

Association of macromolecular prodrugs consisting of adriamycin bound to poly(L-glutamic acid)

Masahiro Nukui^{a)}, Kees Hoes*, Hans van den Berg, Jan Feijen

Department of Chemical Technology, University of Twente, P.O. Box 217, 7500AE Enschede, The Netherlands

(Date of receipt: December 28, 1990)

SUMMARY:

Soluble macromolecular conjugates for the controlled delivery of the strongly hydrophobic antitumor drug adriamycin (ADR) were prepared. The association behaviour of two types of these amphiphilic conjugates consisting of ADR bound to poly(L-glutamic acid) (PGA) in aqueous solution was investigated using absorbance and fluorescence spectroscopy as well as GPC and low-angle laser light scattering (LALLS) measurements. ADR was bound via the aminoribosyl moiety either directly with PGA side-chain groups or via oligopeptide spacer groups. UV/VIS, GPC and LALLS data showed that in buffer solution both ADR conjugates associate intermolecularly. The data of the directly bound ADR conjugates are consistent with the presence of multimers of different degrees of association in equilibrium with single polymer chains. In contrast, spacer-containing ADR conjugates associate to give stable uniform multimers. UV/VIS and fluorescence spectroscopy performed at very low conjugate concentration show that polymer-bound ADR residues associate intramolecularly as well. The degree of intra- and intermolecular association of the conjugate-bound ADR molecules depends on the type of conjugate, ADR load and conjugate concentration as well as on ionic strength of the solvent, the presence of organic cosolvents and temperature. The data indicate that hydrophobic domains in spacer-containing conjugates are more stable compared with directly bound conjugates, probably due to enhanced flexibility and the presence of hydrophobic leucine residues. It is concluded that spacer-containing conjugates of ADR may have a potential for effective drug delivery under *in vivo* conditions due to their solution properties in addition to the biodegradability of the drug-polymer bond.

Introduction

Macromolecular prodrugs consisting of antitumor agents coupled to water-soluble polymeric carriers have been developed to improve chemotherapy with less toxic side effects^{1,2,3}. The design of soluble drug conjugates usually conforms to either one of two general strategies: The drug is bound to the carrier via a hydrolytically labile bond resulting in a sustained release of drug in the circulation, thereby preventing toxic peak concentrations of free drug⁴. Alternatively, the drug is bound to the carrier through a bond which is stable in the circulation and which is cleaved after pinocytic uptake of the conjugate by cells either by lysosomal enzymes or by the acid environment of endosomes and lysosomes. The latter approach may have potential for enhanced selectivity towards certain tumor cells with an enhanced rate of uptake of macromolecules^{5,6}.

Most drugs are hydrophobic, and upon binding to a hydrophilic polymeric carrier a conjugate is obtained having amphiphilic properties in aqueous solution^{7,8,9}. As a result, drug conjugates may associate intramolecularly affecting the conformation of

^{a)} Present address: Research center, Mitsubishi Kasei Corporation, 1000, Kamoshida-cho, Midori-ku, Yokohama 227, Japan.

single chains as well as intermolecularly yielding formation of multimers. Association phenomena have been shown to occur for polymer-bound *p*-nitroaniline used as a model drug⁸⁾ and for polyelectrolyte-bound phenanthryl groups⁹⁾ but have not yet been studied in detail for polymeric conjugates of clinically used drugs. The therapeutic effectivity of macromolecular drug conjugates is dependent on biodistribution, including capture by cells of the reticulo-endothelial (RES) system and binding with plasma proteins, as well as the mode and rate of uptake by cells and the drug release rate. These properties may be altered in drug-polymer conjugates compared with polymeric carriers due to drug-mediated association phenomena.

In previous papers we described macromolecular conjugates of the antitumor drug adriamycin (ADR) using sodium poly(L-glutamate) (PGA), as a soluble biodegradable carrier¹⁰⁻¹²⁾. The design of these drug conjugates aims at lysosomal release of drug after pinocytic uptake by cells. The rate of drug release is regulated by using oligopeptide spacers of specific composition between drug and carrier. We have reported on the syntheses of conjugates having oligopeptides of different composition as spacer units¹⁰⁾, interaction with an *in vitro* cytotoxicity against L1210 leukemia cells^{11, 12)} and enzyme-mediated drug release¹²⁾.

ADR is known to be very prone to adsorption to surfaces and to formation of dimers in solution due to the hydrophobic nature of the aglycone moiety¹³⁾. By limiting the level of substitution of polymer with this hydrophobic drug to low values, the conjugates can be kept soluble¹⁰⁾. The amphiphilic nature of ADR conjugates may also result in intramolecular and/or intermolecular association affecting essential aspects of the performance of a drug conjugate such as biodistribution, transport properties, uptake by cells and rate of drug release.

In this paper the solution properties of ADR conjugates derived from PGA are described using GPC and low-angle laser light scattering (LALLS) to characterize the behaviour of the polymer and UV/VIS absorbance as well as fluorescence spectroscopy to analyze the behaviour of the polymer-bound drug. Two different types of ADR conjugates were prepared by coupling ADR either to the γ -carboxyl groups of PGA or to the Leu carboxyl groups of PGA-GlyGlyGlyLeu grafts¹⁰⁾. The suitability of GlyGlyGlyLeu-containing ADR conjugates for controlled drug delivery is suggested by the observation that free drug as well as amino acid and peptide derivatives of the drug are released upon incubation with lysosomal enzymes¹²⁾. The effects of conjugate structure, concentration and drug load as well as solvent composition and temperature on the association of the conjugates were studied.

Results

Preparation of polymeric carriers and polymer-drug conjugates

Poly(γ -benzyl-L-glutamate), PBLG, was prepared according to the method of Blout and Karlson¹⁴⁾ using dioxane as the solvent. Debenzylation of PBLG was carried out using trifluoroacetic acid (TFA)/methanesulfonic acid (MSA) according to the procedure¹⁵⁾ reported for the preparation of low-molecular-weight PGA. The reaction conditions were optimized in order to prepare PGA having the lowest possible amount of

residual benzyl groups while maintaining a high molecular weight. The results are summarized in Tab. 1.

It was found that benzyl groups were removed very rapidly after the addition of MSA to the TFA solution of PBLG. Scission of polymer main chains took place gradually. As a result high-molecular-weight PGA ($\bar{M}_v = 7,3 \cdot 10^4$) with a low residual amount of benzyl groups (0,4 mol-%) could be obtained after reaction of PBLG for 20 min at 0 °C and 13 min at room temperature. The classical debenzoylation method using

Tab. 1. Syntheses of poly(L-glutamic acid) (PGA) from poly(γ -benzyl L-glutamate) (PBLG) ^{a)}

| Run | Time in min ^{b)} | | Residual benzyl groups in mol-% | \bar{M}_v ^{c)} |
|-----|---------------------------|---------------------|---------------------------------|---------------------------|
| | at 0 °C | at room temperature | | |
| 1 | 0 | 0 | — ^{d)} | — ^{d)} |
| 2 | 10 | 0 | 45 | — ^{e)} |
| 3 | 20 | 0 | 20 | — ^{e)} |
| 4 | 20 | 13 | 0,4 | 73 000 |
| 5 | 20 | 15 | 0,2 | 56 000 |
| 6 | 20 | 30 | <0,1 | 39 000 |

^{a)} Debenzoylation of PBLG using trifluoroacetic acid and methanesulfonic acid (see Exptl. part). Parent PBLG: $\bar{M}_v = 206\,000$ (determined by viscometry in dichloroacetic acid).

^{b)} Time after adding methanesulfonic acid into a trifluoroacetic acid solution of PBLG.

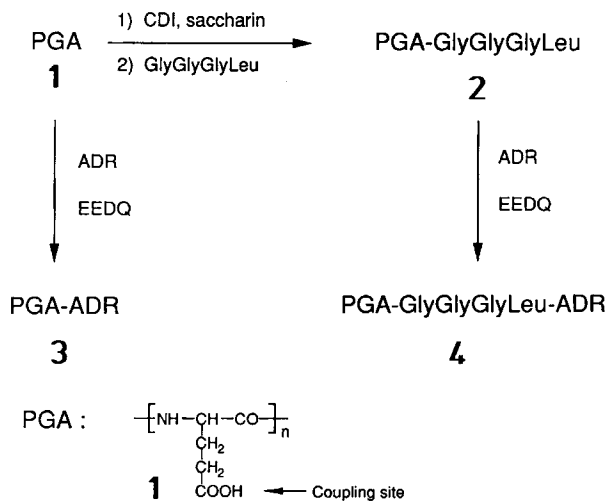
^{c)} Determined by viscometry (see Exptl. part).

^{d)} No water-soluble polymer obtained.

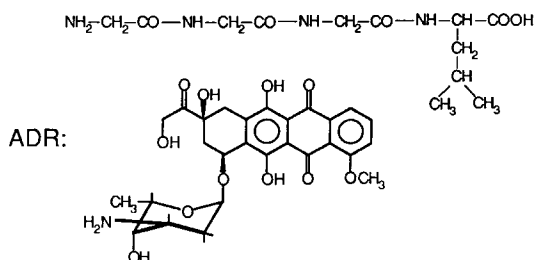
^{e)} \bar{M}_v could not be determined because of a high amount of residual benzyl groups.

hydrogen bromide ¹⁶⁾ in toluene yields PGA having ca 1 mol-% of benzyl groups after a reaction time of 1 day at room temperature ¹⁷⁾. The amount of residual benzyl groups could be suppressed by increasing the reaction time, but the molecular weight of PGA decreased significantly ¹⁷⁾. Therefore, debenzoylation using TFA/MSA is more convenient for the preparation of high-molecular-weight PGA having low residual amounts of benzyl groups.

The syntheses of ADR conjugates were performed as described earlier ¹⁰⁾. Direct coupling of ADR onto PGA was carried out by using *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, EEDQ, as a coupling agent (Scheme 1, PGA-ADR, 3). The attachment of ADR onto PGA through spacer groups was carried out by the following two steps (Scheme 1, PGA-GlyGlyGlyLeu-ADR, 4). First, PGA was grafted with the oligopeptide spacer, GlyGlyGlyLeu ¹¹⁾ by preactivation of PGA with saccharin and *N,N'*-carbonyldiimidazole (CDI) in DMF and subsequent reaction of the saccharin imides with the *N,N,N',N'*-tetramethylguanidinium salt of the oligopeptide. Then, ADR was coupled onto the grafted polymer 2 using EEDQ. The results of the preparations of ADR conjugates are summarized in Tab. 2.



GlyGlyGlyLeu :



Scheme 1: Syntheses of two types of adriamycin conjugates: directly coupled conjugate **3** and spacer-coupled conjugate **4**

Tab. 2. Macromolecular prodrugs of adriamycin^{a)}

| Code ^{b)} | Drug load ^{c)} | | Yield of conjugates in % |
|--------------------|-------------------------|---------|--------------------------|
| | in mol-% | in wt-% | |
| 3a | 1,8 | 6,1 | 93 |
| 3b | 4,0 | 12,7 | 97 |
| 3c | 5,0 | 15,5 | 97 |
| 4a | 1,3 | 1,9 | 90 |
| 4b | 4,8 | 6,6 | 88 |

^{a)} Derived from poly(L-glutamic acid) (PGA, **1**; $\overline{M}_v = 73\,000$, residual benzyl groups 0,4%) and PGA-oligopeptide grafts (PGA-GlyGlyGlyLeu, **2**; spacer load: 77 mol-%, determined by ¹H NMR measurements).

^{b)} **3a-c**: Directly coupled conjugates (PGA-ADR), **4a, 4b**: spacer-coupled conjugate (PGA-GlyGlyGlyLeu-ADR) (see Scheme 1).

^{c)} Determined by UV-VIS measurements (see Exptl. part).

GPC measurements

The intermolecular association behavior of ADR conjugates in buffer solution was investigated by GPC. The effects of ADR load, spacer units and ionic strength of the solvent were studied.

Fig. 1 shows elution curves of the parent polymer PGA (**1**) and directly coupled ADR conjugates **3a** and **3b** in phosphate buffer containing 0,1 M NaCl, pH 7,0. At this pH, PGA is completely ionized¹⁸), and it is assumed that conjugates are also completely ionized. The elution curve of **1** shows a single broad peak at an elution volume (V_e) of 174 mL. The void volume of the column used was determined to be 155 mL using Blue Dextran 2000 with $\bar{M}_w = 2 \cdot 10^6 \text{ g} \cdot \text{mol}^{-1}$. Hence, **1** penetrates into the gel pores. The shape of the elution curves of the conjugates depends on the amount of ADR molecules attached to the polymeric carrier. After the binding of a small amount of drug onto the PGA main chain (**3a**, ADR load 1,8 mol-%), early-eluting new peaks are observed at $V_e = 165$ (shoulder) and 168 mL indicating the presence of high-molecular-weight products. With higher drug loads, as in the case of **3b** (ADR load 4,0 mol-%), the early-eluting peaks ($V_e = 161, 164$ and 166 mL) become predominant. Apparently, the formation of multimeric products is mediated by the polymer-bound drug. Further, multimers of various molecular weights seem to be present indicating 'open-type' association of polymer chains¹⁹).

The shape of the elution curves is also dependent on the type of conjugate. Fig. 2 shows elution curves of the parent polymer PGA-GlyGlyGlyLeu (**2**) and spacer-coupled ADR conjugates **4a** and **4b**. The elution curves of conjugates **4a** and **4b** having 1,3 and 4,8 mol-% of bound ADR, respectively, are similar and show two peaks. V_e of the major peak due to low-molecular-weight material (173 mL) is almost identical with that of the parent polymer **2** ($V_e = 175$ mL). A minor peak due to high-molecular-weight conjugate ($V_e = 160$ mL) appears at the void volume. Qualitatively, the extent of multimer formation by the spacer-bound conjugates **4a** and **4b** is much lower compared with the directly bound conjugates **3a** and **3b**.

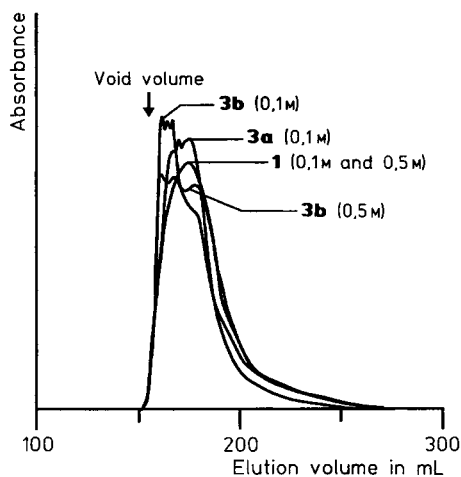


Fig. 1. GPC elution curves of parent PGA (**1**) and directly coupled adriamycin conjugates (**3a** and **3b**) in 0,01 M sodium phosphate solution containing NaCl (0,1 M or 0,5 M), pH 7,0

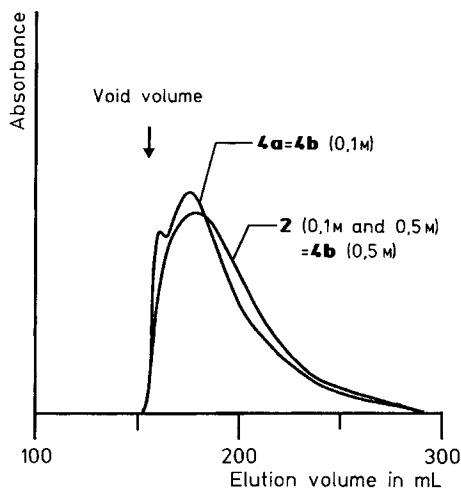


Fig. 2. GPC elution curves of oligopeptide-grafted PGA (**2**) and adriamycin conjugates coupled via oligopeptide spacer (**4a** and **4b**) in 0,01 M sodium phosphate solution containing NaCl (0,1 M or 0,5 M), pH 7,0

Finally, the shape of the GPC curves of ADR conjugates is affected by the ionic strength of the eluent. In a solvent of high ionic strength (0,5 M NaCl-phosphate buffer) the formation of multimers is suppressed for conjugate **3b** and is not detectable any more for conjugate **4b** compared with 0,1 M NaCl phosphate buffer (Figs. 1 and 2). The elution curves of the parent polymers **1** and **2** are independent of the ionic strength of the eluent.

Low-angle laser light scattering (LALLS) measurements

The ADR conjugates were studied by LALLS to obtain molecular weights and additional evidence for intermolecular association. Errors in the determination of molecular weights due to Donnan-type exclusion of salts from the polyelectrolytes were suppressed by dialyzing the polymeric conjugates against the appropriate buffer solution²⁰. Fig. 3 shows the concentration dependence of the reciprocal apparent molecular weight ($1/\bar{M}_{w,app} = Kc/\Delta R$, K is the optical constant, c the concentration of the polymer and ΔR the Rayleigh ratio) observed for the ADR conjugates **3b** and **4b** in 0,01 M phosphate buffer solution at pH 7,0 containing 0,1 or 0,5 M NaCl.

The salt concentration affects the concentration dependence of $1/\bar{M}_{w,app}$ as well as the average molecular weight obtained by extrapolation of the curves to zero concentration ($\bar{M}_{w,extr}$). In 0,1 M NaCl both conjugates show positive values of the second virial coefficients ($2,7-4,9 \text{ mol} \cdot \text{cm}^3 \cdot \text{g}^{-2}$) typical for thermodynamically good polymer-solvent interactions. The values are much smaller than observed for parent PGA, **1** ($47 \text{ mol} \cdot \text{cm}^3 \cdot \text{g}^{-2}$). This may indicate a less expanded polymer conformation due to intramolecular association mediated by the coupling of drug (**3b**) or spacer and drug (**4b**)¹⁹. The initial decrease of $1/\bar{M}_{w,app}$ with increasing concentration observed for **4b** may be explained by the presence of multimeric associates¹⁹.

In 0,5 M NaCl the slopes of the plots are slightly negative (**3b**) and slightly positive (**4b**), respectively. The decrease of $1/\bar{M}_{w,app}$ with increasing concentration, i.e. a

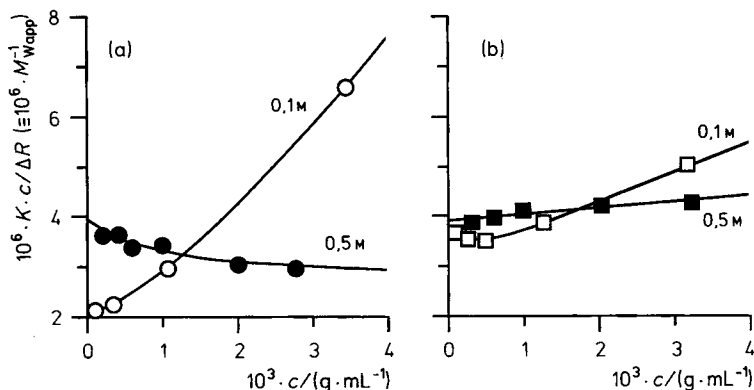


Fig. 3. Low-angle laser light scattering data of adriamycin conjugates in 0,01 M sodium phosphate solution containing NaCl (empty symbols: 0,1 M; filled symbols: 0,5 M), pH 7,0. (a): Directly coupled conjugate **3b**, (b): spacer-coupled conjugate **4b**

negative virial coefficient, observed for **3b** is evidence for the presence of significant amounts of multimeric associates¹⁹). In contrast, the concentration dependence of $1/\bar{M}_{w,app}$ observed for **4b** is very slight, the small positive slope indicating polymer-solvent interaction close to theta-conditions with no evidence of multimer formation.

By extrapolation of the curves to zero concentration the values of the average molecular weight, $\bar{M}_{w,extr}$, of the unimer should be obtained¹⁹). Thus, conjugate **3b** shows an $\bar{M}_{w,extr}$ of $4,8 \cdot 10^5 \text{ g} \cdot \text{mol}^{-1}$ or $2,6 \cdot 10^5 \text{ g} \cdot \text{mol}^{-1}$ in phosphate buffer solution containing 0,1 M NaCl or 0,5 M NaCl, respectively. These values are larger than the unimer molecular weight ($1,5 \cdot 10^5 \text{ g} \cdot \text{mol}^{-1}$) calculated from the molecular weight of the parent polymer **1** determined by LALLS ($1,27 \cdot 10^5 \text{ g} \cdot \text{mol}^{-1}$) and the composition (drug load 4,0 mol-%). This can be interpreted by assuming that multimers of **3b**, presumably trimers (0,1 M NaCl) or dimers (0,5 M NaCl), are present below the lowest concentration used. The $\bar{M}_{w,extr}$ of conjugate **4b** is $2,6 \cdot 10^5 \text{ g} \cdot \text{mol}^{-1}$ or $2,5 \cdot 10^5 \text{ g} \cdot \text{mol}^{-1}$ in buffer solution containing 0,1 M NaCl or 0,5 M NaCl, respectively. These values are smaller than the unimer molecular weight ($3,9 \cdot 10^5 \text{ g} \cdot \text{mol}^{-1}$) calculated from the molecular weight of the parent polymer **1** and the composition (spacer load 77 mol-%, drug load 4,8 mol-%). The smaller $\bar{M}_{w,extr}$ determined by LALLS may be explained by the occurrence of some polymer main chain bond cleavage due to the strongly basic reaction conditions in the preparation of the spacer-grafted carrier¹⁰) **2**. It should be pointed out that the values of $\bar{M}_{w,extr}$ are apparent and at low concentration may still deviate from the true values due to polyelectrolyte effects. Hence, values obtained in 0,5 M NaCl are probably more reliable than those obtained in 0,1 M NaCl.

Concentration dependence of UV/VIS and fluorescence spectra

The ADR conjugates were studied by UV/VIS and fluorescence spectroscopy which are drug-specific optical techniques allowing to obtain information on hydrophobic

domains. Free ADR and related compounds are known to associate in aqueous solution yielding dimers and higher-order oligomers with association constants in the order of 10^3 to $10^4 \text{ mol}^{-1} \cdot \text{L}$. The type and extent of association of free ADR has been shown to depend on pH (amount of free base), salt concentration, solvent composition and temperature using different techniques: UV/VIS absorbance^{21–23}, fluorescence²¹, circular dichroism^{21, 24} and NMR^{22, 24, 25}. In macromolecular ADR-PGA conjugates similar hydrophobic interactions between drug moieties may occur and will compete with solvation of the polymer backbone and side chains and electrostatic interactions between side chain carboxylate groups.

Figs. 4 and 5 show the absorption spectra at different concentrations of free ADR or the conjugate **3b** in 0,01 M phosphate buffer solution (pH 7,0) containing 0,1 M NaCl, respectively. The spectra of free ADR in buffer solution at concentrations of 1,3; 13 and 300 $\mu\text{mol} \cdot \text{L}^{-1}$ correspond with previously published spectra²¹). The spectrum measured at the highest concentration used (300 $\mu\text{mol} \cdot \text{L}^{-1}$) indicates the presence of ADR dimers which show the absorption maximum (λ_{max}) at²¹) 483 nm. On decreasing the concentration of drug (13 $\mu\text{mol} \cdot \text{L}^{-1}$), a shoulder appears at a higher wavelength

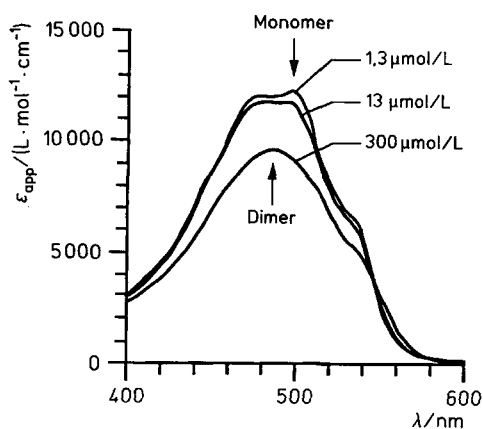


Fig. 4. Absorption spectra of free adriamycin (apparent extinction coefficient ϵ_{app} vs. λ) at different concentrations in 0,01 M sodium phosphate solution containing 0,1 M NaCl, pH 7,0

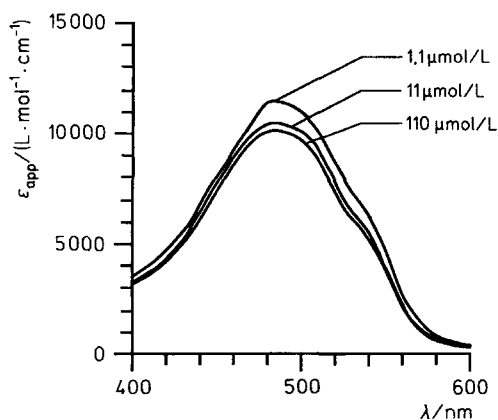


Fig. 5. Absorption spectra of adriamycin conjugate **3b** at different concentrations of bound drug in 0,01 M sodium phosphate solution containing 0,1 M NaCl, pH 7,0

than λ_{\max} . At very low concentration ($1,3 \mu\text{mol} \cdot \text{L}^{-1}$), the shape of the spectrum indicates the presence of monomeric ADR which shows λ_{\max} at ²¹⁾ 498 nm. The association constant (K_a) is calculated from the concentration dependence of the apparent extinction coefficient using a simple dimer model ²¹⁾. The value of K_a is $6\,100 \text{ mol} \cdot \text{L}^{-1}$ similar to previously reported data ^{21,23)}.

The spectra of conjugate **3b** (Fig. 5) show the absorption maximum at 483 nm characteristic of dimeric ADR at all drug concentrations studied down to $1,1 \mu\text{mol} \cdot \text{L}^{-1}$ (conjugate concentration $4,7 \cdot 10^{-6} \text{ g} \cdot \text{mL}^{-1}$). Similar results were obtained for ADR conjugates with oligopeptide spacer (**4a**, **4b**). These data indicate that ADR molecules present in the conjugates associate at very low conjugate concentration probably due to intramolecular interactions. It should be realized that the local drug concentration within polymer chains is generally much higher than in a solution of free ADR at a comparable total concentration of drug.

Figs. 6 and 7 show the concentration dependence of the apparent extinction coefficient (ϵ_{app} at 483 nm) of directly bound or spacer-bound ADR conjugates, respectively. The ϵ_{app} values of the directly coupled conjugates **3a** and **3c** decrease gradually on increasing the conjugate concentration. This indicates the formation of multimers having different degrees of association according to the 'open association type' mechanism. At high concentration no constant value of ϵ_{app} was reached indicating partial multimer formation. The magnitude of the total change in ϵ_{app} ($1\,500 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) for both conjugates is appreciable but smaller than that of free ADR ($3\,000 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). Both, environmental changes and extent of multimer formation contribute to the change in ϵ_{app} . The ϵ_{app} values of the spacer-containing conjugates **4a** and **4b** (Fig. 7) are almost constant up to a certain

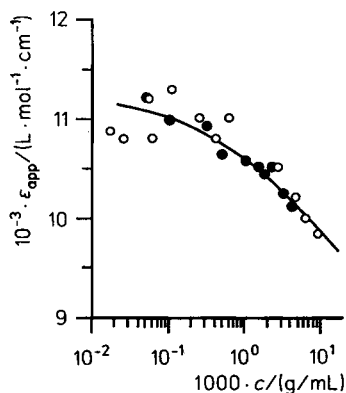


Fig. 6.

Fig. 6. Dependence of apparent extinction coefficient (at 483 nm) on polymer concentration c of directly coupled conjugates **3a** (\circ) and **3c** (\bullet) in $0,01 \text{ M}$ sodium phosphate solution containing $0,1 \text{ M}$ NaCl, pH 7,0

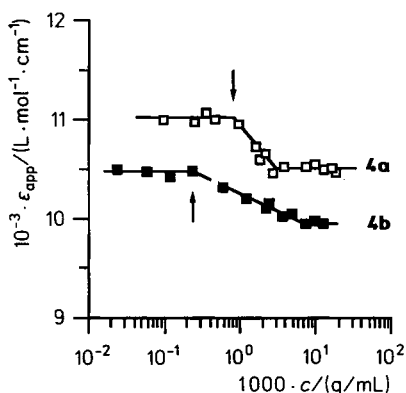


Fig. 7.

Fig. 7. Dependence of apparent extinction coefficient (at 483 nm) on polymer concentration of spacer-coupled conjugates **4a** (\square) and **4b** (\blacksquare) in $0,01 \text{ M}$ sodium phosphate solution containing $0,1 \text{ M}$ NaCl, pH 7,0. Arrows indicate the onset of intermolecular association

concentration, then decrease sharply and attain again constant values at a higher concentration range. The steep transition indicates that the spacer-bound conjugates yield multimers of constant degree of association according to the 'closed association type' mechanism. The critical polymer concentration above which the ϵ_{app} values decrease sharply depends on the ADR load of the conjugates ($8 \cdot 10^{-4} \text{ g} \cdot \text{mL}^{-1}$ for **4a**, $2 \cdot 10^{-4} \text{ g} \cdot \text{mL}^{-1}$ for **4b**). The total change in ϵ_{app} is rather small ($500 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) indicating a minor change in chromophore environment upon multimer formation.

The interaction of polymer-bound ADR molecules was also investigated using fluorescence spectroscopy. Fig. 8 shows the concentration dependence of the normalized fluorescence intensity (observed fluorescence intensity divided by concentration, excitation at 475 nm, emission at 550 nm) of free ADR and the conjugates **3a**–**c**. The fluorescence intensity of free ADR is quenched strongly at concentrations above $3 \mu\text{mol} \cdot \text{L}^{-1}$ due to stacking interaction in the dimer²¹). On the other hand, a remarkable fluorescence quenching was observed for all ADR conjugates even at very low drug concentrations below $1 \mu\text{mol} \cdot \text{L}^{-1}$. This supports the presence of interactions between drug moieties, bound to the same polymer molecule, as concluded from the UV/VIS absorbance data. The fluorescence intensity of the conjugates decreases slightly in the concentration range of 1 – $20 \mu\text{mol} \cdot \text{L}^{-1}$ consistent with the onset of multimer formation observed by UV/VIS absorbance.

Effect of solvent composition and temperature on UV/VIS and fluorescence spectra

The association of ADR conjugates is expected to depend on the hydrophobic interaction between drug molecules as well as on the conformation of the polymeric carrier. At pH 7,0, PGA is anionically charged and assumes an unordered conformation in solutions containing salts, the dimension of PGA being dependent on the ionic strength of the solvent^{18,26}). By adding organic solvent a transition towards an α -helical conformation occurs²⁶). Further, the dimerization equilibrium of anthracyclines is shifted towards free drug in the presence of organic solvents²¹). On this

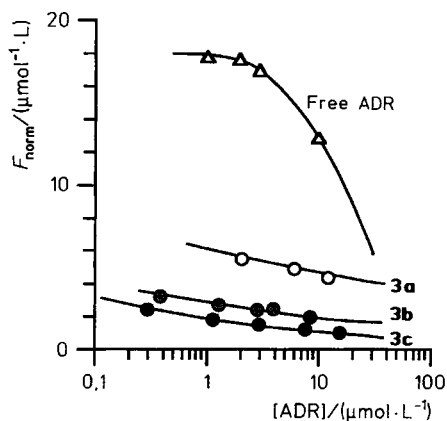


Fig. 8. Dependence of normalized fluorescence intensity F_{norm} on the total drug concentration of free adriamycin and directly coupled conjugates **3a**, **3b** and **3c** in 0,01 M sodium phosphate solution containing 0,1 M NaCl, pH 7,0. $\lambda_{\text{exc}} = 475 \text{ nm}$, $\lambda_{\text{em}} = 555 \text{ nm}$

basis the association of ADR conjugates derived from PGA may be well dependent on the ionic strength and polarity of the solvent.

The effect of salt concentration and methanol addition on the UV/VIS spectra of conjugate **3b** at low concentration ($1 \cdot 10^{-5} \text{ mol} \cdot \text{L}^{-1}$) was studied (Tab. 3). The spectrum measured in a mixture of phosphate buffer (0,01 M sodium phosphate, pH 7,0, no NaCl) and methanol (50/50 v/v) (Tab. 3, run 1) is similar to that of the monomeric form of free ADR. This indicates that covalent linkage of ADR to PGA does not change the spectrum and that no drug-drug interactions in the polymeric conjugate occur. Addition of NaCl to the aqueous methanol solution induces association of polymer-bound drug (Tab. 3, run 2).

Tab. 3. Absorbance spectra data of conjugate **3b** measured in various solvents^{a)}

| Run | Solution composition | | Shape of spectrum ^{b)} | $\epsilon_{\text{app}} \cdot 10^{-3} / (\text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1})$ | |
|-----|--|--------------------|---------------------------------|---|--------|
| | NaCl in $\text{mol} \cdot \text{L}^{-1}$ | Methanol in vol.-% | | 483 nm | 498 nm |
| 1 | 0 | 50 | U | 12,0 | 12,1 |
| 2 | 0,1 | 50 | D | 11,0 | 11,0 |
| 3 | 0 | 0 | D | 11,4 | 11,4 |
| 4 | 0,1 | 0 | D | 10,4 | 10,3 |
| 5 | 1,0 | 0 | D | 9,6 | 9,4 |

^{a)} Measured in phosphate buffer: 0,01 M phosphate, pH 7,0, $[\text{ADR}] = 10 \mu\text{mol} \cdot \text{L}^{-1}$.

^{b)} D: dimer form, U: unimer form.

The effect of increasing ionic strength on the association of the ADR conjugates in purely aqueous solutions is demonstrated by changes in ϵ_{app} . In 0,01 M sodium phosphate, pH 7,0, ϵ_{app} values of **3b** at low concentration ($1 \cdot 10^{-5} \text{ mol} \cdot \text{L}^{-1}$) are lower than in the presence of methanol, thus indicating the presence of drug-drug interactions. With increase of NaCl concentration up to 1 mol/L the ϵ_{app} of **3b** in aqueous solution decreases even further (Tab. 3, runs 3–5).

Fig. 9 shows the dependence of the fluorescence intensity of **3b** and **4b** ($5 \cdot 10^{-6} \text{ mol} \cdot \text{L}^{-1}$) on ionic strength. The fluorescence intensity of both conjugates decreases on increasing the ionic strength indicating enhanced association of polymer-bound ADR molecules. The fluorescence intensity of **3b** is more sensitive to ionic-strength variation than that of **4b** indicating that **3b** is more sensitive to salt-induced polymer contraction and enhancement of hydrophobic interaction. The enhanced association of drug molecules in **3b** at high salt concentration is probably intramolecular because the LALLS data at low conjugate concentration indicate less multimer formation under these conditions (Fig. 3).

The effect of temperature on the association of the ADR bound to the conjugates is illustrated by changes in the normalized fluorescence intensity. Fig. 10 shows that the fluorescence intensity of conjugate **3b** increases with increasing temperature indicating a rather limited stability of the ADR domains. On the other hand, the fluorescence

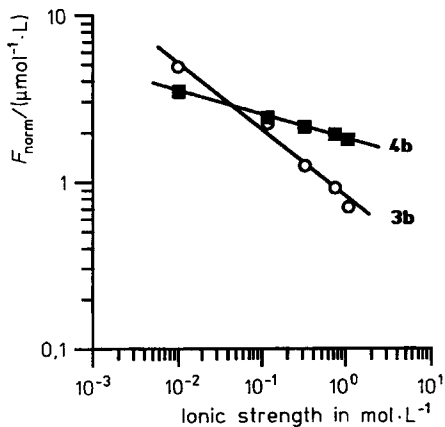


Fig. 9. Effect of ionic strength on the normalized fluorescence intensity of adriamycin conjugates in 0,01 M sodium phosphate solution; pH 7,0; **3b**: directly coupled conjugate (○) and **4b**: spacer-coupled conjugate (■). The ADR concentration for both conjugates was $5 \cdot 10^{-6} \text{ mol} \cdot \text{L}^{-1}$

intensity of conjugate **4b** is almost insensitive to temperature change pointing to a relatively high stability of ADR domains. The temperature effects were found to be reversible.

Discussion

Model for the association of ADR conjugates in solution

From the data presented above we propose a qualitative model for the solution behaviour of each type of ADR conjugate (Schemes 2 and 3). At low ionic strength (0,1 M NaCl) close to physiological conditions the polyelectrolyte-drug conjugates are expanded due to strong electrostatic repulsion between polymer-bound carboxylate groups. The spacer-containing conjugate **4** forms stable intramolecular hydrophobic domains, aided by the flexible spacer arms, at low concentration. At higher concentra-

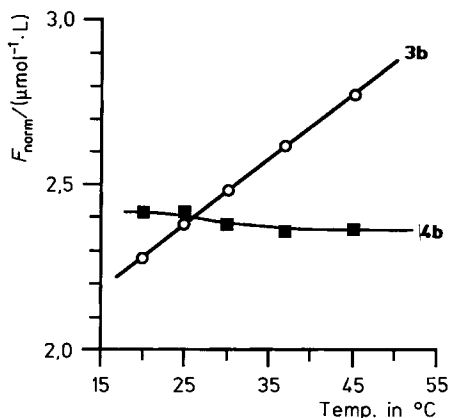


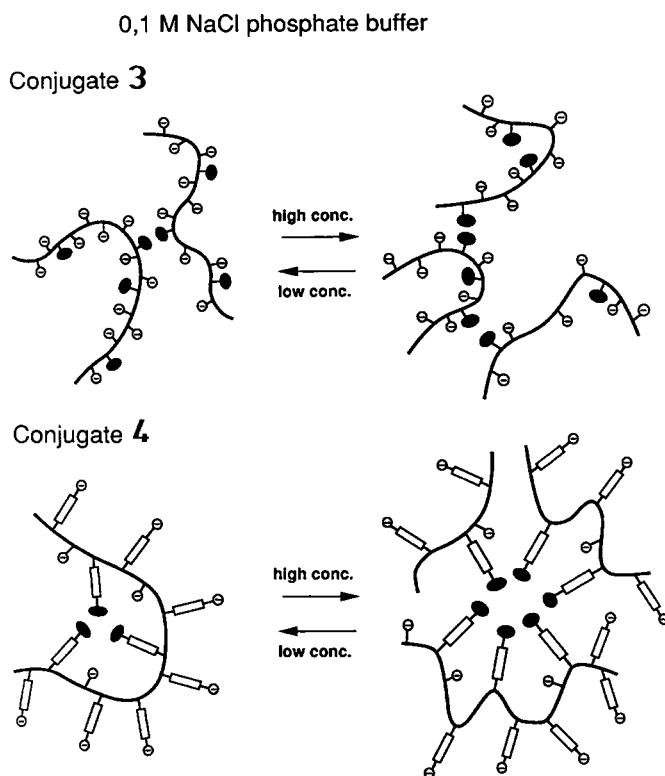
Fig. 10. Effect of temperature on the normalized fluorescence intensity of adriamycin conjugates in 0,01 M sodium phosphate solution containing 0,1 M NaCl, pH 7,0; **3b**: (○) and **4b**: (■). The ADR concentration for both conjugates was $5 \cdot 10^{-6} \text{ mol} \cdot \text{L}^{-1}$

tions the intramolecularly folded structure of **4** is in equilibrium with stable multimers of constant composition. At low concentration the directly coupled conjugate **3** forms weakly stabilized hydrophobic domains partly by intermolecular interaction due to lack of flexibility between ADR molecules and strong repulsive forces. At high concentration the intermolecular association is enhanced, but the limited stability of hydrophobic domains yields randomly associated polymer chains.

By increasing the salt concentration the electrostatic repulsion between polymer-bound charged groups is suppressed, and hydrophobic interaction is enhanced for both conjugates. At low concentration intramolecular hydrophobic domains, presumably of a compact nature, are formed from **3** as well as **4** (Scheme 3). Towards higher concentration, association of polymer chains occurs only for **3** but not for **4** due to its inherently more stable intramolecular structure.

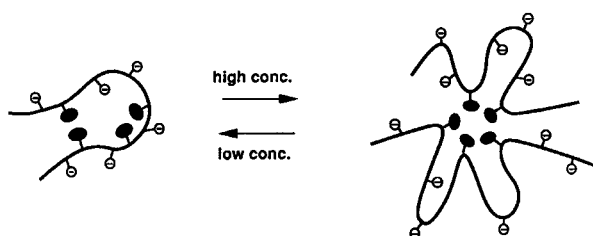
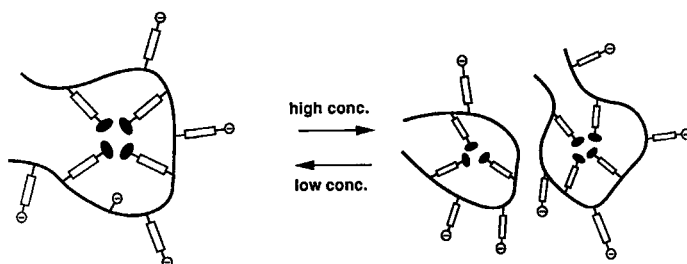
Evidence for multimer formation by ADR conjugates

UV/VIS and fluorescence data on PGA-derived ADR conjugates (Scheme 1) with either 1,3–1,8 mol-% (**3a**, **4a**) or 4–5 mol-% (**3b**, **3c**, **4b**) of bound drug show



Scheme 2: Association behaviour of directly coupled conjugate **3** and spacer-coupled conjugate **4** at low ionic strength (0,1 M NaCl phosphate buffer)

0,5 M NaCl phosphate buffer

Conjugate **3**Conjugate **4**

Scheme 3: Association behaviour of directly coupled conjugate **3** and spacer-coupled conjugate **4** at high ionic strength (0,5 M NaCl phosphate buffer)

evidence for hydrophobic interaction at very low concentrations in 0,1 M NaCl-containing buffer solution. Notably, the shape of the UV/VIS spectra of polymeric conjugates is very similar to that of the dimer of ADR which exists in solution due to the hydrophobic character of the drug aglycone²¹⁻²³). The polymeric conjugates also show a slightly reduced molar absorbance at maximum wavelength as well as strong quenching of drug fluorescence consistent with drug-drug interaction. The tendency of polymer-bound drug moieties to interact may be rationalized by the high local drug concentration within polymer chains. In principle, this interaction between polymer-bound drug moieties can be either intramolecular or intermolecular yielding multimers. The average molecular weights obtained from LALLS data after extrapolation to zero concentration indicate that the directly bound conjugate **3b** associates intermolecularly even at very low concentration, whereas the spacer-containing conjugate **4b** is present as the unimer and drug-drug interaction occurs intramolecularly.

Various techniques provide evidence for intermolecular association of ADR conjugates **3a**, **3b** and **3c** as well as **4a** and **4b**. GPC performed with high conjugate concentration shows the presence of both unimers and multimers in 0,1 M or 0,5 M NaCl-containing solution. Qualitatively, the extent of multimerization is appreciable for conjugate **3b** having ADR bound directly to PGA both in 0,1 M and 0,5 M NaCl solution, but is low (0,1 M NaCl) or undetectable (0,5 M NaCl) for the PGA-GlyGlyGlyLeu-derived conjugate **4b**.

Multimerization of **3b** in 0,5 M NaCl solution is also indicated by the negative value of the virial coefficient from LALLS. This is in accordance with the molecular weight obtained from extrapolation to zero concentration. The presence of unimers of **4b** in 0,5 M NaCl solution, as concluded from the molecular weight value obtained by extrapolation to zero concentration, is corroborated by the almost zero value of the virial coefficient from LALLS. On the other hand, the LALLS data obtained for **3b** and **4b** in 0,1 M NaCl indicate relatively large positive values of the virial coefficient precluding a definite conclusion on multimers. Numerical analysis of the LALLS data¹⁹⁾ is not warranted due to the unknown values of the virial coefficient of the unimer as well as the lack of quantitative data on the unimer-multimer equilibrium.

Both types of conjugate demonstrate concentration-dependent changes in the molar absorbance of the ADR chromophore indicative of multimer formation. Importantly, the multimer structure of the two types of conjugate appears to be different apparently due to the different chemical structure. The directly bound conjugates **3a** and **3c** in 0,1 M NaCl yield open associates involving differently sized multimers as judged from the smooth transition in the concentration dependence of the UV/VIS absorbance and from the GPC elution profile. In contrast, the spacer-containing conjugate **4a** and **4b** in 0,1 M NaCl yield multimers of the closed type characterized by constant association number as indicated by the steep transition in the concentration dependence of UV/VIS absorbance and by GPC.

Stability of hydrophobic domains in ADR conjugates

Various data point to a difference in stability of hydrophobic domains in ADR conjugates. Addition of salt enhances the fluorescence quenching of **3b** to a greater extent than that of **4b**. Increase of temperature enhances the fluorescence intensity of **3b** but not of **4b**. These data indicate that the hydrophobic domains in the spacer-containing conjugate **4b** are more stable compared with the directly-coupled conjugate **3b**. Moreover, the constant ADR absorbance of spacer-containing conjugates **4a** and **4b** at low and high concentration is also consistent with stable ADR domains. Thus, the open-type association of directly bound ADR conjugates **3a** and **3c** may be due to relatively weakly stabilized ADR domains.

Various structural properties of spacer-containing conjugates may contribute to the enhanced stability of ADR domains. The presence of hydrophobic Leu residues in the spacer may well supply additional stability. Further, the spacer can provide enhanced flexibility for interaction of the polymer-bound drug. Also, electrostatic interactions between conjugate carboxylate groups opposing hydrophobic interactions of the drug might be weaker in spacer-containing conjugates due to the lower charge per molar mass and yield more stable ADR domains.

Conclusions

Conjugates of ADR bound to polyelectrolyte carriers yield partly multimeric products in salt-containing solution. For the application as a soluble conjugate for controlled drug delivery such a property is not desirable because the transport of very high-molecular-weight materials in the circulation is slow due to diffusional limitations, and passage between body compartments is restricted due to size barriers^{2,3}). The present results indicate that ADR conjugates having the drug bound to GlyGlyGlyLeu grafts of PGA have a preference for intramolecular folding and may therefore act as better drug carriers compared with directly coupled ADR conjugates which tend to multimer formation. Thus, on the basis of the present results and the previously found lysosomal release of drug from ADR conjugates with GlyGlyGlyLeu spacers¹¹) further studies on this type of conjugate including the therapeutic effectiveness are necessary.

Experimental part

Materials

Tetrahydrofuran (THF) and 1,4-dioxane were stirred with finely powdered potassium hydroxide (10 g/L), filtered and distilled from sodium wire under nitrogen atmosphere. Triethylamine was refluxed with phthalic anhydride and distilled. Trifluoroacetic acid (TFA) was distilled after refluxing with 1 vol.-% of trifluoroacetic anhydride. Methanesulfonic acid (MSA) was used without further purification. All chemicals were obtained from Merck (Darmstadt, F. R. G.). The synthesis of GlyGlyGlyLeu will be described in detail elsewhere²⁷).

Briefly, equimolar amounts of the *N*-hydroxysuccinimidyl ester of *N*-triphenylmethylglycine, GlyGlyLeu (Sigma, USA) and *N*-ethylmorpholine were reacted in DMF for 3 days at room temperature to give, after workup, *N*-triphenylmethyl-GlyGlyGlyLeu, homogeneous as judged from thin-layer chromatography (TLC) and ¹H NMR. The compound was dissolved in 75% acetic acid and stirred for 3 h at room temperature. After workup, GlyGlyGlyLeu was obtained in an overall yield of 68%. The product was homogeneous as judged from TLC, ¹H NMR and amino acid analysis.

Preparation of poly(L-glutamic acid) (PGA)

γ -Benzyl-L-glutamate¹⁰) was reacted with a solution of trichloromethyl chloroformate (TCF) in THF as described²⁸) to give, after two crystallizations from ethyl acetate, the 5-benzyl ester of L-glutamic acid *N*-carboxyanhydride (system. name: 4-(2-benzoyloxycarbonylethyl)oxazolidine-2,5-dione); yield 32%, m. p. 90–91 °C (ref. ¹⁴); 93–94 °C). 13,9 g (52,9 mmol) of the latter was polymerized in 1,4-dioxane (700 mL) using triethylamine (0,15 mL; 1,08 mmol) as the initiator to give poly(γ -benzyl L-glutamate) (PBLG). The weight-average molecular weight of PBLG was determined via intrinsic viscosity at 25 °C in dichloroacetic acid using the following relation²⁹): $[\eta]/(\text{dL} \cdot \text{g}^{-1}) = 2,78 \cdot 10^{-5} \bar{M}_w^{0,87}$.

Debenzylation of PBLG was performed using TFA/MSA¹⁵). PBLG (8,61 g) was dissolved in TFA (70 mL) at room temperature within ca. 1 h. To the solution of PBLG containing anisole (12 mL), MSA was added with stirring at 0 °C. After stirring for 20 min at 0 °C and for 13 min at room temperature (15–18 °C), the reaction mixture was poured into diisopropyl ether. The crude PGA was dissolved in 0,2 M aqueous sodium hydrogen carbonate solution and dialyzed exhaustively against deionized water. The solution was filtered and added to diluted HCl solution (pH 2) with stirring. The precipitate was collected by filtration, washed with water and freeze-dried; the yield was 4,01 g. The ¹H NMR spectrum was in accordance with the structure. The content of residual benzyl groups was calculated from UV absorption (in water, $\lambda_{\text{max}} = 256 \text{ nm}$,

molar extinction coefficient $\epsilon = 176 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). The weight-average molecular weight of PGA was determined by measuring the intrinsic viscosity of the polymer in 0,19 M NaCl, 0,01 M sodium acetate (pH 7,3) at 25 °C using the following relation³⁰: $[\eta]/(\text{dL} \cdot \text{g}^{-1}) = 4,1 \cdot 10^{-5} M_w^{0,94}$.

Preparation of drug-polymer conjugates

The ADR conjugates of PGA were prepared according to the procedure described in the previous paper¹⁰. Conjugate **3** was prepared by direct coupling of ADR onto PGA using *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, EEDQ, as a coupling agent. To a stirred solution of PGA (936 mg, 7,26 mmol) and ADR (203 mg; 0,35 mmol) in freshly distilled DMF (25 mL) containing *N*-ethyl morpholine (99 μL ; 0,776 mmol) was added EEDQ (105 mg; 0,42 mmol), and the mixture was stirred overnight at room temperature. The resulting viscous mixture was diluted with sodium phosphate buffer (0,1 mol \cdot L⁻¹; pH 7,0; 400 mL), and the solution was dialyzed against water and freeze-dried. A solution of the crude conjugate was purified on a polystyrene-based cation exchange column (20 \times 5 cm, Dowex 50W X-8, sodium form) to remove free ADR which binds strongly to the conjugates due to π -complex formation, then filtered (0,45 μm , Flow Pore D) and freeze-dried. To prepare conjugate **4** the oligopeptide spacer was grafted prior to coupling of ADR. To a solution of PGA (224 mg; 1,74 mmol of repeating units) and saccharin (374 mg; 2,04 mmol) in DMF (4 mL) was added *N,N'*-carbonyldiimidazole (380 mg; 2,34 mmol), and the reaction mixture was stirred for 30 min. To this solution was added a solution of the *N,N,N',N'*-tetramethylguanidinium (TMG; 0,23 mL; 1,83 mmol) salt of the oligopeptide GlyGlyGlyLeu (530 mg; 1,75 mmol) in DMF, and the resulting reaction mixture was stirred for 3 days. The mixture was added to a phosphate buffer, and the solution was dialyzed against water, filtered (0,45 μm) and freeze-dried. The product was converted to the pyridinium salt by passing a solution through a column of Dowex 50W X-8, pyridinium form. The eluate fractions containing the polymer were combined and freeze-dried. The coupling of ADR as well as the purification and isolation of spacer-containing conjugate **4** were performed according to the procedure described above for the directly coupled conjugate.

The UV/VIS absorbance spectra of both ADR conjugates, **3** and **4**, are identical with the spectrum of free ADR in the mixture of phosphate buffer (10 mmol \cdot L⁻¹; pH 7,0) and methanol (50/50 v/v). The load of ADR in the conjugates was determined using the molar absorbance at 483 nm, $\epsilon = 12000 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.

Light scattering measurements

Low-angle laser light scattering (LALLS) measurements were carried out using a Chromatix KMX-6 light scattering photometer. Stock solutions of the ADR conjugates for LALLS measurements were prepared by dissolving the sodium salts of the conjugates in phosphate buffer (phosphate 10 mmol \cdot L⁻¹; pH 7,0; NaCl 0,1 mol \cdot L⁻¹), dialyzing exhaustively against the same solvent and passing through filters of pore size 0,45 μm (Flow Pore D). Sample solutions of a certain concentration were prepared by addition of sufficiently filtered dialyzate to the stock solution and were transferred into the light scattering cell through a 0,2 μm filter (Flow Pore D). The concentration of the conjugate solution was determined from the absorbance spectrum using a calibration curve in the same solvent. The refractive index increment of the sample solution was determined by using a Brice Phoenix refractometer.

Gel permeation chromatography (GPC)

GPC was performed on a column of Fractogel TSK HW-55S (Merck, Germany). The column of 85 \times 2,6 cm (inner diameter) was connected to a UV detector (LKB 2138; filter: 278 nm for ADR conjugates and 206 nm for carriers) and differential refractometer (Waters R 403). The eluent was 10 mmol \cdot L⁻¹ sodium phosphate buffer, pH 7,0, containing 0,1 mol \cdot L⁻¹ NaCl; the flow rate was 32,4 mL/h. The sample solution was prepared by dissolving the conjugate (ca. 4 mg) in the eluent (1 mL) and passing the solution through a filter (0,45 μm).

Spectroscopic measurements

Stock solutions for spectroscopic measurements of free ADR and conjugates were prepared in water. Sample solutions of a given ionic strength were prepared by adding an appropriate buffer solution. For example, 1 mL of phosphate buffer (phosphate 0,1 mol · L⁻¹; NaCl 1,0 mol · L⁻¹; pH 6,47) was added to the sample solution in order to prepare 10 mL of phosphate buffer solution of conjugates containing 0,1 mol · L⁻¹ NaCl (phosphate 10 mmol · L⁻¹; pH 7,0). Solutions of the conjugates and ADR were stored at 4 °C in the dark.

UV/VIS spectra were recorded on a Kontron Instruments Uvikon 930 spectrophotometer at room temperature. Fluorescence measurements were carried out with a Perkin-Elmer LS-3 fluorescence spectrometer. The temperature of the cell holder was kept constant by circulating water from a thermostatted bath. The experimental values were corrected for inner filter effects³¹⁾.

- 1) H. Ringsdorf, *J. Polym. Sci., Polym. Symp.* **51**, 135 (1975)
- 2) R. Duncan, J. Kopecek, *Adv. Polym. Sci.* **57**, 51 (1984)
- 3) C. J. T. Hoes, J. Feijen, in *"Drug Carrier Systems"*, ed. by F. H. D. Roerdink, A. M. Kroon, John Wiley & Sons Ltd., Chichester, U.K. 1989, p. 57
- 4) M. Hashida, Y. Takakura, S. Matsumoto, H. Sasaki, A. Kato, T. Kojima, S. Muranishi, H. Sezaki, *Chem. Pharm. Bull.* **31**, 2055 (1983)
- 5) J. Kopecek, in *"Recent Advances in Drug Delivery System"*, ed. by J. M. Anderson, S. W. Kim, Plenum Press, New York 1984, p. 41
- 6) R. Duncan, in *"Controlled Drug Delivery"*, ed. by J. R. Robinson, V. H. L. Lee, Marcel Dekker Inc., New York and Basel, 2nd edition, 1987, p. 581
- 7) H. Bader, H. Ringsdorf, B. Schmidt, *Angew. Makromol. Chem.* **123/124**, 457 (1984)
- 8) K. Ulbrich, C. Konak, Z. Tuzar, J. Kopecek, *Makromol. Chem.* **188**, 1261 (1987)
- 9) Y. Morishima, T. Kobayashi, S. Nozakura, *J. Phys. Chem.* **89**, 4081 (1985)
- 10) W. A. R. van Heeswijk, C. J. T. Hoes, T. Stoffer, M. J. D. Eenink, W. Potman, J. Feijen, *J. Controlled Release* **1**, 301 (1985)
- 11) C. J. T. Hoes, W. Potman, W. A. R. van Heeswijk, J. Mud, B. G. de Grooth, J. Greve, J. Feijen, *J. Controlled Release* **2**, 205 (1985)
- 12) C. J. T. Hoes, W. Potman, B. G. de Grooth, J. Greve, J. Feijen, in *"Innovative Approaches in Drug Research"*, ed. by A. F. Harms, Elsevier, Amsterdam 1986, p. 267
- 13) J. Bouma, J. H. Beijnen, A. Bult, W. J. M. Underberg, *Pharm. Weekbl. Sci. Ed.* **8**, 109 (1986)
- 14) E. R. Blout, R. H. Karlson, *J. Am. Chem. Soc.* **78**, 941 (1956)
- 15) Y. Kato, N. Umemoto, Y. Kayama, H. Fukushima, Y. Takeda, T. Hara, Y. Tsukada, *J. Med. Chem.* **27**, 1602 (1984)
- 16) M. Idelson, E. R. Blout, *J. Am. Chem. Soc.* **80**, 4631 (1958)
- 17) M. J. D. Eenink, *Thesis*, University of Twente, The Netherlands 1987
- 18) R. B. Hawkins, A. Holtzer, *Macromolecules* **5**, 294 (1972)
- 19) H.-G. Elias, in *"Light Scattering from Polymer Solutions"*, ed. by M. B. Huglin, Academic Press, London and New York 1972, p. 397
- 20) E. F. Casassa, H. J. Eisenberg, *Adv. Protein Chem.* **19**, 287 (1964)
- 21) S. R. Martin, *Biopolymers* **19**, 713 (1980)
- 22) J. B. Chaires, N. Dattagupta, D. M. Crothers, *Biochemistry* **21**, 3927 (1982)
- 23) M. Menozzi, L. Valentini, E. Vannini, F. Arcamone, *J. Pharm. Sci.* **73**, 766 (1984)
- 24) V. Barthelemy-Clavey, J. C. Maurizot, J. L. Dimicoli, P. Sicard, *FEBS Lett.* **46**, 5 (1974)
- 25) I. J. McLennan, R. E. Lenkinski, Y. Yanuka, *Can. J. Chem.* **63**, 1233 (1985)
- 26) M. Satoh, J. Komiyama, T. Iijima, *Colloid Polym. Sci.* **258**, 136 (1980)
- 27) C. J. T. Hoes et al., *in preparation*
- 28) M. Oya, R. Katakai, H. Nakai, Y. Iwakura, *Chem. Lett.* **1973**, 1143 (1973)
- 29) P. Doty, J. H. Bradbury, A. M. Holtzer, *J. Am. Chem. Soc.* **78**, 947 (1956)
- 30) M. Morcellet, C. Loucheux, *Biopolymers* **15**, 1857 (1976)
- 31) J. R. Lakowicz, *"Principles of Fluorescence Spectroscopy"*, Plenum Press, New York 1983