

THE SYNTHESIS AND CHARACTERIZATION OF POLYPEPTIDE—ADRIAMYCIN CONJUGATES AND ITS COMPLEXES WITH ADRIAMYCIN. PART I

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Poly(α -L-glutamic acid) (PGA) was grafted with amino acid and oligopeptide spacers up to 5 amino acids with the use of N,N'-carbonyldiimidazole and 2,3-dihydro-1,2-benzisothiazole-3-on-1,1-dioxide (saccharin) as an additive, and these polypeptides were characterized. The antitumor antibiotic adriamycin was covalently coupled via an amide bond onto PGA and onto the grafted polymers with the use of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ); these conjugates were characterized. The conjugates containing Gly—Gly—L-Leu spacer arms did yield free adriamycin upon digestion with papain. Adriamycin gave fairly stable complexes with PGA—adriamycin and branched polypeptide—adriamycin conjugates; these complexes were characterized.

INTRODUCTION

Major problems in the administration of cytostatic agents are the lack of specificity of the drug for the tumor cell, the toxic side effects and the development of resistance of tumors for a particular drug. Extracellular polymeric release systems can be applied to maintain constant levels of the drug in a particular body compartment for extended periods of time. Examples of these systems are the well known DNA—anthracycline complexes developed by Trouet et al. [1]. The release of the drug from these systems is at least partially governed by the clearance of plasma from the free drug, which in turn leads to further dissociation of the DNA complexes. In principle, constant levels of the drug can also be obtained by bioerosion of degradable polymer matrices or by extracellular cleavage of covalently bound polymer—drug conjugates.

Although peak concentrations of the drug can be avoided using these systems and a decrease in systemic toxicity can be expected, a more selective administration of the drug would be advantageous. This could be achieved with the use of endocellular release systems, which release the drug after being incorporated by tumor cells. Only after uptake by tumor cells should the conjugates degrade and release the parent drug.

Tumor cells show a higher degree of uptake of macromolecules by endocytosis than their normal counterparts do. After internalization of the polymer—drug conjugate, a phagosome is formed which fuses with a lysosome, and subsequently the polymer—drug conjugate is exposed to a mixture of at least 40 digestive enzymes at an acidic pH (4–5).

Information on the presence and activity of specific enzymes in the lysosomal compartments can be used to design polymer—

drug conjugates in which the drug is coupled to the macromolecular carrier through spacers which can be easily cleaved by the enzymes involved [2]. Macromolecular prodrugs can be designed which, by virtue of the presence of high levels of specific enzymes at the tumor cell membrane (γ -glutamyl transferase) [3] or near the tumor (plasmin), rapidly release the drug at the membrane or in the immediate surroundings of the tumor [4].

Problems which still exist are the competitive uptake of macromolecular prodrugs by endocytosing cells, e.g. RES, which in principle can be avoided by the coupling of appropriate antibodies against the tumor cells onto the polymer—drug conjugate. In this paper we have concentrated on the development of high molecular weight prodrugs of adriamycin using poly(α -L-glutamic acid) as a biodegradable carrier and different peptides as spacers. These systems were characterized with respect to composition and load of adriamycin by spectroscopic and chemical methods.

RESULTS AND DISCUSSION

a. Prodrug design

Polymeric systems of anthracyclines which have been investigated include: DNA (adriamycin [5] and daunomycin [1]); antibodies and fab dimers (daunomycin [6–8] and adriamycin [9]); bovine serum albumin (daunorubicin [10]) and various other proteins (daunorubicin [11]); poly(D-lysine) (daunomycin [9]); polyglutamic acid (adriamycin salt [12]); poly(N^5 -hydroxypropyl-L-glutamine) (adriamycin hydrazone [13]); polyaspartic acid (daunorubicin [14]); lectins (daunomycin [15]); dextran (daunorubicin [13, 16]); polyglutaraldehyde (adriamycin [12]) and a copolymer of 2-(hydroxy)ethyl methacrylate and vinylpyrrolidone (daunorubicin [17]).

Polyglutamic acid (PGA) and derivatives have also been used as carriers for mustard

compounds [18], cyclophosphamide [19], Ara-C [20] and steroid hormones [21–25]. Poly(α -amino acids) were also used as biodegradable matrices for the release of bioactive compounds [26–28]. PGA possesses factor VIII-like coagulant activity [29] and PGA has been tested as a plasma expander [30]. Adriamycin yields a water-insoluble salt with PGA which showed anti-tumor activity and greatly reduced toxicity in mouse mammary experiments [12].

We have concentrated on the use of PGA as a carrier, because this polymer can be readily degraded by lysosomal enzymes, is rather plasma stable and contains sufficient reactive groups for derivatization. After partial derivatization the conjugate will still be water-soluble owing to residual carboxylate groups. PGA is not expected to be toxic in the quantities required for the use as a carrier and can be readily obtained with different molecular weight distributions.

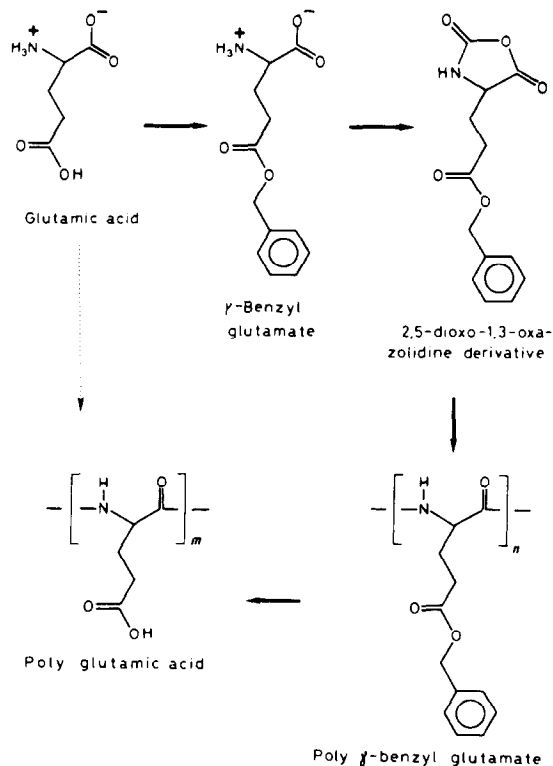
The interpolation of appropriate spacers between carrier and the bioactive compound to meet optimal requirements for lysosomal attack and release of the drug has been applied by several investigators [2, 10, 31]. Thus, Trouet et al. [10] have shown that optimal spacers consisted of tri- and tetra-L-Leu-terminal peptides attached to succinylated ϵ -lysine groups of albumin. Kopecek [2] described the peptide-spacer dependent release of *p*-nitroaniline from *N*-(2-hydroxypropyl)methacrylamide using thiolproteinases (Cathepsin B, L and H) as important representatives of the lysosomal enzyme population. Because both the length and the composition of the spacer are of great importance we have synthesized and characterized a number of conjugates with various spacer arms (1–5 amino acid residues). Tyrosine was incorporated in two of the systems to enable labeling with radioactive tracers which should aid in the study of biodegradation of the conjugate (Table 2).

Adriamycin displays a tendency to self-association in aqueous solution, resulting

in dimers, and even higher oligomers [32, 33] due to the hydroquinone-quinone like structure in the aglycon. It can be expected that free adriamycin might yield relatively strong charge transfer complexes with the covalently bound drug in the conjugates. The application of a negatively charged carrier such as PGA might greatly add to the stability of these complexes.

b. Synthesis and characterization

Polyglutamic acid (PGA) was prepared according to Blout et al. [34-36] (Scheme 1) by triethylamine-initiated polymerization of L-glutamic acid 4-benzyl ester [37] *N*-carboxyanhydride [38] in toluene and subsequent debenzylation of the polymers obtained with the use of hydrogen bromide (Scheme 1). Molecular weights (\bar{M}_v) of the polymers typically ranged from 50,000



Scheme 1. Synthesis of poly(α -L-glutamic) acid.

up to 350,000 daltons. Residual benzyl groups mounted up to 5% (UV spectrometry) (Table 1). Both variables (\bar{M}_v and residual groups) largely depend on the conditions employed during the debenzylation procedure. The material obtained as described (experimental, Table 1) was free of microgel particles and readily dissolved in dimethylformamide.

In our efforts to attach adriamycin directly onto PGA we found that the majority of the well established techniques used in peptide synthesis were not suitable or gave erratic results due to the nature of both the carboxylic component (PGA) and the amine (adriamycin). First, activation of PGA with carbodiimides [39, 40] or Woodward reagent K [41] also gave rise to the incorporation of *N*-acylisourea derivatives, which do not further react with nucleophiles. Secondly, it has been shown [42, 43] that overactivation of lateral carboxylic groups in glutamic and aspartic acid containing peptides may result in the formation of cyclic imides, which, in turn, are likely to isomerise into the corresponding ω -peptides or even may result in chain scission. The latter fragmentation became a serious side reaction with the use of *N,N'*-carbonyl diimidazole (CDI) [44]. Thus, addition of CDI to a stirred

TABLE 1

Molecular weight, \bar{M}_v , and residual benzyl groups of poly(α -L-glutamic acid) (PGA)

Batch	\bar{M}_v^a	Residual benzyl groups ^b (mol %)
PGA ₂	1.3×10^5	5
PGA ₃	3.0×10^4	<0.5
PGA ₄	2.5×10^5	<0.5
PGA ₇ ^c	3.5×10^5	2.5

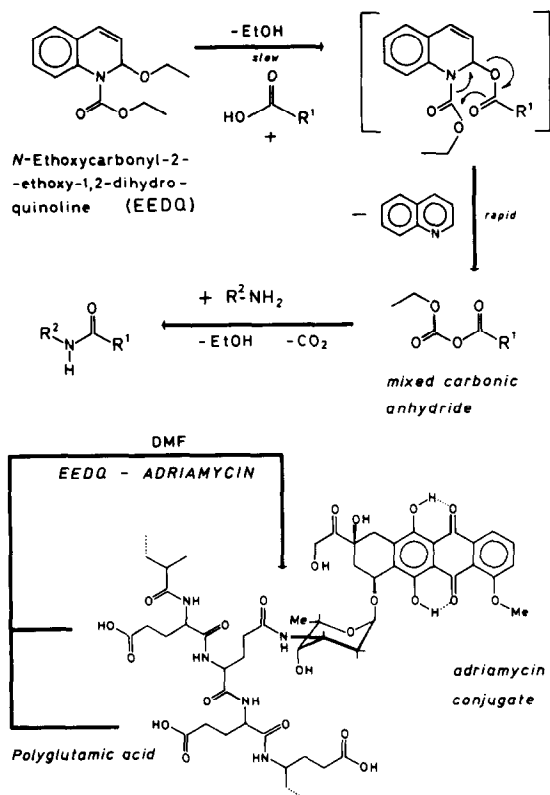
^aDetermined by viscometry using the relation $[\eta] = 3.13 \times 10^{-3} (\bar{M}_v)^{0.965}$ [50-52].

^bDetermined by UV spectrometry using γ -benzyl-L-glutamate as a reference compound.

^cDeprotection was accomplished by transfer hydrogenation of poly- γ -L-benzylglutamate [53].

solution of PGA in dimethylformamide resulted in a rapid decrease in viscosity and poor recoveries of PGA after dialysis. Moreover, in view of the polyfunctionality of adriamycin (amine, ketone, quinone, hydroquinone, phenolic OH, primary, secondary and tertiary OH and the sensitive 2-deoxyglycoside acetal) the conditions of coupling of the drug onto the polymer must be compatible with the structure elements present. This implies that the great majority of methods for the establishment of covalent bonds, including those based on oxidation-reduction reactions [45], is not suitable and lays a number of restrictions during coupling and work-up of products. Selective coupling of the amino group in the daunosamine moiety without concomitant formation of esters also greatly

reduces the number of methods available. We have found that the use of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) [46, 47] did meet our requirements. This compound is known to react with carboxylic acid groups with the formation of mixed carbonic anhydrides (Scheme 2). However, we observed that these mixed anhydrides disproportionate into symmetrical acid anhydrides. Thus, addition of EEDQ to a solution of PGA in dimethylformamide gave rise to a solid transparent gel within 30 min due to the formation of acid anhydride crosslinks. These crosslinks are hydrolysed by water, which ultimately results in redissolution of the gels. In the presence of equimolar amounts of adriamycin, these gels are not formed. This observation provides additional chemical evidence for the formation of both symmetrical acid anhydrides and the amides anticipated. A slight excess of EEDQ in the reaction mixture also gave rise to the formation of transparent gels after 24 h at which time virtually all of the drug has been incorporated into the polymer. However, the crosslinks present in these gels are much more resistant towards hydrolysis than those of the acid anhydride type. It was concluded, that these crosslinks were, at least partly, of an ester-type nature. It was found that these ester bonds are selectively hydrolysed in the course of 2-3 days in the presence of imidazole. The hydrolysed material readily passed a 0.45 μm Millipore filter and the conjugate was purified and isolated by dialysis, ultrafiltration, chromatography and lyophilization. The UV spectrum and the molar extinction were essentially the same as those of the free drug. Both the pure conjugates and mixtures with the free drug did follow Lambert Beer's law. These findings enabled accurate measurements of the bound drug in the conjugate. In a high frequency NMR spectrum (500 MHz) both the C' 6-methyl doublet of the daunosamine moiety and the MeO singlet (aglyconic portion) were discernible.



Scheme 2. Direct conjugation of adriamycin onto PGA via an amide bond using EEDQ.

TABLE 2

Covalently bound prodrugs^a of adriamycin (ADR) derived from poly(α -glutamic acid) (PGA)/oligopeptide grafts

Batch of PGA (Table 1)	Spacer	Spacer load		ADR load		Yield of oligo- peptide graft (by wt%)	Yield of ADR- prodrug (by wt%)
		(mol%)	(wt%)	(mol%)	(wt%)		
PGA ₄	none	—	—	6	16	—	96
PGA ₃	none	—	—	5	15	—	84
PGA ₄	Gly—Leu	100	—	4.2	6.6	85	58
PGA ₃	Gly—Gly—Leu	76	—	4	7	N.D. ^b	N.D. ^b
PGA ₃	Gly—Gly—Leu	91	—	10	12	91	77
PGA ₄	Gly—Gly—Leu	91	—	8.3	11	92	83
PGA ₄	Gly—Gly—Leu	95	—	—	—	77	—
PGA ₂	(Gly)—(Gly—Gly—Leu)	95 ^c /67 ^d	—	10	13	60 ^c /93 ^d	89
PGA ₃	(Gly—Tyr)—(Gly—Leu) ^e	77 ^c /34 ^d	—	3.6	5.0	90	82
PGA ₃	(Gly—Tyr)—(Gly—Gly—Leu) ^e	100 ^c /53 ^d	—	9.1	9.1	73	92
PGA ₇	(Gly—Tyr)—(Gly—Gly—Leu) ^e	100 ^c /67 ^d	—	3.3	3.3	40	56

^aSodium salt.^bNot determined.^cFirst spacer fragment.^dSecond spacer fragment.^eCascade coupling of respective spacers (see Experimental).

Results are given in Table 2. In early preparations we were faced with difficulties in removing small amounts of free (extractable) drug from the conjugates. These amounts seemed to be bound to the conjugate by relatively stable charge transfer complexes (adriamycin itself exists as dimers in solution [32, 33]). By analogy with relatively stable anthracycline—DNA complexes [1, 5], the surprising stability of the present conjugate—free drug complexes can be ex-

plained by the additional formation of ion pairs (COO^- , NH_3^+). Both the presence of covalently bound drug and free carboxylic acid groups are required for the formation of these complexes. Addition of the free drug to PGA led to the formation of a voluminous precipitate. Thus, apart from being a prodrug, the conjugate itself may also serve as a carrier for the free drug. These conjugate complexes are readily prepared either by (a) the mere addition of

TABLE 3

Complexes of adriamycin (ADR) and conjugates of adriamycin derived from PGA and PGA—oligopeptide graft (Table 2)

Conjugate	Covalently bound ADR		Complex bound ADR		Yield of complex (by wt%)
	(mol%)	(wt%)	(mol%)	(wt%)	
PGA ₄ —ADR	6	16	3.3	8.8	51 ^a
PGA ₃ —ADR	5	15	3.3	9.3	93
PGA ₃ —Gly—Gly—Leu—ADR	4	7	2.9	4.4	89

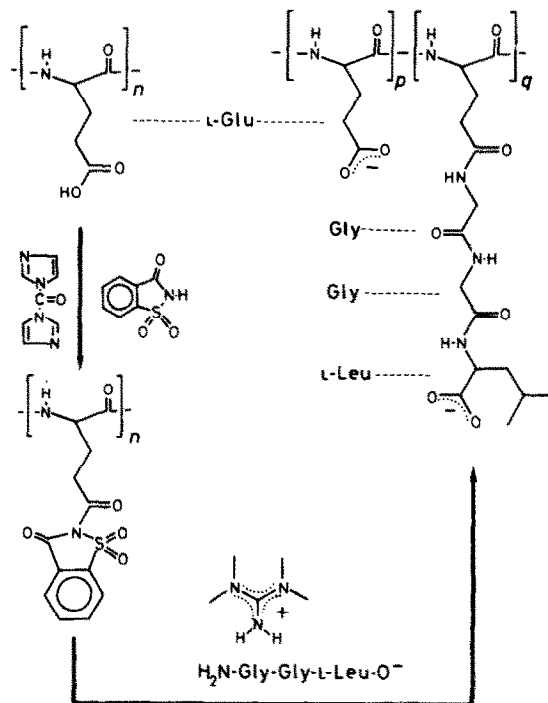
^aLow yield due to gel formation of conjugate.

the free drug to the conjugate or (b) by directly coupling adriamycin to PGA using 0.5–0.6 equimolar amounts of EEDQ with respect to the free drug. In contrast to aqueous solutions of adriamycin hydrochloride, these conjugate complexes were stable at physiological pH for long periods of time. Results are given in Table 3.

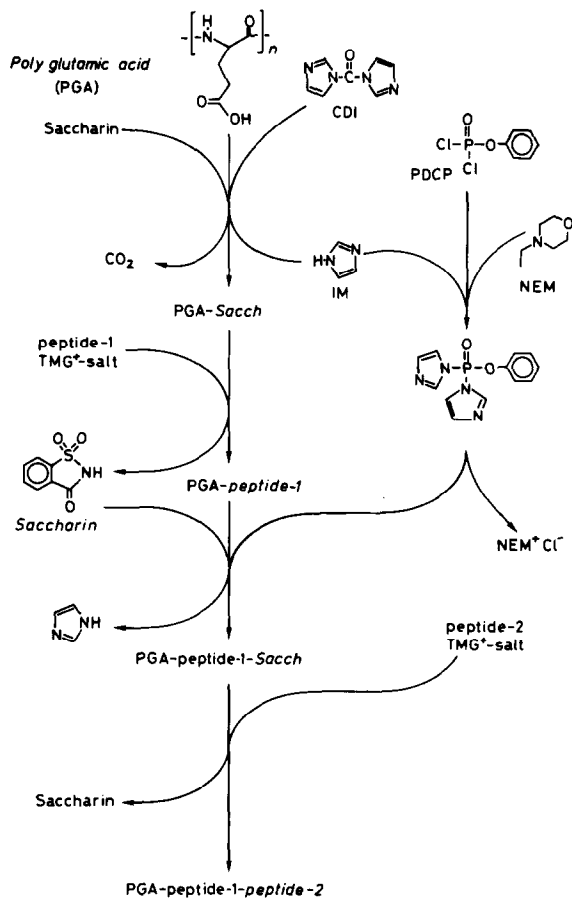
In our approach to interpolate oligopeptide spacers between the carrier and the drug, we preferred a different synthetic strategy than Trouet et al. [10] on the following considerations: Firstly, in view of the expense of the drug, a route which allows coupling of adriamycin in the very last step of the synthesis is more attractive than being engaged in a tedious multistep synthesis in which considerable amounts of the anthracycline are lost. Secondly, the preparation of carriers, which already contain the spacer arms desired will, eventually, be attractive for use in applications with other drugs. In the present approach, we preferred the use of commercially available C-unprotected oligopeptides. To discriminate between the carboxylic groups of PGA and those of the oligopeptides introduced, preactivation of PGA prior to addition of the oligopeptide is essential.

As mentioned above, the preactivation of lateral carboxylic acid groups in PGA by CDI proved to be successful, but gave rise to extensive fragmentation of the peptide chain. By analogy with other methods of preactivation, a number of additives were tried. Saccharin (2,3-dihydro-1,2-benzisothiazole-3-on-1,1-dioxide) proved to be very successful. Saccharin imides [48] have been advocated for use in peptide synthesis but the method has gained little interest, probably due to difficulties in their preparation (i.e. formation of DCC–saccharin adducts or the use of highly reactive chemicals such as thionyl chloride) [48]. We have found that the addition of CDI to a solution containing both the carboxylic component and a slight excess of saccharin resulted in the smooth generation of these imides in

essentially quantitative yield and with only slight concomitant fragmentation of the polypeptide chain. Moreover, these polymeric imides proved to be sufficiently reactive to condense in high yields with the tetramethylguanidinium salts of the oligopeptides of choice in DMF. The chemistry of the method is depicted in Scheme 3, and results are given in Table 2. Both saccharin and imidazole, being the sole non-toxic and water-soluble byproducts of the reaction, were readily removed by dialysis and molecular filtration, whereas the tetramethyl guanidinium ions ($^1\text{H-NMR}$ (D_2O) δ 3.0 ppm) were exchanged quantitatively by sodium ions using ion-exchange chromatography. Theoretically, a partial transfer of the saccharinic imides from PGA to the carboxyl groups of the spacer may result in products in which some of these spacers are dimeric on the PGA-backbone and some of the PGA carboxyl groups are unreacted.



Scheme 3. Grafting of PGA with Gly-Gly-Leu tripeptide using the CDI-saccharin method.



Scheme 4. Stepwise elongation (cascade technique) of oligopeptide spacers with the use of phenyl dichlorophosphate (PDCP).

However, such an imide transfer has not been observed in fragment condensations in which *C*-unprotected substrates were used [48]. The simplicity of the present method also prompted us to investigate the possibility of stepwise elongation of the peptide spacer arms without the isolation of intermediate products. This was accomplished readily with the use of phenyl dichlorophosphate [49] (PDCP) in DMF. The latter compound smoothly reacts with imidazole already present in the reaction mixture, thereby generating the corresponding phosphoryl diimidazolides, which in turn reactivate the *C*-terminal of the spacer arms giving the saccharin imides anticipated.

The chemistry of the cascade coupling method is depicted in Schemes 3 and 4. Oligopeptide spacer arms containing up to 5 amino acid residues (Gly-Tyr-Gly-Gly-Leu) were readily prepared by this method (Table 2) using Gly-Tyr and Gly-Gly-Leu in the CDI-saccharin and PDCP cascade methods, respectively. Degrees of incorporation of both oligopeptide fragments were obtained from the proton integrals attributable to appropriate isolated signals in the $^1\text{H-NMR}$ spectra (e.g. Tyr-Ar-H and Leu- CH_3 vs. Gly- $\beta\text{-CH}_2$ and total of all integrals, Fig. 1). However, in one experiment (Table 2) in which the first spacer fragment (Gly-Tyr) was coupled in 77 mole percent, a part of the second spacer fragment (Gly-Leu) may be coupled directly to the carboxylic groups which are left unreacted in the first coupling step. A slight modification of the method was necessary using glycine. The latter amino acid was applied using the corresponding *t*-butyl ester, rather than its TMG salt. The protecting group was removed quantitatively with the use of trifluoroacetic acid. The grafted polymers were isolated either as the sodium or as the pyridinium salts. In contrast to PGA, the free acids of these polymers were sticky gums which were difficult to handle.

Finally, adriamycin was coupled to the grafted polymers (pyridinium salts) with the use of EEDQ, and isolated, purified and analysed for the drug as described for PGA with slight modifications. Results are given in Table 2.

In contrast to the conjugates in which the drug was attached directly onto the carrier, the prodrugs in which peptide spacers were interpolated between the PGA backbone and the drug (Fig. 2) readily yielded free adriamycin upon digestion with a relatively aspecific protease like papain.

The voluminous coloured conjugates were stable at -20°C over prolonged periods of time. However, the batches derived from high molecular weight PGA (\bar{M}_v 200,000) had a tendency to age, resulting in slow or

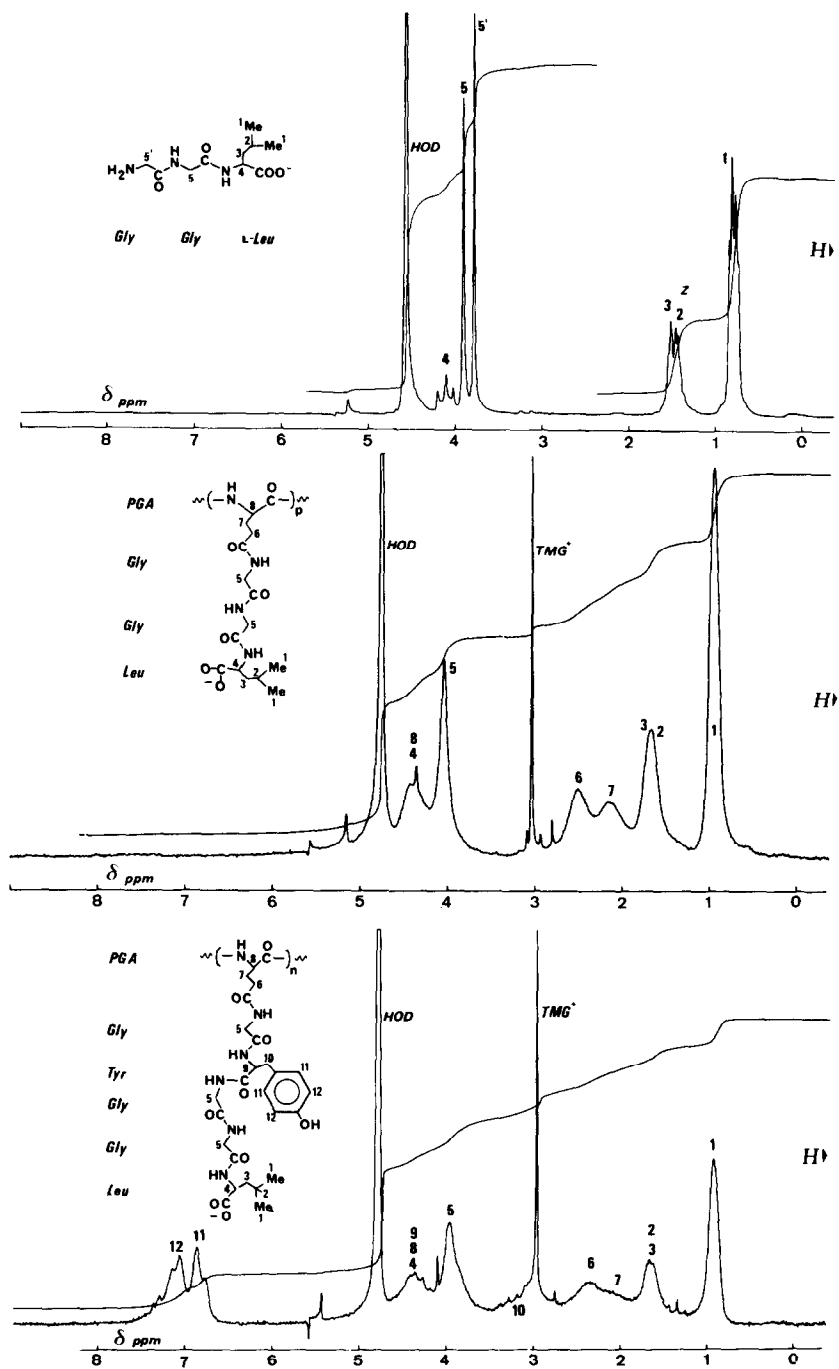


Fig. 1. Interrelated ¹H-NMR (80 MHz, D₂O) spectra and assignments of Gly-Gly-Leu, and the polypeptide grafts PGA-Gly-Gly-Leu and PGA-Gly-Tyr-Gly-Gly-Leu, respectively.

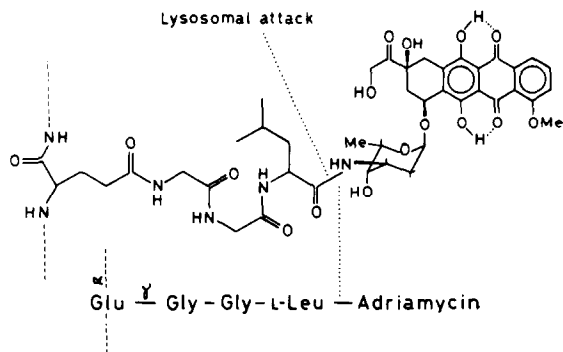


Fig. 2. Monomeric unit in protease-activated macro-molecular prodrug of adriamycin.

even poor solubility in water and the reformation of microgel particles. The branched conjugates also had a tendency to give charge transfer complexes with free adriamycin (Table 3), albeit not with the extreme stability as was found for the conjugates not containing the spacer arms. Surprisingly, these complexes were more stable upon storage and did redissolve more readily than the conjugates from which they were prepared. Thus, apart from being interesting devices both in terms of controlled extracellular release of the (complexed) drug and endocellular release of the (covalently bound) drug, the present complexes are stable formulations at physiological pH for both the prodrug and free adriamycin.

MATERIALS AND METHODS

Tetrahydrofuran (NCA preparation) was stirred with finely powdered potassium hydroxide (10 g/l), filtered and distilled from calcium hydride under a nitrogen atmosphere. Toluene used for the polymerization of NCA was dried azeotropically. Pyridine (1 l) was distilled from chlorosulphonic acid (10 ml) and distilled. Dimethylformamide (spectrograde) was purchased from Janssen (Belgium). All chemicals used, including oligopeptides (Pierce Chem. Comp.) are commercially available. Small-scale reactions were conducted in Reacti-

vials (Pierce Chem. Comp.). $^1\text{H-NMR}$ spectra (internal Me_4Si (CDCl_3 , $\text{DMSO } d_6$) or sodium 2,2-dimethyl-2-silapentane-5-sulfonate in D_2O) were recorded with Bruker WP 80 (80 MHz) and Bruker WM 500 (500 MHz) spectrometers. TLC was performed on silica gel (Merck Kieselgel 60 F254) using n-butanol-acetic acid-water (4:1:1) as the eluent. Spots were visualised with ninhydrin spray and/or UV light (245 nm). Immersible molecular filters (CX-10), filters (HA 0.45 μm) and a stirred pressured cell (XX42.04710) were purchased from Millipore Benelux, Belgium.

Poly(α -glutamic acid)

Glutamic acid 5-benzylester [37] *N*-carboxyanhydride [38] (10 g, 38 mmol, m.p. 92–93.5°C) was polymerized in toluene (1 l) under a blanketing atmosphere (N_2 , room temperature, 5 days) using triethylamine (200 μl , 1.44 mmol) as the initiator. Then, a brisk stream of hydrogen bromide was passed through the reaction mixture under vigorous stirring (20 min) and the mixture was allowed to stand overnight. The toluene was decanted and the polyacid was washed with portions (100 ml) of fresh toluene (2 \times) and acetone (5 \times) until the washings were colorless.

The crude polyacid was stirred in sodium phosphate buffer (100 mM, 100 ml, pH 7) and aqueous sodium bicarbonate solution was added dropwise with stirring until a clear neutral solution resulted, which was dialyzed exhaustively against deionized water (Visking dialysis tubes 9-36/32 in, internal stirring with a slow stream of nitrogen), and filtered (Millipore 0.45 μm) with the aid of a stirred pressure cell (Millipore XX42.04710). The filtered solution was acidified (HCl, pH 2), stirred (40°C, 20 min), and the precipitate was collected and washed with water. The fibrous wet mass was freeze dried (the air-dried compound dissolves less readily in dimethylformamide); yield was 3–4 g. $^1\text{H-NMR}$

and UV (sodium salt) spectra were indistinguishable from those reported [39]. Residual benzyl groups varied up to 5% (molar) as shown by UV measurements (250 nm, using L-glutamic acid 5-benzyl ester as the reference compound). Molecular weights of these polymers were determined by their intrinsic viscosity at 25°C in 0.2 M NaCl solution [50–52] by $[\eta]$ (dl/g) = $3.13 \times 10^3 (\bar{M}_v)^{0.965}$; Table 1.

Poly α -L-glutamic acid anhydride cross-linked gel

Polyglutamic acid (50 mg, 0.39 mmol) was dissolved in dimethylformamide (1.34 ml). Then, EEDQ (8.7 mg, 0.0352 mmol; 0.09 eq) was added with stirring. Viscosity drops rapidly in the course of 2–3 min, and then gradually increases. Stirring is virtually impossible after 20 min, whereas after 45 min a solid rubbery gel results, indicating the formation of interchain symmetrical acid anhydrides. The fragmented gel dissolves completely in the course of a few hours in an aqueous solution of sodium bicarbonate, and even more rapidly in the presence of imidazole (1 M). The formation of a gel, however, does not occur in the presence of a relatively strong amine such as adriamycin (>0.04 mmol), which provides additional chemical evidence for the generation of corresponding amides. The presence of a relatively weak amine, such as prazosine, does not hamper the formation of rubbery cross-linked gels.

Copolymer of L-glutamic acid and 3'-N-(5-L-glutaminy) adriamycin

Polyglutamic acid (200 mg, 1.55 meq) was dissolved in dimethylformamide (2–5 ml, depending on the molecular weight of the polymer). The viscosity must be such that mechanical manipulation and stirring in a small reaction vial is possible. Free adriamycin was generated in a separate reaction vial from its hydrochloric acid salt (54 mg, 0.093 mmol) in dimethylformamide (2 ml) containing N-ethyl morpholine (20 μ l,

0.16 mmol), and subsequently transferred to the polymer solution. Additional dimethylformamide (200 μ l) may be necessary to transfer virtually all of the adriamycin. The resultant solution was stirred for 3 h, EEDQ (26 mg, 0.11 mmol) was added, and the solution was stirred overnight. In preparations when using high molecular weight polyacid or larger excesses of EEDQ a translucent solid red gel containing cross-links of both the acid anhydride and 14-ester types may result. The solution or the gel, which was fragmented mechanically, was stirred into a phosphate buffer (80 ml, 100 mM, pH 7) solution containing imidazole (1 g) and the mixture was kept in the dark at 6°C for 3–5 days with occasional shaking. A diluted sample from the bulk gave the same UV spectrum and molar extinction coefficient for bound adriamycin ($E_{490} = 84900 \text{ l mol}^{-1} \text{ cm}^{-1}$) as found for the free drug. The clear red solution was dialyzed against deionized water, freed from minute amounts of microgel particles (Millipore HA filter 0.45 μ m), concentrated and washed by molecular filtration (Millipore immersible filters CX-10). TLC analysis may reveal small amounts of free adriamycin bound to the polymer by Π -complexes. The conjugate can be purified by passing slowly the solution through an ion exchange column (100 \times 4 cm, Dowex HCRW-2, sodium form). Finally, the pure conjugate is isolated by concentration (molecular filtration) to a small volume and lyophilization giving a brick-red voluminous mass; yield: 80–90% (recovery, by weight), 90–100% (conversion to bound adriamycin; UV).

Attempt to digest the copolymer (5 mg) with papain (5 mg) in phosphate buffer (pH 5.4) did not yield free adriamycin (TLC).

In a 500 MHz (D_2O) $^1\text{H-NMR}$ spectrum a small sharp singlet on the positive slope of the HOD resonance attributable to MeO-CH_3 (aglycone; δ 3.99 ppm) and a doublet (daunosamine; $\text{C}'6\text{-CH}_3$, δ 1.164, $J_{5,6}$, 6.35 Hz) were discernable). Other resonances were obscured by the resonances of polyglutamic acid, Table 2.

Complex of adriamycin and the copolymer of L-glutamic acid and 3'-N-(5-L-glutaminy) adriamycin

Method a

The copolymer above (210 mg) was dissolved in water (10 ml) and the solution was transferred to a small flask fitted with a screw cap holding a molecular filter (Millipore immersible filter CX-10) attached to a vacuum line, a magnetic stirring bar and a septum outlet. Solutions were then introduced into the stirred cell with the aid of a syringe at a very slow rate which was controlled by the efflux from the stirred cell. In this manner a solution of adriamycin hydrochloride (40 mg, 0.069 mmol), water (5 ml), phosphate buffer (2 ml, 100 mM, pH 7) and water (250 ml), respectively, were introduced. No adriamycin was present in the filtrate of the cell, indicating that all of the drug added is accommodated by the polymer in the form of Π -complexes. The solution of the macromolecular complex was filtered (Millipore HA 0.45 μ m) with the aid of a stirred pressure cell to remove any microgel particles, and the filtrate and washings were again concentrated to a small volume with the aid of an immersible molecular filter as described above. The polymeric drug was finally isolated by lyophilization (240 mg, 95%). The material behaved as one entity in dialysis, molecular filtration and gel chromatography (Sephadex G10), but was readily decomposed into the conjugate and free adriamycin by ion-exchange chromatography (polystyrene, sulphonate type, sodium form) and TLC. The UV spectrum of the polymeric complex was virtually the same as that of adriamycin or its conjugate, Table 3.

Method b (incomplete covalent linkage of the drug onto polyglutamic acid)

Polyglutamic acid (200 mg, 1.55 mmol) was dissolved in dimethylformamide (1 ml) and a suspension of adriamycin, generated from its hydrochloric acid salt (108 mg, 0.186 mmol), *N*-ethyl morpholine (25 μ l)

in dimethylformamide (4 ml) was added. The solution was stirred for an hour, EEDQ (25 mg, 0.093 mmol) was added, and stirring was continued overnight. The contents of the vial were dissolved in phosphate buffer (100 ml, 100 mM, pH 7.0) and the solution was dialysed exhaustively with internal stirring by a slow stream of nitrogen. The solution was passed through a Millipore filter (HA 0.45 μ m) and prior to lyophilization the filtrate was concentrated by molecular filtration as described in Method a. The material prepared was indistinguishable from that prepared according to Method a.

Polyglutamic acid glycyglycyl-L-leucine graft (PGA-Gly-Gly-L-Leu), using *N,N'*-carbonyl diimidazole (CDI) [44] as the sole condensing agent

Polyglutamic acid (33 mg, 0.25 mmol) was dissolved in dimethylformamide (1 ml); CDI (60 mg, 0.35 mmol) was added and the solution was stirred under rigid exclusion from moisture. The reaction between the two components was accompanied with the evolution of carbon dioxide and with a dramatic decrease in the viscosity of the solution. Then, a solution of Gly-Gly-L-Leu *N,N,N',N'*-tetramethyl guanidinium salt (TMG⁺, prepared by the slow addition of *N,N,N',N'*-tetramethyl guanidine (TMG; 30 μ l, 0.25 mmol) to a stirred suspension of finely powdered Gly-Gly-L-Leu in dimethylformamide (4 ml)) was added. Stirring was continued for three days. The solution was taken in phosphate buffer (15 ml; 100 mM, pH 7.0), the resultant solution was dialysed against water, concentrated by molecular filtration and freeze dried. Yields turned out to be irreproducible (5–48%), and degrees of substitution erratic (up to 15%, molar). The low yields and poor results are without any doubt attributable to extensive self-fragmentation of the polyacid imidazoles and contrast with coupling experiments in which saccharin is used as an additive.

Polyglutamic acid oligopeptide graft, using saccharin as a CDI-additive (general procedure, Scheme 3)

Polyglutamic acid (129 mg, 1 mmol eq) and saccharin (220 mg, 1.2 mmol) were dissolved in dimethylformamide (1–4 ml, depending on the molecular weight of the polymer). In a separate vial, *N,N,N',N'*-tetramethylguanidine (TMG, 131 μ l, 1.05 mmol) was slowly added to a stirred suspension of the finely powdered amino acid or oligopeptide (1 mmol) selected. After all solids have gone into solution, *N,N'*-carbonyl diimidazole (227 mg, 1.4 mmol) was added to the solution containing both the polymer and additive to generate the polymeric saccharinic acid imides (30 min stirring). To this solution was added the solution containing the TMG salts of the oligopeptide and the resulting reaction mixture was stirred for 3 days. The solution was added to a phosphate buffer (25 ml, 250 mM, pH 7.0) and the resultant solution was dialysed against water, filtered (Millipore HA 0.45 μ m) with the aid of a stirred pressure cell, concentrated by molecular filtration, and freeze dried. A 10–20 mg sample of the product was dissolved in a small volume of deuterium oxide and freeze dried prior to $^1\text{H-NMR}$ analysis. The $^1\text{H-NMR}$ spectra of the products were in accordance with the structures assigned. Degrees of substitution were calculated from proton integrals attributable to structure elements in spacer (e.g. leucine- CH_3) and backbone. See Table 2 and Fig. 1.

Polyglutamic acid glycine graft

Polymeric saccharin imides were generated from polyglutamic acid (129 mg, 1 mmol eq) as described above. A solution of glycine *t*-butyl ester hydrochloride (170 mg, 1.05 mmol) and TMG (131 μ l, 1.05 mmol) in dimethylformamide (2 ml) was added, and the reaction mixture was stirred for 3 days. The clear solution was diluted with dimethylformamide (10 ml) and then added dropwise

to a vigorously stirred aqueous phosphate buffer (600 ml, 100 mM, pH 7.0). Fibrous polyglutamic acid glycine *t*-butyl ester graft precipitates in essentially quantitative yield. The material was washed with water and dried *in vacuo*. A $^1\text{H-NMR}$ spectrum (DMSO d_6) was in accordance with the structure assigned: δ 1.35 (s, 9H, *t*-Bu); 1.7–2.5 (m, 4H, Glu- CH_2); 3.7–4.0 (complex multiplet indicating prochirality of Gly- CH_2 introduced by the polymer, 2 H); 4.2–4.5 (m, 1H, Glu- CH). Degrees of substitution (up to 95%, molar) were obtained from the ratios of the Glu- CH_2 and *t*-Bu- CH_3 proton integrals.

The protected polymer was dissolved in trifluoroacetic acid (3 ml) and stirred (3 min) and rapidly concentrated *in vacuo* (20°C, bath). The solid mass obtained was dissolved in aqueous phosphate buffer (250 mM, pH 7.0, 10 ml), and the pH of the resulting suspension was readjusted to pH 7.0 by the portionwise addition of sodium carbonate and stirring. The clear solution obtained was further processed, purified and subjected to analysis as described for the polyglutamic acid oligopeptide grafts described above.

Polyglutamic acid oligopeptide graft using a two-step cascade coupling procedure without isolation of intermediate coupling products

Polymeric saccharin imides derived from polyglutamic acid (1 mmol eq), *N,N'*-carbonyl diimidazole and saccharin were generated as described above and allowed to react with an oligopeptide (1.05 mmol eq) TMG salt in dimethylformamide solution. After incorporation of the oligopeptide of choice (3 days), the *C*-terminal groups of the spacer arms present can be reactivated by the addition of *N*-ethyl morpholine (152 μ l) and a freshly prepared (<15 s) solution of phenyl dichlorophosphate [49] (180 μ l) in dimethyl formamide (0.5 ml), respective-

ly (Scheme 4). The resulting light yellow solution was stirred for 4 h, and the second oligopeptide of choice (1.05 mmol) acid and TMG (132 μ l) in dimethylformamide (2 ml) was added. The solution was then stirred for 3 days. The polymers containing the elongated oligopeptide grafts (up to 5 amino acid residues) were isolated, purified and subjected to analysis as described above. Degrees of substitution of the oligopeptide fragments were obtained from the proton integrals attributable to appropriate isolated signals in the $^1\text{H-NMR}$ spectra (e.g. Tyr-Ar-H and Leu-CH₃ vs. Glu-CH₂ and total of all integrals). Results are given in Table 2 and Fig. 1.

Polyglutamic acid oligopeptide graft adriamycin conjugates (general procedure)

Polyglutamic acid oligopeptide graft sodium salt (1 mmol eq) was dissolved in a small amount of water and slowly passed through an ion-exchange column (polystyrene sulphonate type, pyridinium form, prepared by neutralization of the acid form by pyridine, and washing with distilled water). The pyridinium form of the polypeptide was collected and freeze dried. The voluminous mass was dissolved in a minimum amount of dimethylformamide (magnetic stirring of the viscous solution should be possible). Then, *N*-ethylmorpholine (0.1 mmol eq) and adriamycin hydrochloric acid salt (0.1 mmol eq) are added. After dissolution of all solids, EEDQ (0.11 mmol eq) was added. The extent of incorporation of the drug can be monitored by TLC analysis (a minute sample is taken in phosphate buffer (50 μ l) and subjected to two-dimensional TLC analysis (same solvent), to account for artefacts caused by the presence of dimethylformamide and salts present). In some experiments the addition of more *N*-ethylmorpholine and EEDQ may improve the coupling yield of adriamycin. The reaction mixture was stirred for an additional 24 h, then added to phosphate buffer, and further

processed, isolated and analyzed for covalently bound adriamycin (UV) as described for the copolymer of L-glutamic acid and 3'-*N*-(5-L-glutaminy) adriamycin. All compounds are believed to be microheterogeneous with respect to molecular weight, degree of substitution of both spacer and drug, but all behave as one entity in dialysis, chromatography and ultrafiltration. All compounds were sterilized by filtration (Millipore HA 0.45 μ m), and should be stored in the dark in a freezing chest. Frozen solutions are more resistant to ageing than the solid compounds. Results are given in Table 2.

Incubation of the PGA-Gly-Gly-Leu (5 mg) with papain (5 mg) in phosphate buffer (pH 5.4) gave rise to the formation of adriamycin (TLC) within 5 min.

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