Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Polymer Therapeutics designed as a Novel Combination Therapy for the Treatment of Hormone-Dependent Cancer**

María J. Vicent,* Francesca Greco, Robert I. Nicholson, Alison Paul, Peter C Griffiths, and Ruth Duncan*

1. Instruments. NMR spectra were recorded on a Bruker AM250 FT-NMR or a Varian 300 spectrometer at 300 MHz for ^{1}H NMR and at 75 MHz for NOE experiments, with TMS as an internal standard. Chemical shifts (δ) are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad singlet). GPC analysis was performed using a JASCO HPLC pump, with two TSK-gel columns in series (G3000 PW followed by G2000 PW) and a guard column (Progel PWXL). The eluent was monitored using a differential refractometer (Gilson 153) and an UV-visible spectrophotometer at 254 nm (UV Savern Analytical SA6504) in series. A flow rate of 1 mL min^{-1}, and a mobile phase 0.1 M PBS buffer was used. PL Caliber Instrument software was used for data analysis. HPLC was performed using one or two JASCO HPLC pumps with a gradient mixer with a μBondapak C18 (150 x 3.9 mm) column. The UV spectra were recorded on a Shimadzu UV-1601 UV-Vis spectrophotometer. Thin-layer chromatography was performed on Merck silica gel plates (DC-60 F254 and Kieselgel ALU 60 F254). Uptake studies were performed with a FACSCalibur flow cytometer (Becton Dickinson, UK) equipped with a single argon laser (excitation wavelength 486 nm).

2. Materials. HPMA copolymer precursors, carrying either a Gly-Gly-*p*-nitrophenol ester (ONp) (5 mol %; Mw~20.000-25.000 g mol^{-1} and Mw/Mn = 1.3-1.5) or Gly-Phe-Leu-Gly-ONp (either 5 or 10 mol %; Mw~20.000-25.000 g mol^{-1} and Mw/Mn = 1.3-1.5) were from Polymer Laboratories Ltd, Shropshire, U.K. The bound ONp content of polymeric precursors was calculated using $\varepsilon_{274\ \mathrm{nm}}$ = 9500 L mol^{-1} cm^{-1} (in DMSO). Anhydrous DMF and DMSO were from Sigma-Aldrich Company Ltd., Dorset, UK and all HPLC grade solvents by Fischer Scientific UK, Manchester. Medical grade O_2, N_2 and CO_2 (all 95 % v/v) and liquid nitrogen were supplied by BOC Gases, Surrey, UK. All other reagents were of general laboratory grade and were purchased from Aldrich unless otherwise stated.

The MCF-7 and MCF-7ca cell lines were from the Tenovus Centre for Cancer Research at Cardiff University. Tissue culture grade DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypan blue solution (0.4%) (cell culture grade), optical grade DMSO and oestradiol were from Sigma (Dorset, UK), 0.25% trypsin-EDTA, fetal calf serum (FCS), RPMI 1640, with L-glutamine and RPMI 1640 without phenol red, with L-glutamine were from Gibco BRL Life Technologies (Paisley, UK). T-octylphenoxypolyethoxyethanol

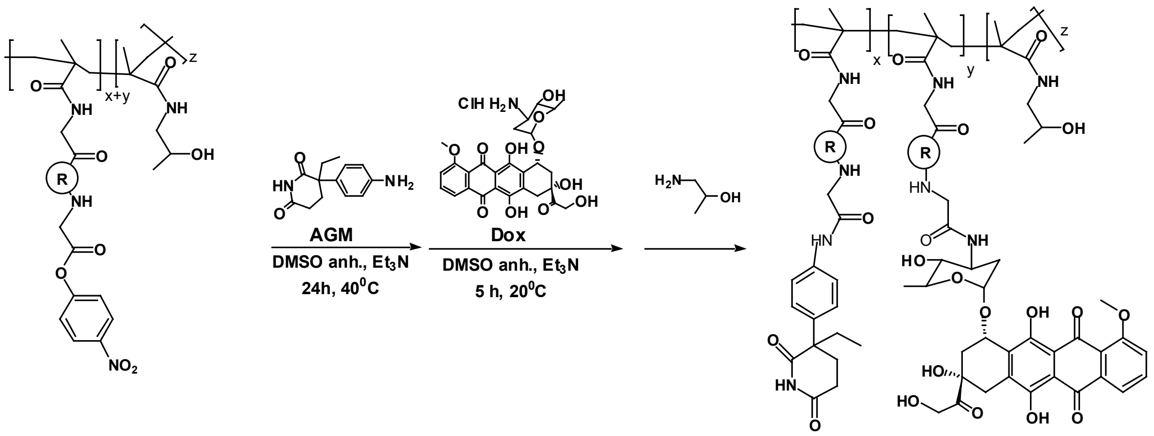
(Triton X-100), dextran (Mw = 74,000 g mol^{-1}), poly(ethyleneimine) (PEI) Mw = 750,000 g mol^{-1} were from Sigma-Aldrich Company Ltd., Dorset, UK. Sephadex^{TM} LH20 was supplied by Amersham Biosciences AB, Uppsala, Sweden.

3. Synthesis of HPMA copolymer-AGM±Dox conjugates.

3.1 By aminolysis reaction.

HPMA copolymer-AGM conjugates **6** – **8**: HPMA copolymer precursors **1** or **5** (1 equiv., calculated as ONp) and **2** (1 equiv.) were dissolved in a minimal volume of dry DMSO. Triethylamine (1 equiv.) was added dropwise to the copolymer solution as basic catalyst. The reaction was monitored spectrophotometrically (release of ONp at 400 nm) and also by TLC using MeOH : CH_2Cl_2 2:98 (v/v) as the mobile phase (the R_f of **2** = 0.54). AGM conjugates remain at the origin. The R_f for ONp = 0.9. The reaction was allowed to proceed for 24 h 40^0C and then quenched with 1-aminopropan-2-ol (1 equiv.).

Scheme 1S. Synthesis of HPMA copolymer–drug conjugates by aminolysis.



1a: R= -PheLeu- x+y= 9.1, z= 90.9
1b: R= -PheLeu- x+y= 4.9, z= 95.1
5: R= ∅ x+y= 4.2, z= 95.8

6: R= -PheLeu- y= 0, z= 95.1
7: R= -PheLeu- y= 0, z= 90.9
8: R= ∅ y= 0, z= 95.8
9: R= -PheLeu- z= 90.9
10: R= -PheLeu- x= 0, z= 95.1
11: R= -PheLeu- x= 0, z= 90.9

HPMA copolymer-AGM-Dox conjugate **9**: HPMA copolymer -Gly-Phe-Leu-Gly-ONp (10 mol % ONp groups) **1b** (100 mg, 0.05 mmol) was dissolved in DMSO anh. (1 mL). Then solutions of AGM (4.8 mg, 0.025 mmol) in DMSO (0.3 mL) and triethylamine (0.04 mmol/0.2 mL DMSO) were added dropwise. The reaction was allowed to proceed for 8 h at 50 ^{o}C and at 40 ^{o}C overnight. After cooling to RT, a solution of Dox·HCl (10.4 mg, 0.017 mmol) in DMSO anh. (0.2 mL) and triethylamine (0.017 mmol/0.2 mL DMSO) were added dropwise. The reaction was left overnight at RT in the dark, and then quenched with 1-amino-2-propanol (5 μL). The reaction was monitored spectrophotometrically by measuring the release of ONp at 400 nm and also by TLC using MeOH : AcOH 98:2 (v/v) as the mobile phase (the R_f of **2** = 0.54; the R_f of **3** = 0.54. HPMA copolymer-drug conjugates remain at the origin; the R_f of ONp = 0.9.

HPMA copolymer-Dox conjugates **10** – **11**. HPMA copolymer precursor **1** (1 equiv., calculated as ONp) and **3** (0.8 equiv., respectively) were dissolved in a minimal volume of dry DMSO. Triethylamine (1 equiv.) was added dropwise. The reaction was monitored spectrophotometrically by measuring the release of ONp at 400 nm and also by TLC using AcOH : n-BuOH: H_2O 0.5: 93:6.5 (v/v) as the mobile phase. The R_f of **3** = 0.54, HPMA copolymer Dox conjugates remain at the origin; the R_f of ONp = 0.9. The reaction was allowed to proceed for 5 h at RT and then quenched with 1-aminopropan-2-ol (1 equiv.).

3.2 By DCC coupling.

HPMA copolymer-COO$^-$Na$^+$ **4**: HPMA copolymer precursor **1** or **5** (1 equiv. ONp groups) was dissolved in 0.1M NaOHaq (3 equiv.) and stirred for 4 h at RT and the reaction was monitored by measuring ONp displacement at 400 nm. The reaction mixture was then purified by dialysis (2 days, 15,000 Mw membrane cut-off) and freeze-dried to yield **4** as a white solid (89%).

HPMA copolymer-AGM conjugates **6'** – **8'**: **4** (1 equiv. COO$^-$ groups) was dissolved in a minimal amount of dry DMF, and DCC (1.5 equiv.) was then added as solid. After 10 min 1-hydroxy-benzotriazole (HOBt) (1.5 equiv.) was also added as solid and the reaction mixture was stirred for 30 min under N_2 atmosphere at RT **2** (1 equiv.) was then added and the reaction was allowed to proceed for 5 - 8 h at RT. The precipitated urea was filtered-off and DMF was evaporated under reduced pressure.

HPMA copolymer-AGM-Dox conjugate **9'**: **4** (1 equiv. COO^- groups) was dissolved in a minimal amount of dry DMF, and DCC (1 equiv.) was then added as solid. After 10 min HOBt (1 equiv.) was also added as solid and the reaction mixture was stirred for 30 min under N_2 atmosphere at RT. **2** (0.5 equiv.) in a minimal amount of dry DMF was added and the reaction was allowed to proceed for 5 h at RT. DCC (1 equiv.) and HOBt (1 equiv.) again as solids, were added 15 min before the Dox·HCl (0.5 equiv.) solution in DMF anh. together with triethylamine (0.5 equiv.). After stirring for 3 h at RT the precipitated urea was filtered off and DMF was evaporated under reduced pressure.

In all cases, the solvent was partly removed under high vacuum and the resulting residue was precipitated into a vigorously stirred mixture of acetone : Et_2O (4 : 1). It was filtered off and washed with acetone and diethyl ether. The polymer conjugate was purified by gel filtration chromatography using Sephadex LH20 (column 5 x 50 cm, eluent MeOH) and the purified compound was then dissolved in a minimal amount of water and freeze-dried. The overall yields based on polymer weight were 70-80 %. The filtration residues and also the impure fractions from the chromatographic column were kept and evaporated in order to analyse the total content of AGM in conjugates by indirect analysis, described below.

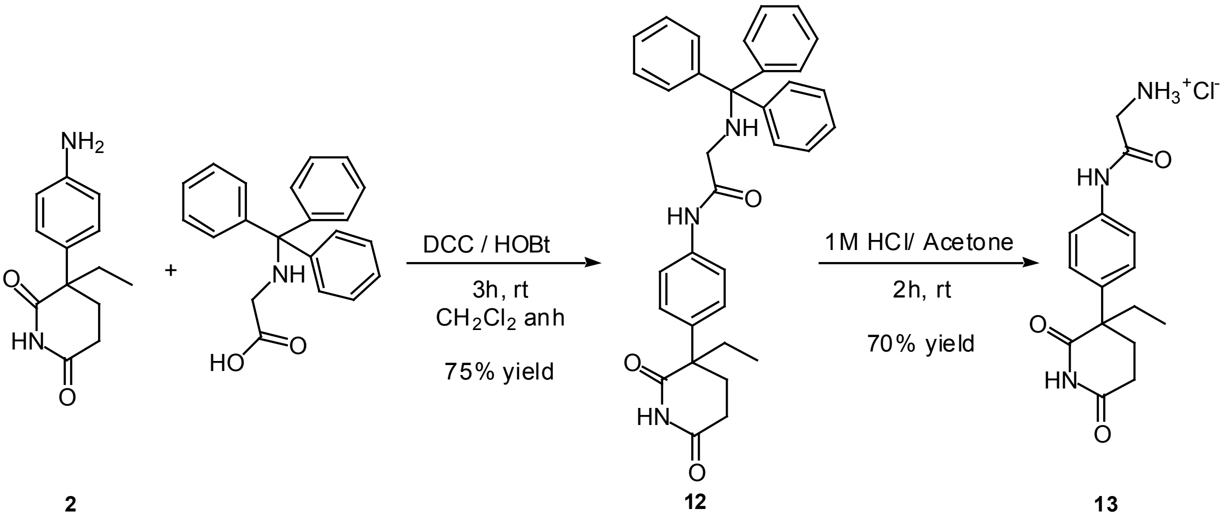
4. Characterization of HPMA copolymer-AGM±Dox conjugates **6** – **11**.

4.1 Determination of total AGM content by UV spectroscopy. An aminoacid-AGM derivative Gly-AGM **13** was first prepared for use as a calibration standard (Scheme 2S). A stock solution of **13** in DMSO was prepared (0.5 mg mL^{-1}). To obtain a calibration curve, samples were diluted using DMSO to give a concentration range of 0 - 500 µg mL^{-1}. The total drug loading of the conjugates was determined by measuring the optical density at 254 nm in DMSO. HPMA copolymer control **14** in the same concentration range as the conjugates analyzed (0 - 5 mg mL^{-1}) was used as blank.

4.2 Synthesis of Gly-AGM (**13**).

N-Trytil-Gly-AGM **12**. Over a homogeneous solution of AGM (30 mg, 0.13 mmol) in anhydrous CH_2Cl_2 (1 mL) under N_2 atmosphere DCC (40.03 mg, 0.195 mmol) was added as solid. After 15 min OHBt (26.21 mg, 0.195 mmol) was also added and the mixture was stirred for 30 min. N-Trytil-Glycine (50 mg, 0.157 mmol) in CH_2Cl_2 anh. (0.2 mL) was then added dropwise and the reaction was allowed to proceed overnight at RT. The precipitated urea was filtered off and CH_2Cl_2 was evaporated under reduced pressure. The crude product was purified by silica gel column chromatography ($CHCl_3$: MeOH; 100 : 0 → $CHCl_3$: MeOH; 90 : 10) to yield N-trytil-Gly-AGM (**12**) (51 mg, 75 %) as a white solid. NMR (δ_H, 300 MHz, DMSO-d^6): 10.9 (s, 1H), 9.9 (s, 1H), 7.6-7.2 (4m, 19H), 7.1-6.4 (m, 8H), 3.0 (d, 2H), 2.5 (br, 1H), 1.8-1.4 (m, 4H), 1.4-0.9 (m, 2H), 0.8 (t, 3H). NMR (δ_C, 300 MHz, DMSO-d^6): 176.1, 175.9, 173.3, 145.9, 145.7, 144.8, 141.0, 134.7, 129.5, 128.7, 128.3, 128.2, 128.1, 127.8, 127.1, 126.9, 126.7, 125.3, 120.0, 68.3, 49.2, 47.8, 33.7, 25.6, 9.26. MS (ESI-positive) m/z: 554 $[M+Na]^+$.

Scheme 2S. Synthetic scheme for Gly-AGM



Deprotection. **12** (40 mg, 0.075 mmol) was dissolved in acetone (3 mL) and the pH adjusted to pH = 2 with 1 M HCl, then the reaction was stirred for 2 h at RT. Acetone was evaporated under reduced pressure and the crude was dissolved in a minimal amount of H_2O and washed with $CHCl_3$ (3x) and Et_2O (3x), then freeze-dried to yield **13** (17 mg, 70 %) as a white solid.

M.p.- 320-325 ºC. NMR (δ_H, 300 MHz, DMSO-d^6): 10.9 (s, 1H), 9.9 (s, 1H), 6.9 (d, 2H, *J =0.2 Hz*), 6.6 (d, 2H, *J =0.2 Hz*), 7.69 (d, 1H, *J= 4.9 Hz*), 7.42 (dd, 1H), 3.6 (d, 2H), 2.4-1.9 (m, 6H), 0.9 (t, 3H). NMR (δ_C, 300 MHz, DMSO-d^6): 176.1, 175.9, 173.1, 143.2, 139.8, 126.9, 126.7, 121.3, 120.9, 46.1, 45.8, 44.3, 33.7, 25.6, 9.26. MS (ESI-positive) m/z: 348 $[M+Na]^+$.

4.3 Determination of total AGM content by HPLC. The dried residue obtained from the conjugation reactions was dissolved in CH_2Cl_2 and all remaining precipitate was filtered off. AGM is completely soluble in CH_2Cl_2. The solvent was evaporated under reduced pressure and MeOH (5 mL) was added to make the stock solution. 100 µl aliquots (3 x) were placed into polypropylene tubes and made up to 1 mL with water. The pH of the samples was adjusted to 8.5 with ammonium formate buffer (100 µL, 1 M, pH 8.5), and then a mixture of chloroform : propan-2-ol at a ratio of 4 : 1 (5 mL) was added. Dnm was used as internal reference standard; 100 µL of a 1 µg mL^{-1} stock aqueous solution was added to each sample. Samples were then thoroughly extracted by vortexing (3 x 10 sec). The upper aqueous layer was carefully removed and the solvent was evaporated under N_2. The dry residue was dissolved in 100 µL of HPLC grade methanol. In parallel the same procedure was carried out for the parent compound **2** (using 100µL of a 1 mg mL^{-1} stock aqueous solution). Addition of 1 mL of methanol to redissolve the product gave a 100 µg mL^{-1} stock from which a range of concentrations were prepared (2 to 60 µg mL^{-1}). The free amount of drug in the conjugates was determined by HPLC using a µBondapak C18 (150 x 3.9 mm) column. Flow rate 1 mL min^{-1} and a gradient elution was used (solvent A: 2-propanol : H_2O 12 : 88 (v/v), solvent B: 2-propanol : H_2O 29 : 71 (v/v) adjusted to pH 3.2 with *o*-phosphoric acid. Total run time was 20 min and the gradient profile was: t = 5 min A 100%, t = 9 min A 0%, t = 14 min A 0%, t = 16 min A 50%, t = 18 min A 100% and t = 20 min A 100%). The retention time was = 4.93 min for AGM.

To monitor **2** an UV detector (Spectroflow 783 Kratos analytical) with a fixed-wavelength filter (254 nm) was used and a Fluoromonitor III fitted with interference filters at 485 nm for excitation and 560 nm for emission was used to monitor the Dnm standard.

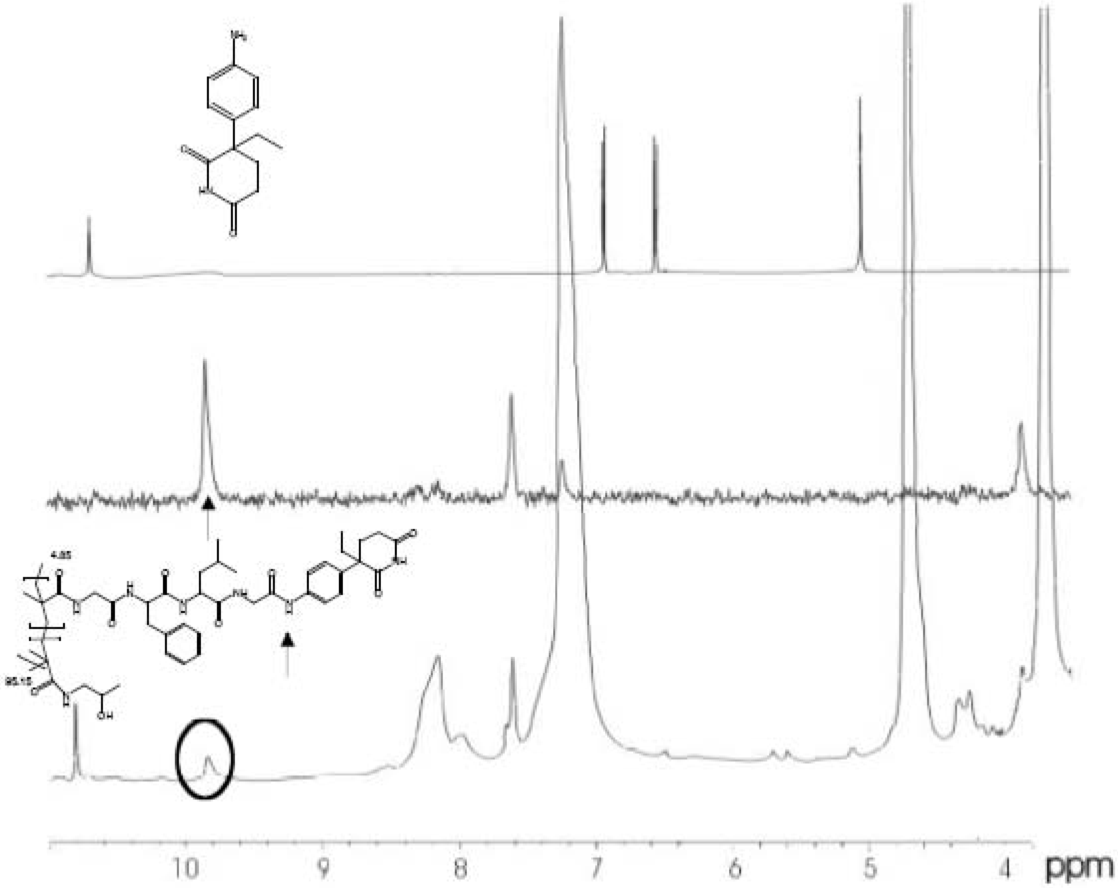
4.4 Determination of total Dox content by HPLC. Aqueous solutions of HPMA copolymer-AGM±Dox conjugates (1 mg mL^{-1}) were prepared, and an aliquot (100 µL) was added to a polypropylene tube and made up to 1 mL with water. Then 1 mL of 2 M HCl was added and the tubes were heated at 80 ^{0}C for 30 min in order to get Dox aglycone. After cooling down to RT. 1 mL 2 M NaOH and the pH of the samples was adjusted to 8.5 with ammonium formate buffer (100 µL, 1 M, pH 8.5). In parallel the same procedure was carried out for the parent compound **3** (using 100 µL of a 1 mg mL^{-1} stock aqueous solution). The extraction procedure and HPLC run as described above. The retention time was 13.3 min for Dox aglycone. Dnm was also used here as internal standard.

4.5 Determination of free AGM/Dox content by HPLC. Aqueous solutions of HPMA copolymer-AGM±Dox conjugates (1 mg mL^{-1}) were prepared, and an aliquot (100 µL) was added to a polypropylene tube and made up to 1 mL with water. The extraction procedure as described above, using for standard stock 10 µg mL^{-1} AGM instead of 100 µg mL^{-1} and also a 10 µg mL^{-1} Dox stock. HPLC conditions also as described for total AGM content characterization, t_r = 10.89 min for Dox.

4.6 Determination of covalent bond in HPMA copolymer-AGM conjugates by ^{1}H-NMR. Covalent linkage of AGM to the polymeric precursor was verified by NMR using the appearance of the characteristic band for aromatic amide H at 9.8 ppm and by the presence of NOE correlation with the *para*-aromatic system of the AGM. At 7.2 and 7.6 ppm this para system is also displaced from the original 6.4 and 6.8 ppm. Correlation of the amide group with the methylene groups present in the polymer linker is also possible using NOE (Figure 1S).

4.7 Use of Small Angle Neutron Scattering (SANS) to characterise HPMA copolymer-Dox±AGM conjugates. SANS experiments were performed on the LOQ time-of-flight diffractometer on ISIS at the Rutherford Appleton Laboratories, Chilton, Didcot, Oxfordshire, UK. The accessible Q-range was 0.008-0.30 Å^{-1}.

Figure 1S. NOE ^{1}H-NMR spectra of HPMA copolymer-GFLG(10 mol%)-AGM (7)



Samples were prepared as 3 wt% solutions in D_2O and placed in 2 mm path-length quartz cells, mounted in a sample changer thermostatted to 37 ± 0.2 °C. Measurement acquisition times were approximately 30 min. Data were corrected for the scattering and transmission of the solvent and cell, and they were placed on an absolute intensity scale by reference to a well-characterized polymer standard. The scattering is described in terms of the scattering vector, Q which is given by $Q=4n\pi/\lambda \sin(\theta/2)$ in which n is the refractive index (~1), λ the wavelength and θ the scattering angle. The scattering intensity, I(Q) is described by equation (1) in terms of the relative contributions of the form factor, P(Q) (which describes the size and shape of the scattering body, considered as a solid particle), the structure factor, S(Q) (which describes interactions between different scattering bodies), and B_{inc} which is a flat background arising from incoherent scattering, principally from hydrogenated material within the sample.

$$I(Q) = \phi V_p (\Delta\rho)^2 P(Q)S(Q) + B_{inc} \qquad (1)$$

The terms $\phi V_p(\Delta\rho)^2$ is the scale factor dependent on the sample volume fraction, ϕ, particle volume, V_p, and contrast term $(\Delta\rho)^2$ which describes the difference in scattering length density between the solvent and the polymer, $\Delta\rho = \Delta_{solvent} - \Delta_{polymer}$. Since hydrogen and deuterium are at opposite ends of the scattering length density scale, in these systems contrast is achieved by dissolving polymer in D_2O. At 3 wt% the samples are below the critical overlap concentration, (a point verified by a concentration scan experiment performed previously, which gave identical shape scattering curves, scaled only by the change in ϕ)[i]. Hence the scattering depends only on the scale factor, background and P(Q)

$$\mathbf{I(Q) \propto P(Q) + B_{inc}.} \qquad (2)$$

In order to obtain information of the size adopted by the polymer in solution, the scattering is considered in terms of equation 2, which describes a simple Gaussian polymer coil. This gives the polymer radius of gyration, Rg, where p is the polydispersity (for these polymers p was assumed to be at 1.3) (Figure 2S).

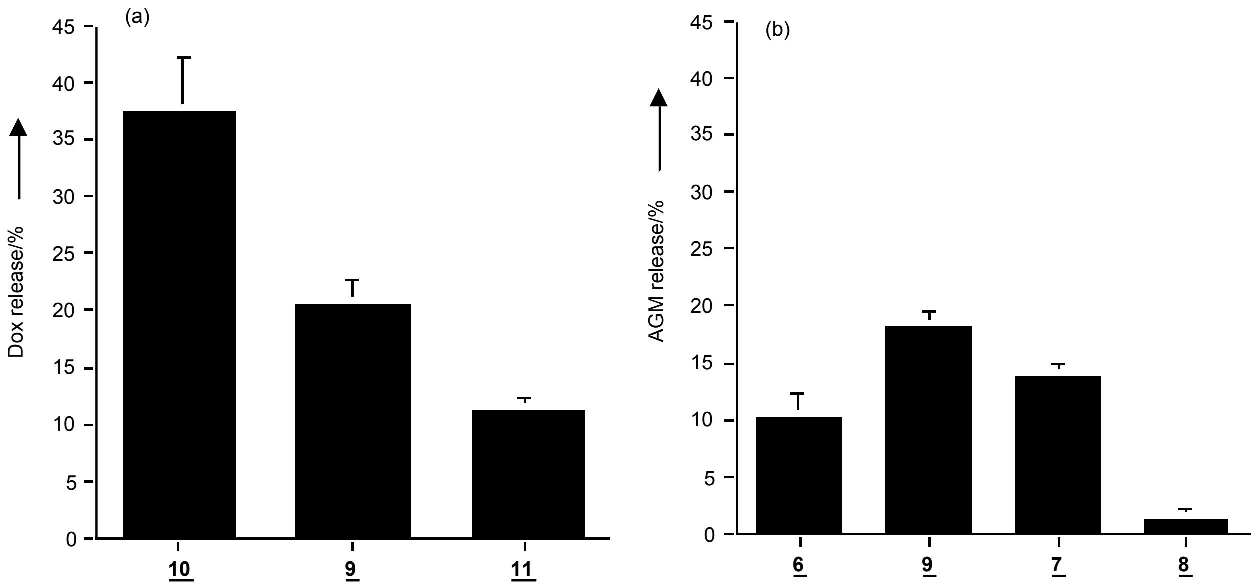
5. Evaluation of the release of AGM and Dox from HPMA copolymer conjugates on incubation with Tritosomes. Rat liver lysosomal enzymes (Tritosomes) were prepared in sucrose according to the method of Trouet.[1] Their protein content was determined using bicinchoninic acid protein assay and the protein content of the Tritosomes used in this study was 2.3 mg mL^{-1}. Their proteolytic activity was determined by measuring the release of *p*-nitroanilide (NAp) from N-benzoyl-Phe-Val-Arg-NAp and in this case activity was 62.4 nM min^{-1} mg of protein^{-1}.

---

[1] A. Trouet, **1974** *Methods in enzymology* (Eds.: S. Fleischer, L. Packer), Academic Press, Vol. XXXI, pp 323-329

HPMA copolymer-AGM±Dox conjugates (50 µg mL^{-1} drug-equiv.) were incubated at 37 °C in citrate phosphate buffer (pH 5.5, 1 mL total volume) with 0.2% w/v Triton X-100, EDTA (100 µL, 10 mM) and GSH (100 µL, 50 mM). To begin the degradation study Tritosomes were added (300 µL) and the eppendorf tubes thoroughly mixed. Aliquots (100 µL) were taken at times up to 24 h, immediately frozen in liquid nitrogen, and stored frozen in the dark until assayed by HPLC. In control experiments conjugates were incubated in buffer alone (without addition of Tritosomes) to assess non-enzymatic hydrolytic cleavage. In addition, free drug **2** or **3** (50 µg mL^{-1}) was also incubated under same conditions and later used as the reference control.

Figure 2S. Liberation of Dox or AGM from HPMA copolymer-drug conjugates after a 5 h incubation with tritosomes. Panel **(a)** shows Dox release and panel **(b)** shows AGM release expressed as a percentage of the total bound. The data represent mean ± S.E., n = 3



6. Cytotoxicity of free Dox, HPMA copolymer-AGM±Dox conjugates and combinations

Cytotoxicity of free AGM and Dox, and the conjugates was evaluated using the MTT cell viability assay (72 h incubation) with MCF-7 human breast cancer cells MCF-7ca cells which were transfected with the aromatase gene in order to overexpress P450 aromatase enzyme.

Cells were seeded in sterile 96-well microtitre plates at a seeding density 4 x 10^4 cells mL^{-1} in WRPMI 1640 with 5.0 mM L-glutamine and 5 % (v/v) charcoal-stripped (steroid-depleted) fetal calf serum (SFCS) plus additional geneticin (1 mM) in MCF-7ca cell line. Plates were incubated for 5 days and compounds (0.2 μm filter sterilized) were then added to give a final concentration of 0 - 1 mg mL^{-1} drug-equiv. After 67 h of incubation, MTT (20 μL of a 5 mg mL^{-1} solution in PBS) was added to each well, and the cells were incubated for a further 5 h. After removal of the medium, the precipitated formazan crystals were dissolved in optical grade DMSO (100 μL), and the plates were read spectrophotometrically at 550 nm after 30 min using a microtitre plate reader. Cell viability was expressed as a percentage of the viability of untreated control cells.

It is important to note that in order to obtain an oestrogen-controlled medium in all experiments involving MCF-7 cells, phenol red-free RPMI 1640 (WRPMI) with 5 % (v/v) SFCS supplemented with L-glutamine (5 mM) and 10^{-9} M oestradiol was used. Oestradiol solution was prepared from a stock initially prepared in ethanol with subsequent dilution for experiments of more than 1 : 1000 (for 10^{-5} M); the presence of ethanol at such dilutions has previously been shown to have no effect on cell growth.

Preparation of SFCS. 500 mL of FCS were adjusted to a pH of 4.2 adding HCl 5 M and equilibrated to a temperature of 20 ^{o}C. A charcoal solution was prepared adding 18 mL of ddH_2O, 0.2 g of Norit A charcoal and 0.01g of dextran T-70. 25 mL of the charcoal solution were added to the acidic FCS and the suspension was stirred for 16 hours at 4 ^{o}C. Then, the suspension was centrifuged (40 min at 12000 g) and coarsely filtrated with celite to remove the charcoal. The pH was adjusted to 7.2 with NaOH 5 M, the suspension was filtered with millipore filters 0.2 μm and stored in appropriate containers at -20 ^{o}C

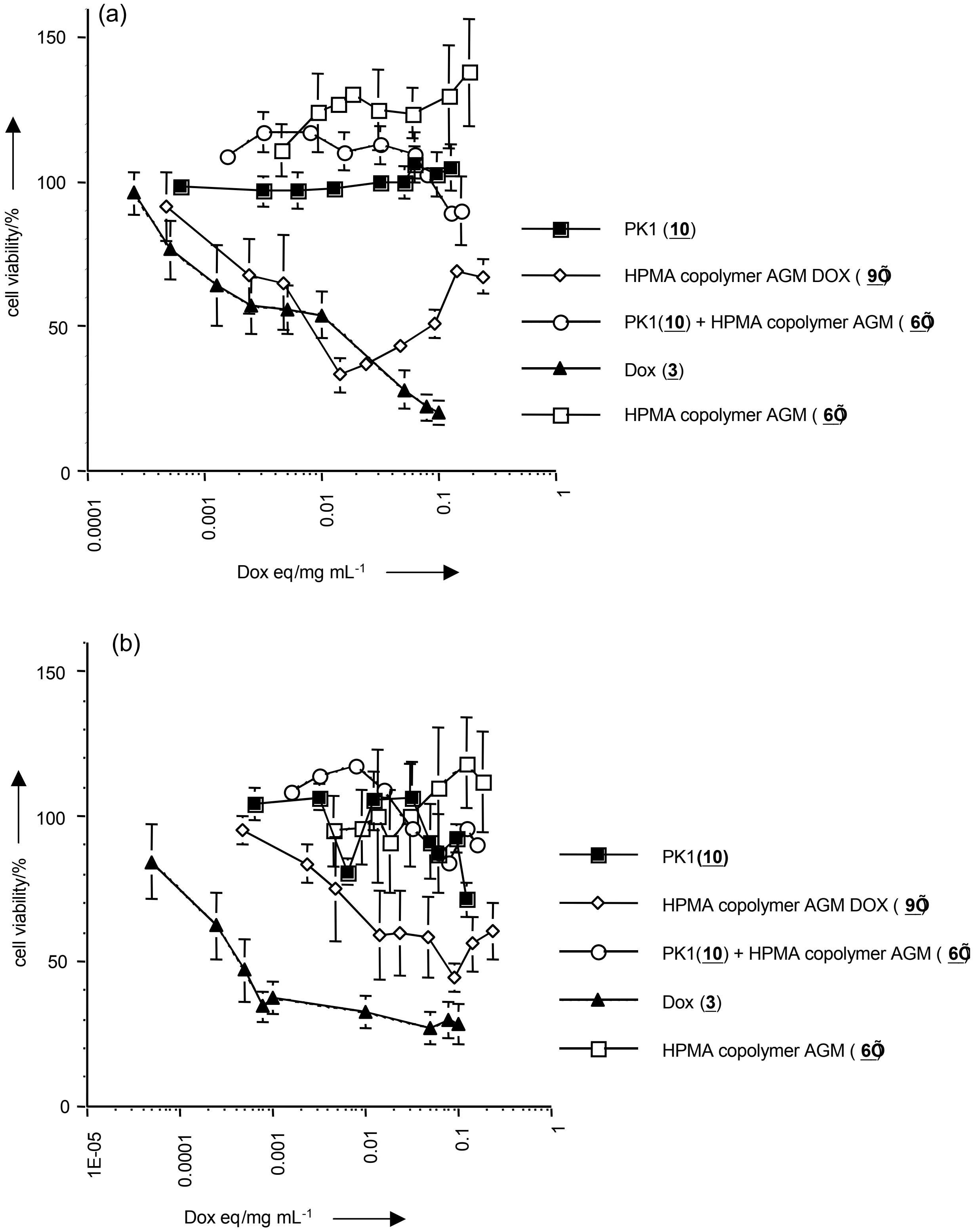


Figure 3S: *In vitro* cytotoxicity of Dox, AGM and HPMA copolymer conjugates against **(a)** MCF-7ca and **(b)** MCF-7 human breast cancer cell lines. Data expressed as mean ± S.E., (n = 3)

7. *Cellular association of HPMA copolymer conjugates measured by flow cytometry.* MCF-7 and MCF-7ca cells were seeded in 6-well plates at a density of $1x10^6$ cells mL^{-1} and allowed to adhere for 24 h. Then, HPMA copolymer-Dox ±AGM conjugates (**9'** and **10**) were added at a concentration of 0.01 mM (DOX-equivalent). Cells were incubated for times up to 60 min at 37 ˚C or 4 ˚C (to assess binding).

At the end of the incubation period, the plates were placed on ice to prevent further uptake and kept at 4 ˚C. The cells were washed three times with ice-chilled PBS (5 ml). Then PBS (1 ml) was added and cells were scraped from the plate and collected in falcon tubes and centrifuged at 4 ˚C, 600 x g for 5 min. Finally, cells were then re-suspended in ice-chilled PBS (200 μl) and analyzed using Becton Dickinson FACSCalibur cytometer (California, USA) equipped with an argon laser (488 nm) and emission filter for 550 nm. Data were collected with 25,000 events per sample and processed using CELLQuest™ version 3.3 software. Control cells were used in all cases to adjust the background fluorescence output (M1 region). Data was acquired in 1024 channels with band pass filters FL2 (585 nm ± 42 nm). Results are expressed as (geometric mean x % of positive)/ 100.

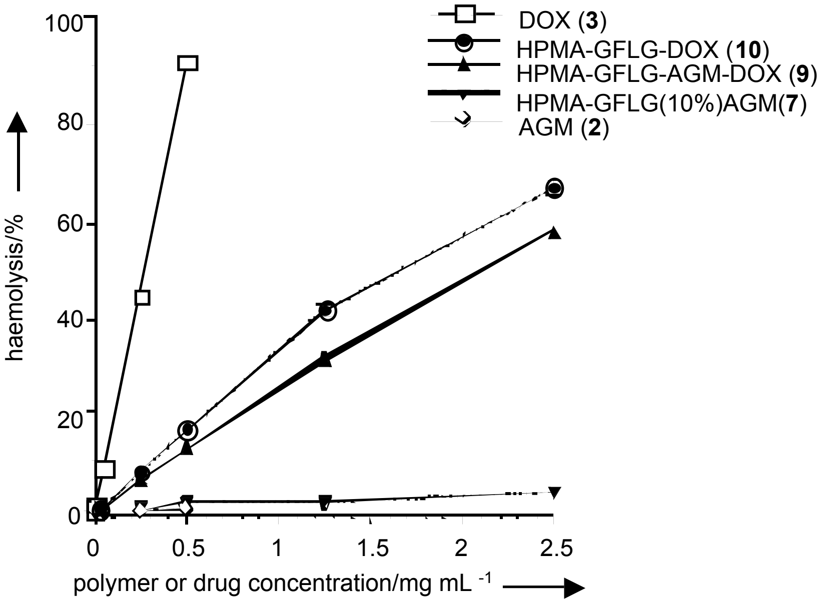
8. Hemolytic activity of free AGM, Dox and HPMA copolymer-AGM-Dox conjugates.[2] Freshly prepared HPMA copolymer-AGM±Dox conjugates, dextran (Mw = 74000 g mol^{-1}) and poly(ethyleneimine) (PEI; Mw = 750000 g mol^{-1}) solutions (range of concentrations 0 – 2.5 mg mL^{-1}), Dox and AGM (0 – 0.5 mg mL^{-1}) in phosphate buffered saline (PBS) at pH 7.4 were plated (100 μL) into non-sterile 96-well microtitre plates. Blood was taken from an adult male Wistar rat (~ 250 g), by cardiac puncture immediately after death (by 4% CO_2 asphyxiation) and placed in a lithium/heparinised tube (10.0 mL) on ice. Erythrocytes (RBC) were isolated by centrifugation at 1500 x g for 10 min at 4 ^{0}C (repeated 3 times). Using the final pellet, a 2 %

---

[2] R. Duncan, M. Bhakoo,M-L Riley, A. Tuboku-Metzger **1991** *Progress in membrane biotechnology* (Eds.: Gomez-Fernandez, Chapman and Packer) Birkhauser Verlag, Basel, pp 253-265.

w/v RBC solution was prepared with pre-chilled PBS and it was added (100 µL) to the previously prepared microtitre plates containing the test compounds. The plate was then incubated for 1 h at 37 °C before centrifugation at 1500 x g for 10 min at RT. The supernatant was then placed in another 96-well microtitre plate and hemoglobin (H_b) release measured spectrophotometrically (OD_{550}) using a microtitre plate reader (Tecan) using PBS as the blank. H_b release for each sample was expressed as a percentage of the release produced by 0.5 % w/v Triton X-100, used as a reference control to produce 100 % lysis. PEI and dextran were also used as reference polymers.

Figure 4S Red blood cell lysis caused by free drugs and HPMA copolymer conjugates. Data expressed as mean ± S.E., (n =3)



9. Statistical analysis

Statistical significance was estimated at $P < 0.05$ using one-way ANOVA followed by Bonferroni post hoc test.