Drug Design

Polymer Therapeutics Designed for a Combination Therapy of Hormone-Dependent Cancer**

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Over the last decade, polymer therapeutics have emerged as first-generation nanomedicines.[1] The term polymer therapeutics was coined to include water-soluble polymers that are polymeric drugs,[2] hybrid polymer–drug[1,3] and polymer–protein conjugates,[4] polymeric micelles that contain cova-

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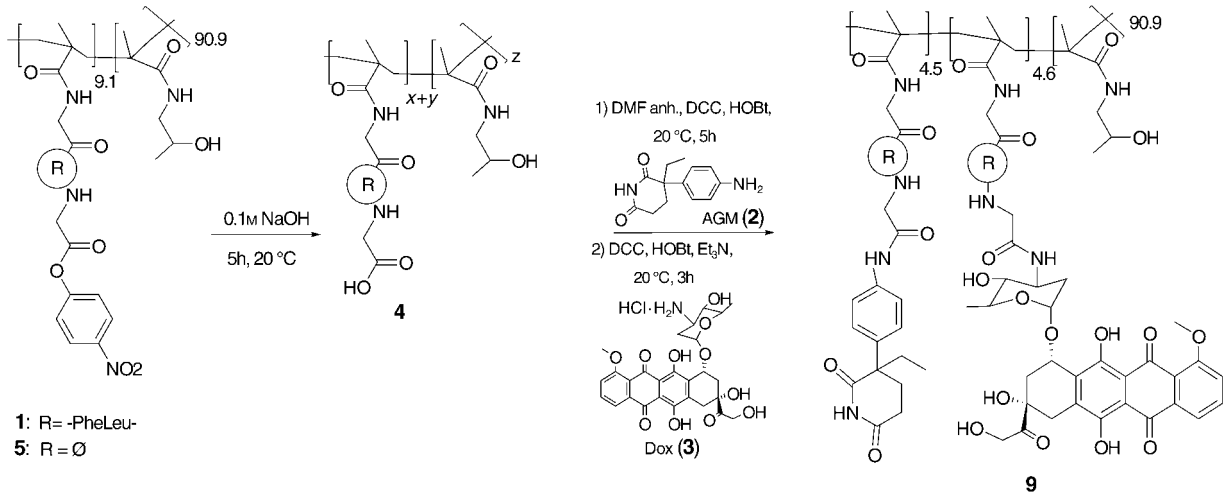
lently bound drug,[5] and multicomponent polyplexes used as nonviral vectors[6] to deliver genes and proteins into the cytosolic compartment of the cell. Of particular relevance to this study are those polymer–drug conjugates now finding acceptance as a new class of antitumor agents.[1,3,6] Polymer conjugates have distinct advantages over conventional chemotherapy. These include 1) passive tumor targeting owing to the enhanced permeability and retention (EPR) effect, a phenomenon that arises from the hyperpermeability of angiogenic tumor blood vessels,[7] 2) lower toxicity of bound drug,[8] and 3) after cellular uptake by the endocytic route, the potential to bypass mechanisms of drug resistance, including p-glycoprotein-mediated multidrug resistance (MDR).[9] So far, only conjugates derived from polyethyleneglycol (PEG),[10] polyglutamic acid (PGA),[11] polysaccharides,[12] and *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugates[13] have progressed to clinical trials. The HPMA copolymer–doxorubicin (Dox) conjugate PK1 (also named FCE28068) was the first synthetic polymer conjugate to enter Phase I trials in 1994.[8] Since then, HPMA copolymer conjugates with established chemotherapy drugs (such as taxol, platinates, or camptothecins)[6,13] and two gamma-camera imaging agents derived from HPMA copolymer have also progressed to clinical testing. Conjugates are now being synthesized to contain experimental drugs[14] and drugs directed towards new therapeutic targets (e.g. antiangiogenic HPMA copolymer–TNP470[15]). Further developments in this field include chemotherapy with two-step polymer–drug combinations (e.g. polymer-directed enzyme prodrug therapy (PDEPT)[16]).

The aim of this study was to design a polymer conjugate that would for the first time combine endocrine therapy and chemotherapy with the hope of eliciting improved antitumor activity in breast cancer. Arrival of the selective oestrogen receptor antagonist tamoxifen led to a 28% reduction in mortality of breast cancer patients at 5 years.[17] Even so, the prognosis for patients with metastatic breast cancer is still poor, the survival rate at 5 years being $<20\%$. The mixed antagonist/agonist activity of tamoxifen and the acquired resistance that can develop over time limit its therapeutic potential.[18] To circumvent this problem, there has been growing interest in the use of aromatase inhibitors. They prevent estrogen biosynthesis by inhibiting P450 aromatase present in normal tissue and in breast-cancer cells of postmenopausal women (77% of the new cases of breast cancer diagnosed each year).[19] Recent clinical trials in these patients showed that the aromatase inhibitors letrozole and anastrozole were more effective in treating estrogen receptor positive breast cancer than tamoxifen.[20]

In our case, we hypothesized that the combination of endocrine therapy and chemotherapy by simultaneous attachment to the same polymer would bring significant advantages. The combination could be administered as a single dose; benefits would be the manufacture of a single conjugate and improved patient compliance. After EPR-mediated targeting, the arrival of both pendant drugs within the tumor cells at the same time is guaranteed. It also provides the opportunity to tailor polymer–drug linkers to impart different rates of drug release for each compound, thus allowing agents to act synergistically. As HPMA copolymer–Dox has already shown activity in chemotherapy refractory breast-cancer patients[8] and this polymer has proven clinical safety, HPMA was chosen as the polymeric carrier (**1** and **5**), and the anthracycline antibiotic Dox (**3**) (widely used as a first-line treatment for breast cancer) was selected as the model chemotherapeutic. The first-generation aromatase inhibitor, aminoglutethimide (AGM; **2**), was chosen as the endocrine model.

A library of conjugates **6**–**11** was synthesized to contain Dox, AGM, or both drugs attached to the same polymeric chain (Scheme 1, Table 1, Supporting Information). HPMA copolymer intermediates ($M_w \approx 20\,000$–$25\,000\ g\,mol^{-1}$; $M_w/M_n = 1.3$–1.5) were used that contained either the tetrapeptide linker Gly–Phe–Leu–Gly (known to be cleaved by lysosomal thiol-dependent protease cathepsin B to release Dox)[6,13] or a Gly–Gly linker (known to be nondegradable and as such to provide a reference control for biological experiments). First, conjugates were prepared by aminolysis of polymeric intermediates in which the C-terminus of the



Scheme 1. Synthesis of HPMA copolymer-AGM-Dox conjugate (**9**′) using DCC coupling reactions. DMF = *N*,*N*-dimethylformamide, DCC = 1,3-dicyclohexylcarbodiimide, HOBt = 1-hydroxybenzotriazole.

Table 1: Characteristics of the HPMA copolymer conjugates.

	Conjugate	Side-chain content [mol%]	Total drug [% w/w][a]		Free drug [% total drug][a]		Rg (±0.3) [nm][d]
			AGM	DOX	AGM	DOX	
6[b]	HPMA copolymer–GFLG–AGM	5	6.2	–	0.75	–	ND
6′[c]	HPMA copolymer–GFLG–AGM	5	6.0	–	0.58	–	7.9
7[b]	HPMA copolymer–GFLG–AGM	10	5.1	–	0.25	–	ND
7′[c]	HPMA copolymer–GFLG–AGM	10	10.4	–	0.59	–	16.5
8[b]	HPMA copolymer–GG–AGM	5	3.4	–	1.17	–	ND
8′[c]	HPMA copolymer–GG–AGM	5	5.4	–	0.61	–	4.1
9[b]	HPMA copolymer–GFLG–AGM–Dox	10	3.1	4.2	0.60	0.10	ND
9′[c]	HPMA copolymer–GFLG–AGM-Dox	10	5.4	7.2	0.73	0.23	12.8
10[b]	HPMA copolymer–GFLG–Dox	5	–	6.6	–	0.39	7.7
11[b]	HPMA copolymer–GFLG–Dox	10	–	14.1	–	0.78	12.4

[a] Total drug and free drug content determined by HPLC. Free drug content expressed as a percentage of total drug. [b] Conjugates synthesized by aminolysis (see supporting information). [c] Conjugates synthesized by DCC coupling (see Supporting Information). [d] Determined by SANS. Fit to a Gaussian coil with Schultz polydispersity. Polydispersity considered 1.3 in all cases. ND = not determined.

peptide side-chains were esterified with *p*-nitrophenol.[21] The lower reactivity of the aromatic amine of AGM, however, favored the use of DCC-mediated coupling. The improved yield of conjugation was particularly noticeable when AGM was bound to the polymeric precursor containing pendant side chains (10 mol%) (Table 1). FTIR and NMR spectroscopy confirmed the identity of the product, and NOE measurements verified the covalent binding of AGM (see Supporting Information). These observations were consistent with previous NOESY and TOCSY experiments, which established the structure of HPMA copolymer–Dox.[22]

Accurate characterization of the bioactive content of any polymer–drug conjugate destined for biological or clinical testing is essential. It is well known that the extinction coefficient of a bound drug can change significantly upon conjugation.[23] In this instance, measurement of the total and free Dox was straightforward, and a validated HPLC method was used.[24] For example, the Dox content of conjugate **9′** was 7.2% w/w (free Dox $< 0.8\%$ w/w total drug) (Table 1). The AGM content was more difficult to determine. Values were obtained either by UV/Vis spectroscopy with an *N*-Gly-AGM (**13**) derivative as standard or by an indirect HPLC method established to quantify residual AGM left after reaction (Table 1 and Supporting Information). Overall, the AGM content of the conjugates was in the range 3.1–10.4% w/w (free AGM content $< 1.2\%$ w/w total AGM) and in conjugate **9′** it was 5.4% w/w (Table 1). The molecular weight of the conjugates was in the range $M_w \approx 25\,000$–$30\,000\ g\,mol^{-1}$.

It has been postulated that polymer–drug conjugates exist in solution as unimolecular micelles and that conformation is influenced by drug loading. At present, however, there is almost no direct physical evidence to support this hypothesis.[25] We have recently demonstrated the value of small-angle neutron scattering (SANS) in providing insight into the solution conformation of polymer therapeutics, particularly in relation to the pH-triggered conformational change of endosomolytic polymers.[26] SANS was used in this case for the first time to define conjugate structure in solution (Table 1 and Supporting Information). The Gaussian coil model for polymer conformation showed the best fit to the raw scattering data. Whereas HPMA copolymer–GFLG–Dox (**10**) and HPMA copolymer–GFLG–AGM (5 mol%) (**6′**) conjugates prepared from the same polymeric intermediate had similar Rg (radius of gyration) values (7.7 and 7.9 nm, respectively; truly nano-sized medicines), an increase in the side-chain content to 10 mol% led to an increase in Rg to 16.5 nm for the AGM conjugate **7′**, but only to 12.5 nm for the Dox conjugate (**11**). Interestingly the HPMA copolymer–GFLG–AGM–Dox conjugate **9′** also displayed an Rg value of 12.8 nm. This might be explained by the tendency of Dox to display π–π stacking, thus leading to the formation of more-compact unimolecular micelles. It is clear that polymer–drug conjugate conformation is affected by both the drug loading and chemical nature of the drug.

Effective functioning of polymer–drug conjugates relies on 1) the stability of the polymer–drug linker in the circulatory system and, after arrival in the tumor tissue and cellular uptake by endocytosis, 2) lysosomal enzyme cleavage of the linker to release the active drug. All the HPMA copolymer conjugates were completely stable in buffer alone, as was the HPMA copolymer–GG–AGM (**8′**) in the presence of isolated rat liver lysosomal enzymes (Figure 1c and Supporting Information). However, in the presence of lysosomal enzymes, Dox and AGM were released from conjugates containing the Gly–Phe–Leu–Gly linker. The drug-release profile displayed marked differences depending on conjugate composition. For conjugates **10** and **11**, Dox release began immediately after addition of enzyme (Figure 1b) and was linear with time; it was apparent that the lower the Dox loading (cf. **10** and **11**) the greater the drug release after 5 h (see Supporting Information). This is in agreement with the solution conformation predicted by SANS (Table 1). Enzyme accessibility would be limited by a more compact coil structure. AGM release (conjugates **6** and **7**) also showed dependence on drug loading with a lower initial release for the highest loading (**7**) (Figure 1a). After 30 min, however, AGM liberation from **7** accelerated and release from **6** started to plateau (see supplementary information for comparison at 5 h).

When incubated with lysosomal enzymes, the conjugate containing both AGM and Dox as the combination (**9′**)

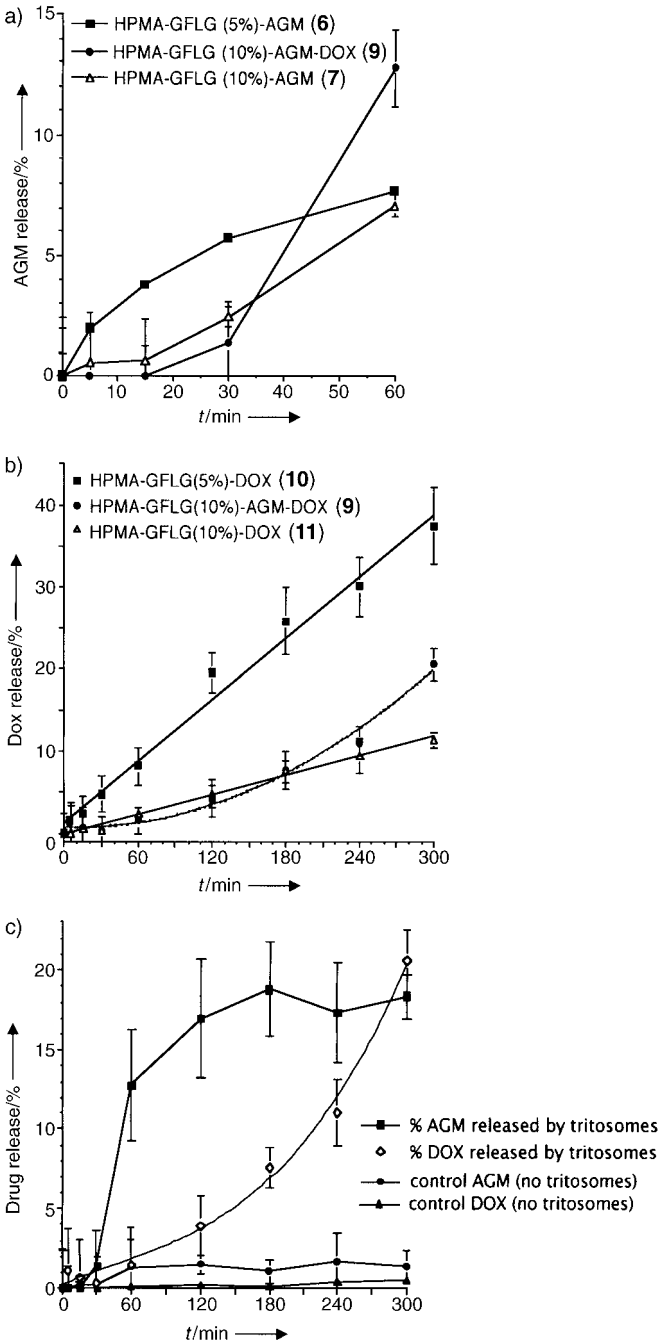


Figure 1. Liberation of Dox or AGM from HPMA copolymer conjugates during incubation with rat liver lysosomal enzymes (tritosomes). Release of a) AGM, b) Dox, and c) Dox and AGM from the HPMA copolymer–GFLG–AGM–Dox conjugate (**9′**). In all cases, drug release is expressed as a percentage of the total drug bound, and data represent the mean ± SEM ($n=4$). In specific cases, the release of drug in the absence of enzymes (control) is also shown.

displayed different profiles of drug liberation (Figure 1c) in comparison with that seen for the single-drug-pendant conjugates (at both 5 and 10 mol % loading). Both Dox and AGM release show a marked lag phase with little release over the first 30 min. However, after this time, AGM release was enhanced relative to **6** and **7**, and the Dox release rate was initially slower but then returned to that seen for **10**. The dynamic and complex geometry of this unimolecular micelle structure determines enzyme access leading to these complex release kinetics. To investigate how the release profile would translate into cytotoxicity, a human estrogen-dependent breast-cancer cell line MCF-7 and the aromatase-transfected derivative MCF-7ca were used (Figure 2 and Supporting Information). As expected, as a result of its slow rate of cellular uptake by endocytosis and the relatively prolonged duration needed for drug liberation, HPMA copolymer–GFLG–Dox (**10**) was much less active than free Dox (**3**). For example, their IC_{50} values against the MCF-7 cell line were >150 μM and 0.74 μM Dox-equivalents, respectively. Neither the HPMA copolymer–GFLG–AGM (**6′**), nor individual mixtures of drug conjugates bearing AGM (**6′**) or Dox (**10**) caused an elevation in cytotoxicity in either cell line MCF-7 or MCF-7ca (see Supporting Information).

In contrast, when AGM and DOX were covalently linked to the same polymeric backbone, that is, in conjugate **9′**, the in vitro cytotoxicity was significantly greater than seen for **10**, **3**, or **6′** against MCF-7 cells. The enhanced cytotoxicity of **9′** was even more evident for the aromatase expressing MCF-7ca cells (see Figure 2 and Supporting Information). The IC_{50} values obtained for MCF-7ca cells incubated with **3**, **6′**, or **9′** were 5238 μM, >1377 μM, and 40.9 μM AGM-equivalents, respectively. Conjugate **9′** displayed approximately tenfold enhancement in cytoxicity in the aromatase cell line, irrespective of whether the data are expressed in terms of Dox- or AGM-equivalents Although it is necessary to understand the precise molecular mechanism of action of these novel conjugates better, it has been reported that aromatase inhibitors can also promote apoptosis, so they may potentiate Dox cytotoxicity in this way.[19]

To conclude, the HPMA copolymer–GFLG–AGM–Dox conjugate (**9**) is the first polymer therapeutic to combine chemotherapy and endocrine therapy. The fact that AGM and Dox can act synergistically to produced markedly enhanced cytotoxicity in vitro relative to that of the HPMA copolymer–GFLG–Dox conjugate (**10**) (PK1), which has already shown activity in breast-cancer patients clinically, underlines the potential importance of this polymer–drug combination. It should be emphasized that mixtures of polymer conjugates containing only AGM or only Dox did not show synergistic benefit. The studies reported confirm cellular uptake of conjugate by endocytosis (verified by flow cytometry as shown in Figure 2b and in the Supporting Information), and also demonstrate the requirement of lysosomal thiol-dependent protease degradation of the polymer–drug linker to liberate drug and thus promote cytotoxicity. All the components (HPMA copolymers, Dox and AGM) are well established clinically, so further preclinical studies are warranted to establish the antitumor potential of this novel anticancer strategy in vivo. The observation that HPMA copolymer conjugates displayed lower hemolytic activity (10 % release at 1 h; $p<0.001$) than Dox (**3**) (92 % hemoglobin release at 1 h) (see Supporting Information) establishes the suitability for intravenous administration. Tailoring of the linker structure and drug loading within this novel nano-sized medicine (Rg in solution of 12.8 nm) may further optimize the drug-release

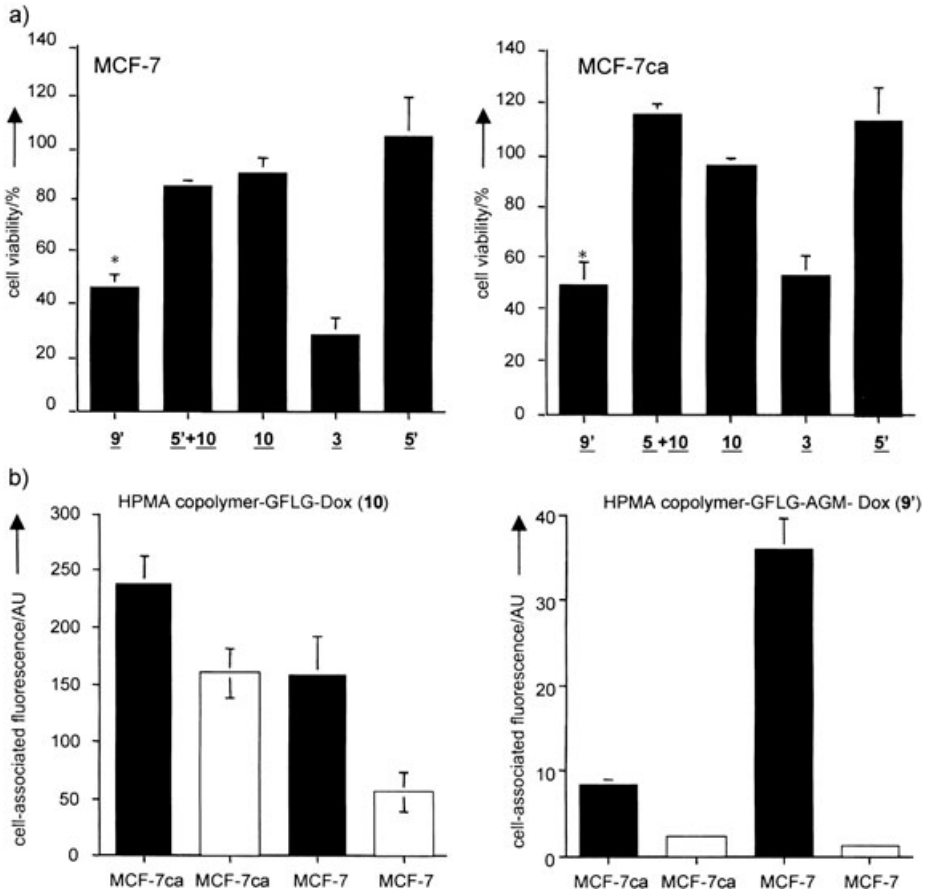


Figure 2. a) Cytotoxicity of Dox and HPMA copolymer conjugates against MCF-7 and MCF-7ca cell lines at the IC_{50} value (0.08 mg mL^{-1} and 0.009 mg mL^{-1} Dox-equivalents, respectively) in the presence of 10^{-9} M estradiol. Measured by MTT assay after 72 h incubation. Data are expressed as mean ± SEM ($n = 3$). The asterisk* indicates statistical significance ($P < 0.05$). b) Cell-associated fluorescence at 37 °C (total association, ■) and 4 °C (external binding, □) of PK1 and HPMA copolymer–AGM–Dox conjugate in MCF-7 and MCF-7ca cells after 1 h. Data are expressed as mean ± SEM ($n = 6$).

profile and cytotoxicity. Ongoing studies to define the precise molecular mechanisms of conjugate action will be used to steer the choice of chemistry used to achieve optimal release kinetics.

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