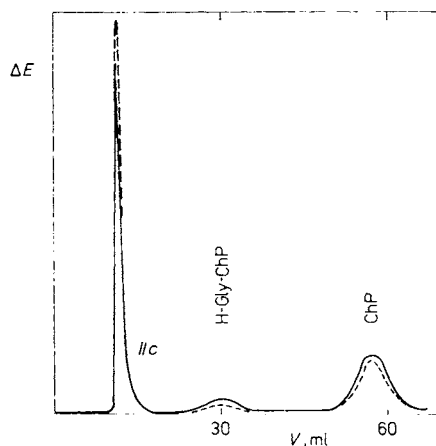

FIG. 2

GPC record of polymer IIc after incubation with Cathepsin B (37°C, 0·2M-phosphate buffer pH 6·0, enzyme concentration $1·9 . 10^{-7}$ mol 1^{-1}, incubation time 11 h and 24 h). Column 1·30 cm, Sephadex G-15, UV detection at 280 nm, 0·2M-phosphate buffer pH 6·0, flow rate 0·45 ml/min

ous[3] structure of the spacer used in binding. The results also demonstrate that the rate of enzymatic drug release from the carrier does not depend too much on the kind of spacer used in binding. With Gly-Phe-Gly as the spacer (sample *IIc*), however, not only chloramphenicol, but to a certain extent its glycine derivative (ester) is released (Fig. 1*b*), i.e. partial enzymatic degradation occurs also inside the oligo-peptidic sequence. The GPC record of the polymer prior to degradation has only a polymer peak, while the GPC record of sample *IIc* during the degradation contains both a decreasing polymer peak and increasing peaks of chloramphenicol and of its glycine derivative (Fig. 2). In sample *IIb* the glycine derivative of chloramphenicol is released to a very small extent only (1·5% in 24 h), in other words, by an order of magnitude more slowly than this is the case with sample *IIc*. If we compare the rate of drug release from sample *IIb* with the rate of release of the drug model bound by the amide bond from the carrier of the same structure and by using the same enzyme[9] (Cathepsin B), we can see that the rates are very close to each other, particularly bearing in mind that in the case of the ester bond we have the sum of purely hydrolytic and enzymatic degradation (47% of the released compound in the case of the amide bond[9] and 53% of the compound in the case of the ester bond within 24 h). From sample *IIb*, 3% of the compound was released by hydrolysis under the same conditions, but in the absence of enzyme (Table II).

CONCLUSION

Three types of polymer derivatives of chloramphenicol were prepared, in which the antibiotic is bound by means of a biodegradable bond to the poly[N-(2-hydroxy-propyl)methacrylamide] carrier. Measurements of the hydrolytic release of chloramphenicol from the polymer carrier (hydrolysis only in buffers and enzymatically catalyzed hydrolysis) have led to a conclusion that the poly[N-(2-hydroxypropyl)-methacrylamide] carrier can be used in binding chloramphenicol: the carrier not only raises considerably the solubility of the drug in water, but also allows chlor-

TABLE II

Amount of hydrolytically released chloramphenicol (mass %) from polymers *IIa—IIc* in 0·2M--phosphate buffer pH 7·4 and 6·0 at 37°C

Time h	*IIa*		*IIb*		*IIc*	
	pH 7·4	pH 6	pH 7·4	pH 6	pH 7·4	pH 6
2·25	3	1	8	0	9	4
24	17	8	37	3	27	6

amphenicol to be released from the carrier at a rate depending on the structure of the oligopeptidic spacer between the drug and the polymer. The samples investigated in the study were submitted to biological testing.

REFERENCES

1. Duncan R., Kopeček J.: Adv. Polym. Sci. *57*, 51 (1984).
2. Rejmanová P., Kopeček J., Duncan R., Lloyd J. B.: Biomaterials (Guildford, England) *6*, 45 (1985).
3. Rejmanová P., Kopeček J., Pohl J., Baudyš M., Kostka V.: Makromol. Chem. *185*, 2009 (1983).
4. Šubr V.: *Thesis*. Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Prague 1986.
5. Duncan R., Cable H. C., Lloyd J. B., Rejmanová P., Kopeček J.: Makromol. Chem. *184*, 1997 (1983).
6. Šubr V., Kopeček J., Duncan R.: J. Bioact. Compat. Polymers *1*, 133 (1986).
7. Gros L., Ringsdorf H., Schupp H.: Angew. Chem. *93*, 311 (1981).
8. Solovskii M. V., Ulbrich K., Kopeček J.: Biomaterials (Guildford, England) *4*, 44 (1983).
9. Ringsdorf H., Schmidt B., Ulbrich K.: Makromol. Chem. *188*, 257 (1987).
10. Ulbrich K., Zacharieva E. I., Kopeček J., Hume I. C., Duncan R.: Makromol. Chem. *188*, 2497 (1988).
11. Duncan R., Kopečková P., Strohalm J., Hume I. C., Cable H. C., Pohl J., Lloyd J. B., Kopeček J.: Br. J. Cancer *55*, 165 (1987).
12. Duncan R., Kopečková P., Strohalm J., Hume I. C., Lloyd J. B., Kopeček J.: Br. J. Cancer, *57*, 147 (1988).
13. Strohalm J., Kopeček J.: Angew. Makromol. Chem. *70*, 109 (1978).
14. Kopeček J.: Makromol. Chem. *178*, 2169 (1977).
15. Rejmanová P., Labský J., Kopeček J.: Makromol. Chem. *178*, 2159 (1977).
16. Pohl J., Baudyš M., Tomášek V., Kostka V.: FEBS Lett. *142*, 23 (1982).

Translated by L. Kopecká.