SYNTHESIS OF POLY[N^5-(2-HYDROXYETHYL)-L-GLUTAMINES] WITH MODIFIED SIDE CHAIN*

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The procedure for synthesis of water-soluble polymers on the basis of poly[N^5-(2-hydroxyethyl)-L-glutamine] was developed. It allows binding of various groups of interest (reactive spacers, biologically active compounds, tracers etc.) to these polymers using side chains modifications. A multi-step procedure involves partial debenzylation of poly(γ-benzyl-L-glutamate), conversion of resulting carboxyl groups to reactive succinimido ester groups, which in turn are aminolyzed by the compound of interest. The final step of the synthesis, viz., aminolysis of residual benzyl ester groups with 2-aminoethanol, leads to water-soluble modified poly[N^5-(2-hydroxyethyl)-L-glutamines].

The recent development in novel drug delivery systems calls for new materials specifically serving a particular purpose. Synthetic polymers have a great potential in this respect, because their structure, physicochemical properties and drug-binding capabilities can be modulated by a rationally designed synthesis. On the other hand, lack of biodegradability of many synthetic polymers limits the spectrum of materials which can be safely used for parenteral applications in humans. Synthetic polymers of amino acid derivatives represent a group of materials in which both requirements, i.e., possibility for a rational synthesis as well as a potential for controlled biodegradability, can be combined[1,2]. Poly[N^5-(2-hydroxyethyl)-L-glutamine] (PHEG) and poly[N^5-(2-hydroxypropyl)-L-glutamine] are hydrophilic, neutral and water-soluble polymers. In our previous paper it has been shown that they are degradable in the main chain essentially down to the level of monomer units by either isolated enzymes or homogenates of living tissues[3]. The objective of the present paper is to suggest a method yielding modified poly[N^5-(hydroxyalkyl)glutamines] carrying various functional groups, e.g., drugs, reactive groups, tracer groups etc. in side chains. Poly[N^5-(hydroxyalkyl)glutamines] are usually prepared by the aminolysis of γ-alkyl ester groups of polyglutamates with the corresponding hydroxyalkylamines, where amine is being used in great excess[4,5]. Such a direct aminolysis is unconvenient for the binding of most drugs and other compounds of interest. Therefore, the feasi-

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bility of a partial replacement of γ-alkyl ester groups by more reactive γ-succinimido ester groups and the use of the latter for binding of various side chains was investigated.

EXPERIMENTAL

Materials

4-(2-Aminoethyl)phenol (tyramine, Trn), dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (HONSu) and HBr (33% in acetic acid) were purchased from Fluka AG, N,N-dimethylformamide (DMF) was dried by azeotropic distillation with benzene, and rectified. Sodium methoxide was prepared by the reaction of sodium with methanol. 6-(3-Aminopropionamido)-fluorescein (F) and 2-(4-aminobenzamido)ethylamine monohydrochloride (ABEA.HCl), were prepared according to ref.[6] and ref.[7] respectively.

N-(6-aminohexyl)-2,4-dinitroaniline (ADNA) was prepared by a reaction of 2,4-dinitrofluorobenzene (18·6 g, 0·1 mol) with 1,6-diaminohexane (232·4 g, 2 mol) in ethanol (2 800 ml). The reaction mixture was acidified to pH 4·0 by 6M-HCl, extracted with chloroform, and the crude product (hydrochloride) was obtained from the oily aqueous layer. Recrystallization from ethanol (2×) yielded 20·9 g (65·6%) of ADNA.HCl, m.p. (Kofler, uncorrected) 167–168·5°C. For $C_{12}H_{19}ClN_4O_4$ calculated: 45·21% C, 6·01% H, 17·58% N, 11·12% Cl; found: 45·00% C, 6·31% H, 16·42% N, 10·86% Cl.

Poly(γ-benzyl-L-glutamate) (PBG) was prepared by the polymerization of γ-benzyl-L-glutamate N-carboxyanhydride (for its synthesis cf. ref.[8]) in 1,4-dioxane with sodium methoxide as initiator (monomer concentration: 0·1 mol l^{-1}; monomer/initiator ratio: 200/1).

Modification of PBG

Partly debenzylated poly(γ-benzyl-L-glutamate) (PBG(OH)), i.e., poly(γ-benzyl-L-glutamate-*co*-L-glutamic acid), was prepared by mixing 9 parts of a benzene solution of PBG (1 g in 180 ml) with one part of 33% HBr in glacial acetic acid. The reaction was carried out at 30°C and terminated by precipitation of the polymer in diethylether. The precipitate was washed with ethanol, dissolved in 1,4-dioxane, and freeze-dried. For kinetic measurements, samples of the reaction mixture described above were withdrawn at appropriate time intervals, the polymer was isolated as mentioned above, and the content of carboxylic groups was determined by the titration with sodium methoxide in benzene–methanol (85 : 15, v/v).

Poly(γ-benzyl-L-glutamate) with succinimido ester groups (PBG(ONSu)), i.e. poly(γ-benzyl-L-glutamate-*co*-γ-succinimido-L-glutamate) was prepared by a modified procedure described in ref.[9]. The solution of PBG(OH) in DMF was mixed with DMF solutions of DCC and HONSu in order to form a reaction mixture containing 60 mg of PBG(OH) per one ml of DMF and 2 moles of DCC and HONSu per one mole of PBG(OH) carboxylic groups. The mixture was stirred for 24 hours at ambient temperature and the resulting precipitate of dicyclohexyl urea was removed by centrifugation. The polymer in solution was either used in further reactions without isolation, or it was isolated by precipitation in diethylether and then freeze-dried from 1,4-dioxane.

Modified poly(γ-benzyl-L-glutamates), i.e. copolymers of γ-benzyl-L-glutamate with a L-glutamine, PBG(ADNA), PBG(Trn), PBG(F) and PBG(ABEA), were prepared by the aminolysis of succinimido ester groups of PBG(ONSu) with ADNA, Trn, F and ABEA, respectively. The

reaction was carried out in DMF at room temperature. The ratios of reactants are indicated in Table I. The time course of the succinimido esters aminolysis was measured as follows: 15 mg of PBG(ONSu) in 0·5 ml of DMF was reacted with ADNA.HCl in an amount equimolar with respect to the amount of succinimido ester groups. The reaction was carried out at 22°C in the presence of an equivalent of triethylamine. The reaction was terminated by adding a sample of the reaction mixture to the 1,4-dioxane–acetic acid (5 : 1, v/v) mixture. The polymer bound ADNA and the unbound low-molecular-weight ADNA were separated by GPC in a Sephadex-LH 20 column in dioxane–acetic acid. Their ratio was determined from the areas under the corresponding peaks of the elution curve monitored at 359 nm.

Water-soluble modified poly[N^5-(2-*hydroxyethyl*)-L-*glutamines*] were prepared by a reaction of the corresponding modified poly(γ-benzyl-L-glutamates) with 2-aminoethanol. Three procedures were tested. Procedure without solvent (A): 1·5 ml of 2-aminoethanol per 100 mg of the polymer. Procedure with DMF as a solvent (B): 1·5 ml of 2-aminoethanol was added to 1·5 ml of DMF solution containing 100 mg of the polymer. One-step procedure (C): 2-aminoethanol was added in the same ratio as above to the reaction mixture after the aminolysis of succinimido esters without isolation of the polymer. In all cases the reaction was carried out for 48 hours at 60°C. The reaction mixture was then neutralized with 15% acetic acid, dialyzed against diluted acetic acid (Visking Dialysis Tubing, Serva), and the polymer was purified by GPC in a Sephadex G-25 column in water. All water-soluble polymers were isolated by freeze-drying.

Measurements

Spectrophotometry was used for the estimation of the tyramine, ADNA, ABEA and fluoresceine content in modified polymers. Free carboxyl groups of water-soluble polymers were determined by titration with 0·01M-NaOH.

Molecular weight distribution of all water-soluble polymers was determined by GPC; molecular weight averages were calculated from the GPC data as described previously[3].

RESULTS AND DISCUSSION

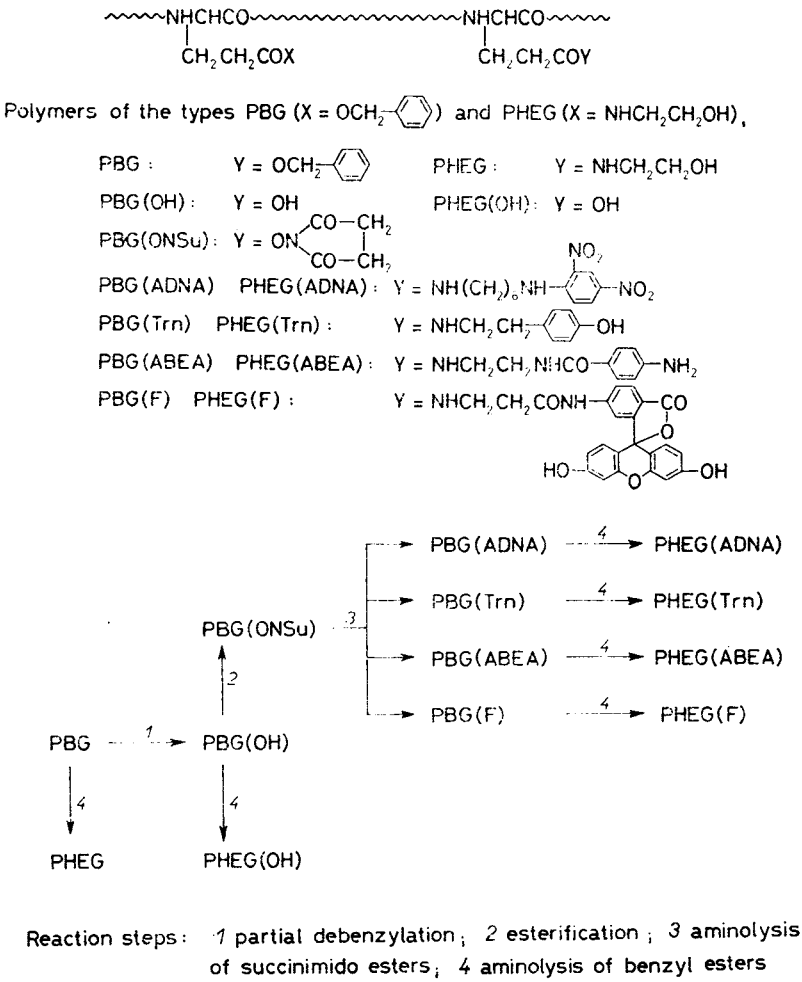
Derivatives of poly[N^5-(2-hydroxyethyl)-L-glutamine] were prepared from PBG by polymer-modification reactions. These reactions include the partial debenzylation

TABLE I

Reaction of poly(γ-benzyl-L-glutamate-*co*-γ-succinimido-L-glutamate) (1 mol of succinimido ester groups) with modificators (DMF, room temperature) in the presence of triethylamine (TEA)

Modificator		TEA mol	Polymer product
type	mol		
N-(6-Aminohexyl)-2,4-dinitroaniline	2	2	PBG(ADNA)
4-(2-Aminoethyl)phenol (tyramine)	0·5	0	PBG(Trn)
6-(3-Aminopropionamido)fluorescein	0·5	1	PBG(F)
2-(4-Aminobenzamido)ethylamine	0·5	0·5	PBG(ABEA)

of PBG and the conversion of the resulting free carboxyl groups to reactive succinimido esters, which in the following step were used for binding of amino compounds by aminolysis. After that, the total aminolysis of residual benzyl ester groups with 2-aminoethanol yielded water-soluble poly[N^5-(2-hydroxyethyl)-L-glutamines] with modified side chain, i.e., copolymers of N^5-(2-hydroxyethyl)-L-glutamine with a different L-glutamine (Scheme 1).



SCHEME 1

The partial debenzylation of PBG in benzene with HBr solution in acetic acid is a well reproducible reaction, the conversion of which, i.e., the content of carboxyl groups in the product, can be readily controlled by the reaction time. The reaction

kinetics (Fig. 1) is more complex than that of the pseudo-first order. Contrary to Nakajima et al.[10], we did not observe an acceleration of the reaction with conversion. It is possible that acetic acid present in the reaction mixture in our case affects the helical conformation of PBG in benzene, and consequently modifies the reactivity of polymer side chains. The effect of the polymer conformation on the reactivity of side chains has been observed in the aminolysis of poly(γ-alkylglutamates)[11], and it was also assumed in the case of debenzylation reaction[10].

The results of polymer modification are summarized in Table II. The effective degree of PBG(OH) activation, i.e., a conversion of carboxyl groups to succinimido ester groups, was determined from the amount of ADNA, which can be bound to the polymer by the aminolysis of the activated polymer, PBG(ONSu), with ADNA, thus producing a polymer product PBG(ADNA). It can be perceived that the carboxyl groups were activated virtually with conversion approaching 100%. When the aminolysis followed immediately, without isolation of the activated polymer, the degree of modification and the content of carboxyl groups of the starting polymer PBG(OH) were in a good accord. The isolation of PBG(ONSu) and its redissolution usually resulted in some loss of NSu esters. In a typical example, the yield of modification reached 85–90%. On the other hand, it is advantageous that the activated polymer, once isolated, can be stored in a dry state for a period of several months without further loss of the reactive groups, being thus available for modification.

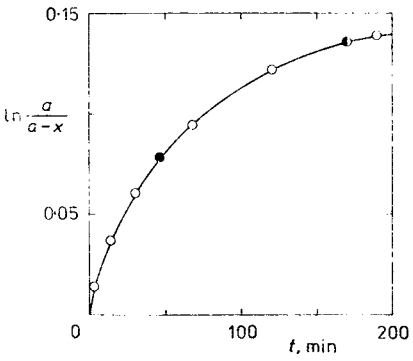


FIG. 1

Debenzylation of PBG (5 g) in benzene solution (900 ml) with HBr (100 ml 33% HBr in glacial acetic acid) at 30°C (different points represent three independent experiments). Molar amounts of groups: a initial benzyl ester, x resulting carboxyl

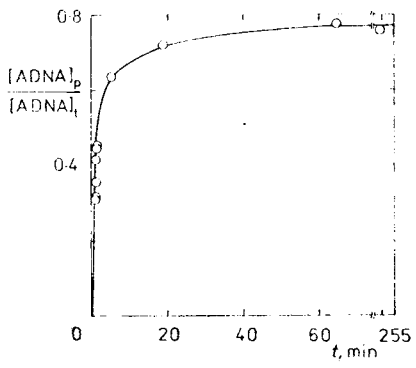


FIG. 2

Aminolysis of PBG(ONSu) with model chromophore ADNA at 22°C. System: 0·5 ml of polymer solution in DMF (30 g l^{-1}), equimolar amounts of ADNA and TEA (both amounts related to succinimido groups). Concentrations of ADNA: $[ADNA]_p$ polymer-bound, $[ADNA]_t$ total in the reaction mixture

The reactivity of succinimido esters towards aliphatic amines is made clear by the time course of the aminolysis with ADNA (Fig. 2). The reaction at 22°C is practically completed within 20 minutes. Under the conditions adopted for measurements, the yield of the reaction approached 75%. For preparative purposes the modification yield can be increased by dissolving the activated polymer in a reaction mixture which already contains the amine and by using the latter in amounts higher than equimolar with respect to the amount of succinimido ester groups. The molecular weight of modified poly(γ-benzyl-L-glutamates), i.e., copolymers of γ-benzyl-L--glutamate with a L-glutamine, controlled by GPC in non-aqueous media, was almost identical with that of the original PBG. This suggests that no significant degradation in the polymer main chain occurs during the operations discussed above.

In order to obtain modified water-soluble poly[N^5-(2-hydroxyethyl)-L-glutamines] the modified poly(γ-benzyl-L-glutamate) were reacted with 2-aminoethanol. The aminolysis of ester bonds in side chains was always accompanied by splitting of

TABLE II

Characterization of polymers (see Scheme 1). Procedures of aminolysis with 2-aminoethanol (A, B, C) see Experimental. Symbols: y content of modified side chain with Y grouping different from X (see Scheme 1), z content of residual carboxyl groups. Abbreviation n.e. means not estimated

Polymer	Procedure	Yield %	y	z	M_n	M_w
			mole %			
PBG	—	—	—	—	n.e.	n.e.[a]
PBG(OH)	—	99	7·5	—	n.e.	n.e.
PBG(ONSu)	—	—[b]	7·5	—	n.e.	n.e.
	—	75	6·6	n.e.	n.e.	n.e.
PBG(ADNA)	—	90[b]	7·5	—	n.e.	n.e.
	—	90	6·6	n.e.	n.e.	n.e.
PHEG(ADNA)	A	50	n.e.	1·5	28 800	56 600
	B	24	2·6	1·0	7 300	12 500
	C	51	3·3	0·7	21 600	79 700
PHEG(Trn)	C	57	3·5	1·2	24 200	44 800
PHEG(F)	C	30	2·5	n.e.	17 500	49 000
PHEG(ABEA)	C	71	1·9	1·4	9 800	18 600
PHEG	A	69	—	—	37 000	83 000
	B	37	—	—	8 700	16 400
PHEG(OH)	A	70	6·5	—	43 400	95 400

[a] M_η = 300 000 (dichloroacetic acid, 25°C); [b] without isolation of the activated polymer.

some amide bonds forming the main polymer chain. As can be seen from different values of molecular weight of the individual polymers, the degradation extent is probably affected by several factors: the presence or absence of solvent, the quality of modifying groups in the polymer side chains, and, in the case of the one-step procedure, by the presence of other reagents remaining in the reaction mixture after the aminolysis of succinimido esters. Although for an exact evaluation of all factors a more detailed study would be needed, from the results in Table II it might be deduced that the decrease of the polymerization degree is more pronounced when the reaction is carried out in the presence of DMF (or N,N-dimethylacetamide) as a solvent. A similar phenomenon was also observed during the aminolysis of PBG with 3-amino-1-propanol in the presence of various solvents[4]. Taking into account that $\overline{M}_n$ decreases to one half by splitting of one bond per average polymer chain, the observed decrease of the polymerization degree corresponded to about 2–4 backbone cleavages per the average polymer chain, i.e., to about 0·2–0·4 mole %. Amide bonds in side chains appear to be thus more susceptible to transamidation with 2-aminoethanol than bonds in the main chain. Fig. 3 shows the time course of the release of ADNA from side chains of PBG(ADNA) during the reaction with 2-aminoethanol. After 48 hours at 60°C, i.e., under the conditions required for the aminolysis of all residual benzyl esters, about 45% of ADNA originally bound to the polymer was released. The rate of this reaction is lower in the presence of free ADNA in the reaction mixture.

The described procedure was developed for polymer modifications with amino compounds soluble in organic solvents. It can be used to produce intermediate side chains which, in turn, are suitable for binding compounds requiring aqueous environment, such as proteins, to the polymer. An example is represented by ABEA, providing the aromatic amine groups, which proved to be a convenient group for the binding of peptides and proteins via the diazotization method[12].

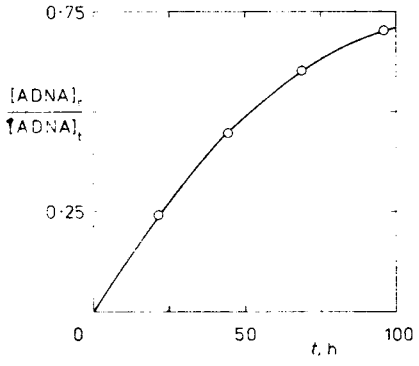


FIG. 3

Release of ADNA from PBG(ADNA) (100 mg) in DMF solution (1·5 ml) by transamidation with 2-aminoethanol (1·5 ml) at 60°C (plotted points are average values from two analyses). Concentrations of ADNA: $[ADNA]_r$ released, $[ADNA]_t$ total in the reaction mixture

In this work ADNA was chosen as drug model with an aliphatic spacer, because of its convenient analytics. The modification of the polymer with tyramine side chains introduces groups which can bind iodine (^{125}I or ^{131}I) as a radioactive tracer. Similarly, the fluorescein derivative can be used for the fluorescence labelling of polymers. Both ways have already been found useful for the investigation of the polymer pharmacokinetics[13]; a modification degree lower than 1 mole % was found to be sufficient for these purposes.

The total aminolysis of the residual benzyl ester groups with hydroxyalkylamines represents the most severe step in the whole procedure, being attended by an undesirable cleavage of the backbone chain and transamidation reaction in side chains. Nevertheless, it is possible to prepare water-soluble polymer products with a defined structure and fairly high molecular weight. The final yield of their modification is more than sufficient for the introduction of groups for various purposes.

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