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Improved plasma stability and sustained release profile of gemcitabine via polypeptide conjugation

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ABSTRACT

To enhance the stability of the anticancer drug gemcitabine (2′-deoxy-2′,2′-difluorocytidine), it was conjugated to poly-L-glutamic acid (PG-H) via a carbodiimide reaction. The synthesised poly-L-glutamic acid-gemcitabine (PG-G) was purified and characterised by using SDS-PAGE to estimate its molecular weight, HPLC to determine its purity and degree of drug loading, and NMR to elucidate the structure. *In vitro* aqueous hydrolytic studies showed that the gemcitabine release from the polymeric drug conjugate was pH dependent, and that the conjugation to PG-H improved its stability in human plasma. The release of the bound gemcitabine from PG-G in plasma was mediated by a hydrolytic process. It began with a lag phase, followed by linear release between 12 and 48 h, and reached equilibrium at 72 h with 51% of the gemcitabine released. *In vitro* cytotoxicity studies using MCF-7 and MDA-MB-231 human mammary cancer cells, as well as human dermal fibroblasts (HDF), showed that PG-G displayed a lower dose dependent cytotoxic effect with respect to the parent drug gemcitabine. On the other hand, in 4T1 mouse mammary tumour cells, PG-G and gemcitabine showed similar toxicities. Gemcitabine was more than likely released hydrolytically from PG-G and taken up by MCF-7, MDA-MB-231 and HDF, whereas both released gemcitabine and PG-G were taken up by 4T1 to mediate the observed cytotoxicities. The improved stability and extended sustained release profile may render PG-G a potential anticancer prodrug.
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1. Introduction

The anticancer efficacy of gemcitabine, a water soluble antimetabolite nucleoside analogue that has broad spectrum cytotoxicity against solid human tumours (Abratt, 1995; Fowler and Van Le, 2003; Haller, 2003; Harper, 2003), is often limited by its short *in vivo* half life (8–17 min) (Immordino et al., 2004). This short half life is the result of extensive deamination of gemcitabine by cytidine deaminase, present in the blood, liver, kidney and other tissues, which metabolise gemcitabine to the inactive form 2′-deoxy-2′,2′-difluorodeoxyuridine (dFdU), which is then rapidly excreted in the urine (Immordino et al., 2004). To achieve a therapeutic drug concentration *in vivo*, gemcitabine is often administered in high doses, for example 1000 mg/m^2 as a 30 min intravenous infusion (Immordino et al., 2004). However, the use of low molecular weight anticancer drugs in high doses often causes non-specific tissue toxicity in patients due to uncontrolled and random dissemination of the drugs in the body. Such high doses account for most of the adverse effects in chemotherapy, for example, myelosuppression and reversible transaminase elevation with gemcitabine (Fossella et al., 1997). To date, many approaches have been attempted to improve the *in vivo* stability of gemcitabine and to reduce its toxicity. These approaches include the encapsulation of gemcitabine in liposomes of different formulations (Celano et al., 2004; Immordino et al., 2004) and attachment of gemcitabine to both saturated and monounsaturated C18 and C20 acyl-chains (Myhren et al., 1998). While providing gemcitabine partial protection against rapid plasma inactivation, the loading of gemcitabine on liposomes and short chain hydrocarbons may not prevent random distribution of the drug in the body and rapid renal excretion, as these small size drug–carrier complexes readily cross the fenestrations in the vascular bed and the glomeruli in the kidney. Hence, considerable tissue degradation and renal loss of gemcitabine still occurs, in addition to the non-specific tissue toxicity due to the increased plasma membrane permeability of the lipophilic encapsulated or derivatised gemcitabine.

The conjugation of drugs to water soluble macromolecular biocompatible polymers increases drug stability in blood (Duncan et al., 2005). This also prevents *in vivo* random dissemination of the drug and thus reduces non-specific drug toxicity on normal tissues (Duncan et al., 2005). The enhanced permeability–retention

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(EPR) effect of the tumour vasculature promotes accumulation of the macromolecular polymeric drug conjugates, and the drug is then released from the polymeric backbone in selected biological environments (Duncan and Spreafico, 1994; Garnett, 2001). To date, the *in vivo* stability and therapeutic properties of numerous anticancer drugs have successfully been improved through polymeric conjugation of the drug (Kopecek, 1990; Maeda, 1991; Li et al., 1998, 1999; Duncan et al., 2001).

Conjugation of gemcitabine onto a polymeric carrier was attempted by Cavallaro et al. (2006). In this study, gemcitabine was conjugated to an α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) polymer together with folate via succinnyl and deocycolyl linkers. The preliminary evaluation of the synthesised PHEA based polymer-gemcitabine-folate conjugates, revealed that the aqueous and the plasma stability of gemcitabine were significantly improved upon its conjugation to the PHEA polymer (Cavallaro et al., 2006).

However, indirect conjugation of gemcitabine via linkers to the polymeric backbone increases the complexity of the synthetic steps. It is conceivable that the drug loading onto the polymer may be reduced due to the bulk and steric hindrance of the linkers. Furthermore, the increase in structural complexity of the polymeric drug conjugate may cause unpredictability in terms of the conjugate's *in vivo* disintegration pattern, fate and efficacy.

To reduce complexity, the synthesis of a polymer-gemcitabine conjugate can be achieved without the use of linkers by attaching the drug directly to a biodegradable water soluble polymer, such as poly-L-glutamic acid that has multiple –COOH side groups, using a simple esterification reaction (Li, 2002; Li et al., 2002). In an effort to generate a polymeric drug conjugate with improved plasma stability through simpler synthetic methods, and to elucidate the mechanism of drug release from the polymeric backbone. Herein, we report the preliminary findings on the use of poly-L-glutamic acid as an alternative polymeric carrier for gemcitabine.

2. Apparatus and common methods

Thin layer chromatography (TLC) of the polymeric drug conjugate synthesis was carried out by developing the TLC strips (Kieselgel 60G F_{254}, Merck, Darmstadt, Germany) spotted with reaction mixture (1 μl) in 100% methanol for 15 min. The R_f value of free gemcitabine was 0.7 while that of poly-L-glutamic acid (PG-H) and poly-L-glutamic acid-gemcitabine (PG-G) was 0.

Ultraviolet (UV) spectra of gemcitabine, PG-H and PG-G were measured on a Shimadzu 1095 UV/vis spectrophotometer. One-dimensional ^{1}H and ^{13}C NMR spectra of gemcitabine, PG-H and PG-G were recorded at ambient temperature on a JEOL JNM LA400 FT-NMR spectrometer (Akishima, Tokyo, Japan) operating at 400 MHz (^{1}H) or 100 MHz (^{13}C). A COSY experiment was also conducted for gemcitabine. The sample concentration in deuterated water (D_2O) was 20 mg/ml and tetramethylsilane (TMS) was used as the internal standard.

High performance liquid chromatography (HPLC) analyses to evaluate the purity and gemcitabine content of the synthesised polymeric drug conjugate, and to assess gemcitabine release in *in vitro* incubation studies, were carried out on a system consisting of a Waters 2690 solvent delivery module, a Waters 996 PDA (Waters Co., Milford, MA, USA), a Microsorb-MV 100-5 C18 RP-HPLC column (4.6 mm × 250 mm, 280 Å, 5 μm ODS coated silica gel particles, Varian Inc., Palo Alto, CA, USA) and a Waters millennium v3.02 workstation. The composition of the mobile phase to determine the content of gemcitabine in the synthesised PG-G, purity of PG-G and *in vitro* aqueous incubation studies was 0.02 M ammonium acetate (0.02 M AMN)/acetonitrile (ACN) (1:1) with a flow rate of 0.5 ml/min. For the *in vitro* plasma incubation studies, the mobile phase was 0.02 M AMN/ACN (9:1). The sample volume was 10 μl and monitored at 268 nm. The retention times for gemcitabine and PG-G peaks were 4.14 min and 3.17 min, respectively, for 0.02 M AMN/ACN (1:1) and 6.75 min and 3.18 min, respectively, for 0.02 M AMN/ACN (9:1).

The molecular weight distribution of the synthesised PG-G was estimated using SDS-PAGE electrophoresis, according to the method of Laemmli (1970) with minor modifications. PG-G sample (15 μl) mixed with sample loading buffer was denatured at 95 °C for 90 s and electrophoresed in a 12.5% SDS-PAGE gel fitted to an electrophoresis tank (Hoefer SE 600, San Francisco, CA, USA). The gel was electrophoresed at 120 V for 0.5 h, followed by 200 V for 24 h (Biorad power pack, Hercules, CA, USA). BenchMark™ Protein Ladder (molecular weight range from 10 kDa to 220 kDa, Invitrogen, Carlsbad, CA, USA) was used as the molecular weight marker. After electrophoresis, the SDS-PAGE gel was subjected to silver staining (Merrill et al., 1983). The image of the stained gel was captured with a scanner, and the molecular weight distribution of PG-H and PG-G were estimated with reference to the molecular weight markers' positions on the gel.

3. Materials

Deuterated water (D_2O), tetramethylsilane (TMS), phosphate buffered saline (PBS), poly-L-glutamic acid (in the form of sodium salt, PGNa, molecular weight by viscosity = 97,800, number-average molecular weight by multi-angle laser light scattering = 41,400) were obtained from Sigma–Aldrich (St. Louis, MO, USA), while gemcitabine was supplied by 21st Century Global E-commerce Network (Westham, Pevensey, East Sussex, UK). Hydrochloric acid, sodium hydrogen carbonate, sodium hydroxide, methanol, chloroform, acetonitrile, ammonium acetate and sodium hydroxide were purchased from BDH Ltd. (Poole, Dorset, UK), while *N*,*N*-dimethylformamide, dicyclohexylcarbodiimide and dimethylaminopyridine were from Fluka (Buchs, Switzerland). The Snakeskin™ dialysis tube (molecular weight cut off point = 10,000) was purchased from Pierce (Rockford, IL, USA). All general glassware used was from Schott Duran (Wertheim/Main, Germany).

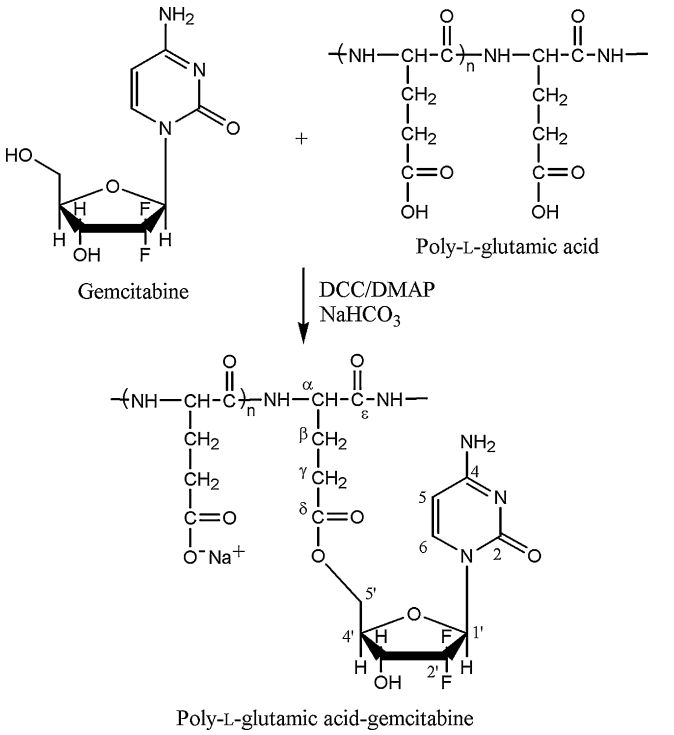
4. Methods

4.1. PG-G synthesis and preliminary characterisation

The aqueous solution of the sodium salt of poly-L-glutamic acid (PGNa) (50 mg/ml; 200 ml) was adjusted to pH 2.0 using 0.2 M HCl to convert PGNa into the proton form (PG-H) to generate sufficient –COOH groups at the polymer's side chains for the subsequent conjugation reaction. The solution was then dialysed against distilled water (5 × 5 L, 1 h per round of dialysis) in a Snakeskin™ dialysis tube (10,000 molecular weight cut off) and lyophilised.

The synthesis of poly-L-glutamic acid-gemcitabine (PG-G) was carried out using the method proposed by Li et al. (2002), with modifications (Scheme 1). 65 mg PG-H (0.5 mmol), 30 mg gemcitabine (0.1 mmol), 41.28 mg dicyclohexylcarbodiimide (DCC, 0.2 mmol) and 0.14 mg dimethylaminopyridine (DMAP) were added to 12 ml dry *N*,*N*-dimethylformamide (DMF) in a small dried reaction flask under a dry nitrogen atmosphere and stirred. The reaction was allowed to proceed at room temperature for 24 h with continuous stirring under a nitrogen atmosphere and monitored by TLC.

At 24 h, 20 ml of chloroform was added to the reaction mixture to precipitate the poly-L-glutamic acid-gemcitabine (PG-G, in the proton form). 60 ml of 1 M $NaHCO_3$ was gently added to the reaction mixture. The mixture was left sitting for 5 min to allow for the separation of the organic and aqueous phases, as well as the partitioning of the hydrophilic PG-G molecules into the aqueous sodium



Scheme 1. Synthesis of poly-L-glutamic acid-gemcitabine.

hydrogen carbonate solution layer. The aqueous sodium hydrogen carbonate upper layer containing PG-G was carefully removed, placed in a Snakeskin™ dialysis tube (10,000 molecular weight cut off) and dialysed against distilled water (5 × 5 L, 1 h per round of dialysis). The PG-G (sodium salt) solution was lyophilised and stored at −20 °C, and the yield of PG-G was calculated.

The molecular weight distribution of the synthesised compound was estimated using SDS-PAGE techniques. The product was further characterised by ultraviolet (UV) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The purity of the PG-G and gemcitabine content in PG-G was determined using HPLC, as described earlier.

The aqueous solubility of PG-G at different pH was determined by dissolving the PG-G (1 mg) in double distilled water (1 ml) at different pH, ranging from 2 to 13, followed by a visual examination of the mixture using a compact IV inspection light box (Health Care Logistics, Inc., Circleville, OH, USA). The maximum PG-G concentration achievable in water at pH 7.4 was determined by gradually increasing the amount of PG-G in 0.1 ml double distilled water at pH 7 (1 mg at a time) until the PG-G no longer dissolved in the solution by light box visual examination. The maximum PG-G concentration achievable in water was then calculated.

4.2. Stability of gemcitabine and poly-L-glutamic acid-gemcitabine (PG-G) in aqueous solution

The aqueous stability of gemcitabine and PG-G, as well as the release kinetics of gemcitabine and related molecular species from PG-G was studied in PBS at 37 °C and pH 5.5 and 7.4. 3 mg gemcitabine and 10 mg PG-G were dissolved in 10 ml PBS at pH 5.5 and 7.4 and incubated at 37 °C for 5 days. Aliquots (500 μl) were sampled from the respective incubation vials at different time points (0, 0.5, 1, 2, 4, 6, 8, 12, and 24 h and 2, 3, 4 and 5 days). The aliquots were analysed directly using the HPLC techniques described earlier (refer to Section 2) and a mobile phase of 0.02 M AMN/ACN (1:1).

4.3. Stability of gemcitabine and poly-L-glutamic acid-gemcitabine (PG-G) in plasma

The *in vitro* plasma stability of gemcitabine and PG-G, as well as the release kinetics of gemcitabine and related molecular species from PG-G, was studied in human plasma at 37 °C. EDTA anticoagulated whole blood was centrifuged at 1300 × *g*, at 4 °C for 10 min to extract the human plasma. Gemcitabine (3 mg) and 10 mg PG-G were dissolved in 10 ml human plasma at pH 7.4 and incubated at 37 ± 1 °C for 5 days. Aliquots (500 μl) were sampled from the respective incubation vials at different time intervals (0, 0.5, 1, 2, 4, 6, 8, 12, and 24 h and 2, 3, 4 and 5 days).

The plasma aliquots were then subjected to an optimised, prevalidated filter-extraction protocol prior to HPLC analysis. Briefly, 350 μl of PG-G or gemcitabine-containing plasma aliquots was spiked with 50 μl 1.6 mM deoxycytidine (dC, as an internal standard) and filtered using an Amicon Microcon centrifugal filter device (Millipore, Billerica, MA, USA, 10,000 molecular weight cut off point, centrifugal filtration at 14,000 × *g* for 1 h) to remove any macromolecules that might block the HPLC column, and any UV absorbing macromolecules that might interfere with the gemcitabine content determination. The filtrates were then analysed using HPLC with a mobile phase composition of 0.02 M AMN/ACN (9:1).

The protocol for the centrifugal-filter extraction-HPLC assay for the analysis of plasma degradation of gemcitabine was validated using six replicates. The limit of quantification (LOQ) of gemcitabine in plasma was 0.01 mM. The calibration curve, between 0.01 and 1.00 mM gemcitabine, was constructed using the internal standard (deoxycytidine) method, and its linearity determined. The precision of the concentrations of gemcitabine at 0.10, 0.50 and 1.00 mM was between 1.2 and 2.2 RSD, whereas the recoveries of gemcitabine at the same concentrations were above 97%. The retention times for gemcitabine, gemcitabine degradation product (Dgp) and deoxycytidine (internal standard) were 6.71 min, 9.12 min and 5.34 min, respectively.

4.4. In vitro cytotoxicity assay

The *in vitro* cytotoxic activities of gemcitabine, PGNa and PG-G on MCF-7 and MDA-MB-231 human breast cancer cells, 4T1 mouse mammary tumour cells and human dermal fibroblasts (HDF) were determined. The average content of gemcitabine in the synthesised PG-G was 10% (0.1 mg gemcitabine/mg PG-G), as measured by HPLC.

Briefly, cells (1.25×10^4 cells/ml) were suspended in 200 μl of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated foetal calf serum, 4 mM glutamine, 100 IU/ml penicillin, 100 g/ml streptomycin and 0.25 g/ml amphotericin B, and they were then seeded into the wells of a 96-well microplate. The microplate was incubated for 24 h at 37 °C in a 97% humidified atmosphere of 5% CO_2. Thereafter, the spent culture medium was replaced with 200 μl of fresh cell culture medium containing 0.0001–100 μg/ml of gemcitabine or PG-G with an equivalent amount of gemcitabine. PGNa was included as a control for the polymeric carrier, and the amount of PGNa tested was equivalent to that of the PG-G used for each treatment concentration in PG-G group, while the negative control consisted of fresh culture medium. All tests were performed in triplicate. Cells were incubated for 72 h at 37 °C in a 97% humidified atmosphere of 5% CO_2. 20 μl of 5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT, 5 mg/ml) was added to each well and the plates were incubated for 5 h at 37 °C. The medium was then carefully removed from each well, 100 μl of dimethylsulfoxide (DMSO) was added and mixed, to dissolve the formed dark blue formazan crystals. Then, the absorbance at 570 nm was measured using a Biotek ELx808™

absorbance microplate reader (Winooski, VT, USA). Cell viability was expressed as a percentage of the test samples/negative control absorbance ratio. Cell viability was expressed as a percentage of the test samples/negative control absorbance ratio, and the 50% growth inhibitory concentrations, IC_{50}, of the test samples were estimated from the graphs of cell viability against concentrations of test samples. Statistical analysis was performed using 2-way ANOVA with the Bonferroni *post hoc* test (Prism 4, Graphpad Software, San Diego, CA, USA).

4.5. Pulse-chase in vitro cytotoxicity assay

To evaluate the uptake of bound and unbound gemcitabine, the effect of exposure time of gemcitabine and PG-G on MCF-7 human and 4T1 mouse mammary tumour cells was determined using a pulse-chase cytotoxicity assay. The time dependent cellular uptake of test samples in the cells was reflected by the degree of cytotoxicity exerted by unbound gemcitabine accumulated in the cells, and cell viability was estimated using the MTT assay. To ensure the presence of sufficient gemcitabine or PG-G to induce cytotoxicity, culture medium containing 10 μg/ml gemcitabine or PG-G with an equivalent amount of gemcitabine was exposed to the cells. This concentration was several folds higher than that of the IC_{50} of gemcitabine or PG-G in the respective cell lines as determined by the *in vitro* cytotoxicity assay described in the earlier section [MCF-7: IC_{50}, 0.061 μg/ml (gemcitabine), 0.104 μg/ml (PG-G); 4T1: IC_{50}, 0.003 μg/ml (gemcitabine), 0.004 μg/ml (PG-G); also refer to Section 5.5].

Cells (1.25×10^4 cells/ml), suspended in 200 μl of cell culture medium, were seeded in 96-well microtitre plates and incubated for 24 h as described above. Thereafter, the spent culture medium was replaced with 200 μl fresh culture medium containing gemcitabine (10 μg/ml) or PG-G with an equivalent amount of gemcitabine. The negative control consisted of fresh culture medium, and the tests were carried out in triplicate.

Cell culture medium supplemented with gemcitabine or PG-G was removed from the appropriate wells and replaced with fresh cell culture media at 0, 0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72 h after initiation of incubation at 37 °C in a 97% humidified atmosphere of 5% CO_2. At 72 h, the cells in the 96-well microtitre plates were subjected to an MTT assay, as previously described. Cell viability was expressed as a percentage of the test samples/negative control absorbance ratio at different time points, and statistical analysis was performed as described earlier.

5. Results and discussion

5.1. PG-G synthesis and preliminary characterisation

In the current study, PG was used as a polymeric carrier for the anticancer drug gemcitabine. In the optimised PG-G synthesis reaction, lyophilisation of the final dialysate yielded 78 mg of product as a white powder (yield = 82%). The molecular weight distribution of the synthesised PG-G was similar to the polymeric carrier (PG-H) at 60–70 kDa, as determined by the SDS-PAGE technique. PG-G (1 mg/ml) was found to be insoluble only under acidic conditions (at pH 3.0 and below), and the maximum PG-G concentration dissolvable in water at pH 7.0 was 180 mg/ml.

UV spectrum analysis of PG-G showed absorption in the range between 230 and 350 nm, with a distinct absorption peak at 268 nm. This absorption coincides with that of the PHEA-gemcitabine conjugate (Cavallaro et al., 2006) and partially resembles that of the gemcitabine (absorption maximum at 232 and 268 nm) (Merck, 2001). The absorption peak at 232 nm was masked by the PG moiety in PG-G.

HPLC analysis using a mobile phase of 0.02 M AMN/ACN (1:1) showed no free gemcitabine or other UV light-absorbing contaminants present in the synthesised PG-G conjugate (Fig. 1B). Under these HPLC conditions, the free gemcitabine was represented by a HPLC peak with a retention time of 4.14 min, while the bound gemcitabine in PG-G was represented by a HPLC peak with a retention time of 3.17 min (Fig. 1A and B). Poly-L-glutamic acid (PGNa) (1 mg/ml) did not afford any chromatography peaks (not shown). The content of bound gemcitabine in the polymeric drug conjugate (PG-G), estimated by HPLC, was 11.4 molar percent, which was higher than that of the PHEA-gemcitabine conjugates, which had an approximately 9.4 molar percent of drug loading (Cavallaro et al., 2006). It is possible that the maximum gemcitabine loading onto the poly-L-glutamic acid can be enhanced through further synthetic optimisation, based on the higher drug loading found for similar polymeric drug conjugates (Li et al., 1998; Singer et al., 2001).

5.2. NMR analysis

^1H and ^{13}C NMR analyses were performed on the PG-G conjugate that was synthesised, as well as on gemcitabine and PG polymer in the proton form, to elucidate the molecular structure of PG-G and to identify the side group that was involved in the conjugation reaction (Scheme 1). The ^1H and ^{13}C chemical shifts of the gemcitabine and PG (proton form; PG-H) NMR spectra were assigned to the various proton and carbon atoms and were consistent with previously published values (Jansen et al., 2000; Cavallaro et al., 2006). Then, the ^1H and ^{13}C chemical shifts of PG-G NMR spectra were assigned with reference to the assignments for the PG-H and gemcitabine.

^1H NMR (D_2O) of gemcitabine, δ (ppm): 6.11 (d, 1H, H-5), 7.85 (d, 1H, H-6), 6.08 (t, 1H, C-1′), 4.23 (m, 1H, H-3′), 3.96 (m, 1H, H-4′), 3.70, 3.73, 3.85, 3.88 (dd, 2H, H-5′); ^{13}C NMR (D_2O), δ (ppm): 149.18 (C-2), 160.22 (C4–NH_2), 96.35 (C-5), 144.67 (C-6), 85.35 (C-1′), 69.88 (C-3′), 81.78 (C-4′), 60.04 (C-5′).

^1H NMR (D_2O) of PG-H, δ (ppm): 2.07 (m, 2H, H-γ), 1.74, 1.84 (dt, 2H, H-β), 4.10, 4.12 (dd, 1H, H-α); ^{13}C NMR (D_2O), δ (ppm): 174.30 (C-δ), 34.42 (C-γ), 28.92 (C-β), 54.24 (C-α), 182.34 (C-ε).

^1H NMR (D_2O) of PG-G, δ (ppm): 6.13 (d, 1H, H-5), 8.07 (d, 1H, H-6), 6.08 (t, 1H, H-1′), 3.54 (m, 1H, H-4′), 3.80 (m, 2H, H-5′), 2.11 (m, 2H, H-γ), 1.78, 1.88 (dt, 2H, H-β), 4.15, 4.17 (dd, 1H, H-α); ^{13}C NMR (D_2O), δ (ppm): 161.02 (C4–NH_2), 65.84 (C-5′), 174.35 (C-δ), 34.42 (C-γ), 28.83 (C-β), 54.30 (C-α), 182.31 (C-ε).

The NMR spectra indicated that the conjugation of gemcitabine to PG-H occurred through the formation of an ester bond between the –OH at the C-5′ position of gemcitabine and the –COOH at C-δ position of PG-H. This was supported by the downfield shift of the C-5′ gemcitabine peak from 60.04 in the ^{13}C NMR gemcitabine spectrum to 65.84 ppm in the ^{13}C NMR PG-G spectrum. This downfield shift was probably due to the deshielding effect of the neighbouring ester group formed by the conjugation reaction, and was consistent with that of the PHEA-gemcitabine conjugate (Cavallaro et al., 2006).

5.3. PG-G hydrolytic degradation study

HPLC analysis showed that the level of free gemcitabine in PBS at pH 5.5 and 7.0 and 37 °C did not change significantly over 120 h (2-way ANOVA; $P > 0.05$) (Fig. 2A). No other UV-absorbing compound was detected in the incubation mixture. These results suggest that gemcitabine is relatively stable and concurred with the findings of Xu et al. (1999) and Cavallaro et al. (2006) on the stability of gemcitabine in aqueous solution. However, a pH dependent gemcitabine release from PG-G conjugate was observed, and the release of gemcitabine over time was higher at pH 7.4 compared to 5.5 (Fig. 2B). A representative HPLC chromatogram is as shown in Fig. 1C. The

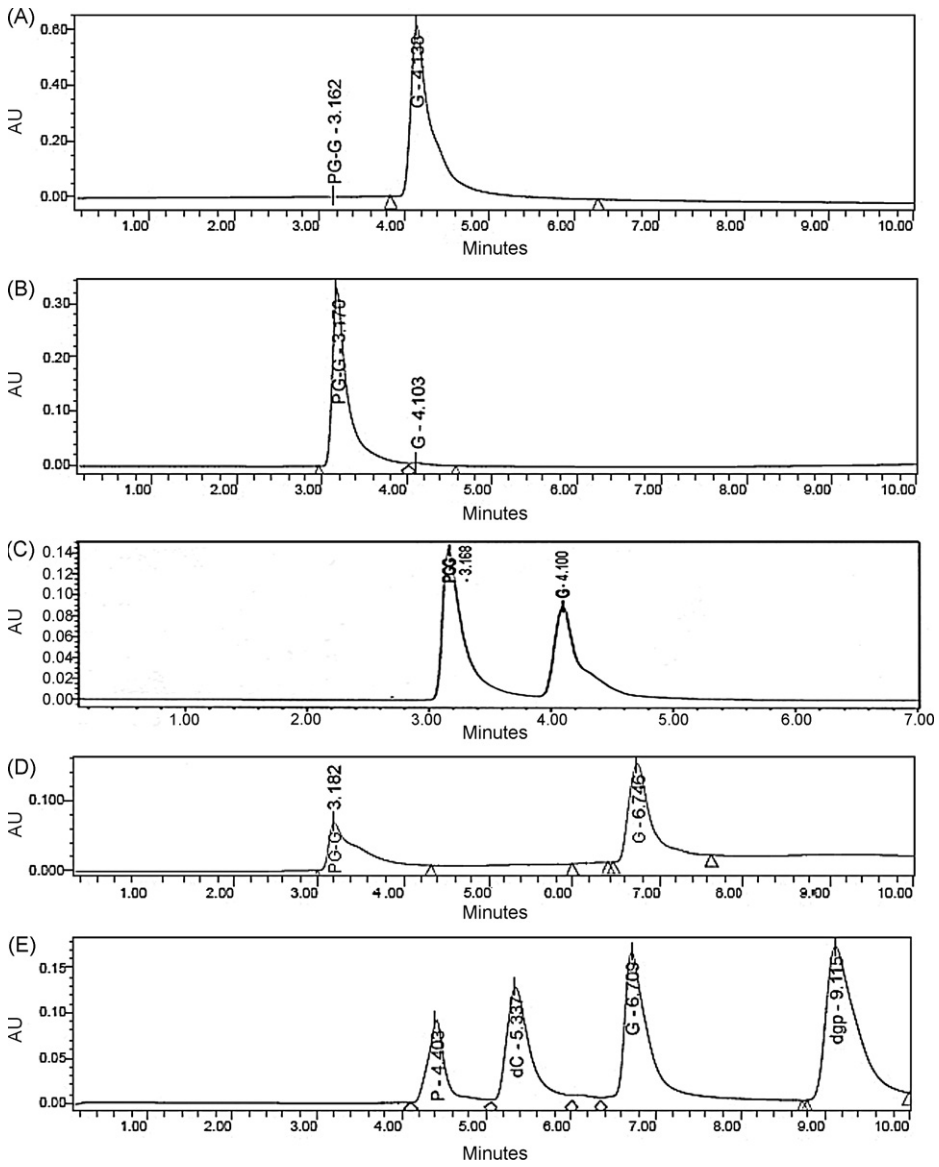


Fig. 1. (A) HPLC profile of gemcitabine (G) (1 mM). (B) HPLC profile of the poly-L-glutamic acid-gemcitabine conjugate (PG-G) (1 mg/ml). (C) A representative HPLC profile of how the poly-L-glutamic acid-gemcitabine conjugate (PG-G) (1 mg/ml) in PBS at pH 5.5 or 7.4 and 37 °C sampled at time intervals to show the release of gemcitabine (G) from PG-G looks. This PG-G sample was incubated in PBS at pH 7.4 and 37 °C, and sampled at day 4. (D) HPLC profile of poly-L-glutamic acid-gemcitabine conjugate (PG-G) and gemcitabine (G) without filtration with an Amicon Microcon centrifugal filtration device (10,000 molecular weight cut off). (E) A representative HPLC profile of poly-L-glutamic acid-gemcitabine conjugate (PG-G) (1 mg/ml) in human plasma at pH 7.4 and 37 °C sampled at time intervals and filtered with an Amicon Microcon centrifugal filtration device showing the deoxycytidine peak (dC; added internal standard) and the release of gemcitabine (G) and subsequent formation of gemcitabine degradation product (Dgp) via gemcitabine from PG-G. The peak (P) was from human plasma (below 10,000 molecular weight). The samples (chromatograms A–C) were eluted using 0.02 M ammonium acetate/acetonitrile (1:1) with a flow rate of 0.5 ml/min and monitored at 268 nm. Chromatograms D and E were eluted using 0.02 M ammonium acetate/acetonitrile (9:1) with a flow rate of 0.5 ml/min and monitored at 268 nm.

release of gemcitabine from the polymeric conjugate began at 2 h post-initiation of incubation. At 30 h, the percentage of released gemcitabine was 25% (w/w) at pH 7.4, representing plasma and extracellular compartments; it was 9% (w/w) at pH 5.5, mimicking the lysosomal compartment, and are consistent with the findings on the PHEA-gemcitabine conjugate and the folate conjugated PHEA-gemcitabine derivative (Cavallaro et al., 2006).

The *in vitro* aqueous hydrolytic studies also suggest that gemcitabine release is a result of the hydrolytic cleavage of the ester bond between gemcitabine and poly-L-glutamic acid in the polymeric drug conjugate, because only the characteristic gemcitabine and PG-G HPLC peaks were detected. This result also supports the restoration of the C5′–OH moiety in the gemcitabine molecule upon its detachment from the polymeric carrier backbone, which is critical for its antimetabolic activity through a masked chain termination mechanism (Abbruzzese et al., 1991; Plunkett et al., 1996; Noble and Goa, 1997).

5.4. Plasma degradation study

Unlike the high stability in aqueous solution, free gemcitabine is degraded rapidly in human plasma at pH 7.4 and 37 °C, with an estimated degradation half life of 15 h (Fig. 3A). This degradation product (Dgp) has a HPLC retention time of 9.12 min, compared to 6.71 min for gemcitabine, when eluted using 0.02 M ammonium acetate/acetonitrile (9:1) at 0.5 ml/min. The HPLC chromatogram is similar to that of Fig. 1E without the presence of the peak due to plasma proteins at a retention time of 4.40 min. The content of Dgp increased synchronously with the decrease of gemcitabine content in the plasma sample suggesting that it is probably 2′-

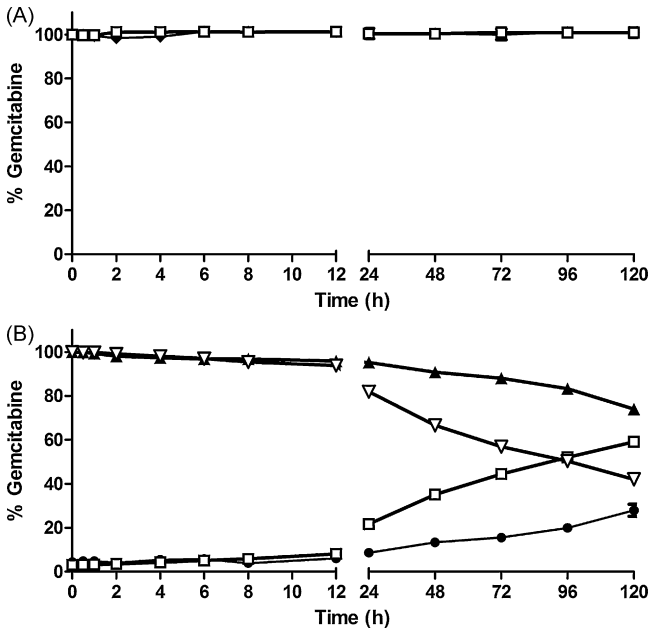


Fig. 2. (A) Stability of gemcitabine (0.3 mg/ml) in PBS at 37 °C and pH 5.5 (♦) and 7.4 (□). (B) Release of gemcitabine from poly-L-glutamic acid-gemcitabine (PG-G) (1 mg/ml) in PBS at 37 °C and pH 5.5 and 7.4. The lines represent the percentage of bound gemcitabine in PG-G at pH 5.5 (▲) and 7.4 (▽), and the percentage of gemcitabine released from PG-G at pH 5.5 (●) and 7.4 (□). The representative HPLC chromatogram is as shown in Fig. 1C. Each data point is expressed as the mean ± SE (n = 3).

deoxy-2′,2′-difluorodeoxyuridine (dFdU), an inactive metabolite of gemcitabine, formed by the action of cytidine deaminase present in the plasma. These results are also consistent with the behaviour observed in an earlier study (Egorin et al., 2002). However, due to the unavailability of this gemcitabine metabolite standard during the study, the identity of Dgp remains unconfirmed.

In the *in vitro* PG-G plasma degradation study, HPLC analysis indicated that the onset of gemcitabine release from PG-G was at about 6 h and reached an estimated apparent maximum concentration of 24.4% of gemcitabine at 72 h (Fig. 3B) (see later; release of total gemcitabine from PG-G). In the first 6 h of incubation, no Dgp or very little Dgp was detected in the PG-G-containing plasma samples. However, after 6 h, traces of Dgp (0.3 ± 0.02%; mean ± SE (n = 3)) were present in the plasma and increased over time. At 24 h, the amount of Dgp found was 9.2 ± 0.1% of the total estimated gemcitabine bound in the PG-G-containing plasma sample (Fig. 3B and C), and is an amount that is five times lower than that found in the free gemcitabine-plasma incubation mixtures (Fig. 3A). At 72 and 120 h, the levels of Dgp in PG-G-containing plasma were 25.0 ± 0.5% and 36.6 ± 0.1%, respectively (Fig. 3C), and they were lower than that of free gemcitabine-containing plasma, which showed 67.0 ± 0.1% and 70.0 ± 0.5% of Dgp, respectively (Fig. 3A). This result demonstrated the ability of PG-G to protect bound gemcitabine from degradation in plasma.

The appearance of Dgp is likely a result of the degradation of the gradually released gemcitabine bound to PG-G, and the pattern of Dgp appearance is consistent to that of consecutive chemical reactions. Therefore, the total amount of gemcitabine released from the PG-G in plasma is the sum of the levels of both gemcitabine and Dgp (Fig. 3B). The release of gemcitabine began with a lag phase of up to 6 h, followed by a linear release between 12 and 48 h and

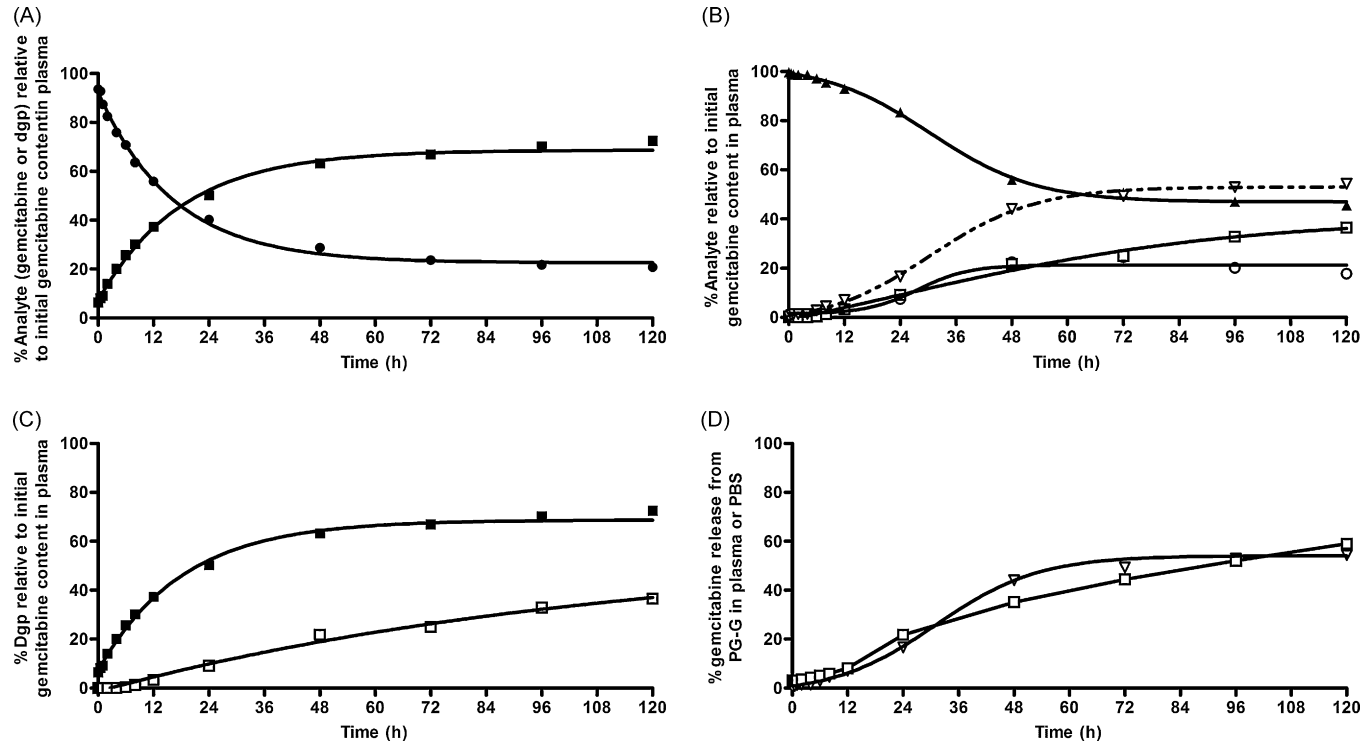


Fig. 3. (A) Time dependent degradation of gemcitabine (0.3 mg/ml) in human plasma at pH 7.4 and 37 °C. The lines represent the percentage of gemcitabine (●) and the percentage of the formation of the degradation product of gemcitabine (Dgp) (■). (B) Time dependent release of gemcitabine from poly-L-glutamic acid-gemcitabine (PG-G) (1 mg/ml) in human plasma at pH 7.4 and 37 °C. The lines represent the percentage of gemcitabine released from PG-G (○), the percentage of Dgp formed (□), the percentage of gemcitabine bound to PG-G (▲) and the estimated total amount of gemcitabine released from PG-G, including gemcitabine which had been degraded into Dgp (▽). A representative HPLC chromatogram is as depicted in Fig. 1E. (C) A comparison of the formation of gemcitabine degradation product (Dgp) from gemcitabine (■) and PG-G (□) in human plasma at pH 7.4 and 37 °C. (D) A comparison of the release of gemcitabine (G) from PG-G (1 mg/ml) in human plasma (▽) or PBS (□) at pH 7.4 and 37 °C. Each data point is expressed as the mean ± SE (n = 3).

reached equilibrium at 72 h with 51% gemcitabine released and an estimated 49% gemcitabine remaining bound in the PG-G conjugate.

Although the pattern of total gemcitabine released from PG-G in plasma at the time intervals measured is statistically different to that of gemcitabine released from PG-G in aqueous solution (PBS) at pH 7.4 and 37 °C (Fig. 3D) (2-way ANOVA; $P < 0.05$), the trend is similar. This result suggests that the release of gemcitabine from the polymeric backbone in plasma is a hydrolytic process without the involvement of plasma esterases for the cleavage of the ester bond between gemcitabine and the polymer. The gemcitabine released was then degraded into Dgp (2′-deoxy-2′,2′-difluorodeoxyuridine) by enzymes, such as cytidine deaminase, present in the plasma. The involvement of enzymes in the formation of Dgp is supported by the lack of degradation of gemcitabine in aqueous solution (Fig. 2A), as compared to the significant degradation in plasma (Fig. 3B), and is consistent with earlier reports (Abbruzzese et al., 1991; Immordino et al., 2004; Lund et al., 1993).

In contrast to the immediate onset of gemcitabine release and attainment of the maximum plasma concentration of gemcitabine at 2 h for the PHEA-(5′succinylgemcitabine)-(1′-carboxypentyl)-folamide conjugate (Cavallaro et al., 2006), PG-G showed a late onset of gemcitabine release of 6 h post-initiation of the incubation and the attainment of the maximum plasma concentration of gemcitabine at 72 h (24.4%) (Fig. 3B). Although the gemcitabine plasma concentration (gemcitabine only without Dgp) at equilibrium was similar to that of the PHEA-(5′succinylgemcitabine)-(1′-carboxypentyl)-folamide conjugate (20%, w/w, attained at 2–4 h post-initiation of incubation) (Cavallaro et al., 2006), the time for PG-G to achieve gemcitabine release equilibrium was significantly longer. These results suggest the presence of an extended sustained drug release property of PG-G as compared to the PHEA based polymer-drug conjugates.

In chemotherapy with gemcitabine, high doses of 1000 mg/m^2 are given to patients through intravenous infusion over 30 min per treatment period to maintain an adequate concentration of gemcitabine in the body, due to the rapid loss of gemcitabine from the patient's body via blood degradation and renal excretion (Immordino et al., 2004). This type of drug administration incurs extra cost and discomfort. Furthermore, it causes an inconvenience to the patient, and exposes the patient to the risk of non-specific acute drug toxicity. The use of a biodegradable PG-G conjugate may alleviate the abovementioned shortcomings of gemcitabine treatment. This is due to the sustained gemcitabine release characteristics of PG-G that may maintain the blood concentration of gemcitabine at a consistent level via the gradual release of the drug into the blood circulation upon detachment from PG. The sustained release reduces the need for prolonged infusion of gemcitabine into the patient's body. Furthermore, the delayed drug release property of PG-G may prove to be important in providing a prolonged and constant supply of active drug to patient after administration. It may also help in giving sufficient time for the macromolecular polymer-drug conjugate to penetrate into the tumour tissue by the enhanced permeability–retention effect, before releasing the bulk of the bound drug. This may reduce premature release of drug into the blood circulation system and thus avoid non-specific tissue toxicity.

5.5. In vitro cytotoxicity studies

The cytotoxicity of PG-G conjugate was studied using oestrogen receptor positive (ER+) MCF-7 and oestrogen receptor negative (ER−) MDA-MB-231 cells as the *in vitro* human breast tumour models (Bates et al., 1990; Van Dijk et al., 1997). Murine 4T1 cell line was included as an *in vitro* animal breast cancer model (Aslakson & Miller, 1992; Pulaski & Ostrand-Rosenberg, 1998), while human dermal fibroblasts (HDF) represented normal human cells. The use of these cells allowed for the evaluation of *in vitro* targeting property of PG-G and its biological activity.

The effects of gemcitabine, PG-G and PGNa on the viability of different cell lines after 72 h incubation are depicted in Fig. 4A–D. The polymeric carrier PGNa did not display any cyto-

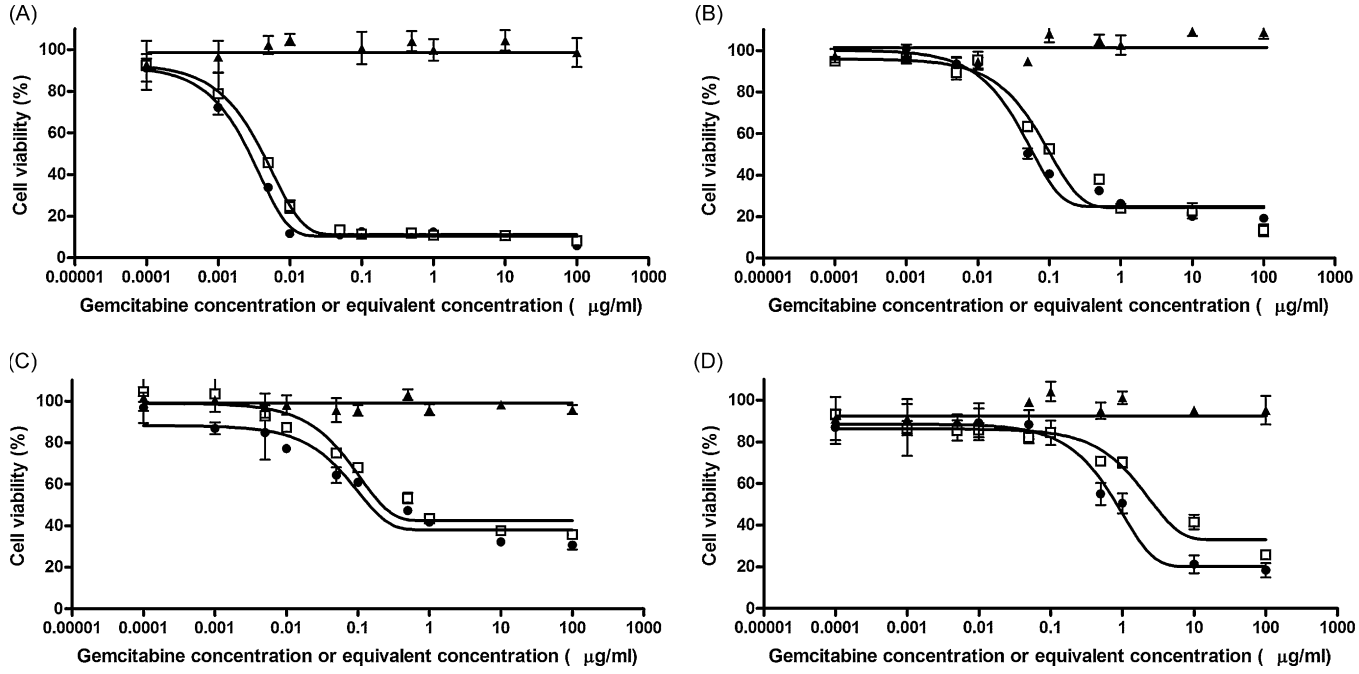


Fig. 4. The *in vitro* cytotoxicity profiles of poly-L-glutamic acid-gemcitabine (PG-G) in 4T1 (A), MCF-7 (B), MDA-MB-231 (C) and HDF (D) cell lines, with the *y*-axis representing % of cell viability relative to the negative control and the *x*-axis is the gemcitabine concentration or gemcitabine equivalent concentration (μg/ml), in log scale. The profiles were generated from the results of MTT assays performed on the respective cell lines after incubating the cells in DMEM-GM containing either PG-G (□) or gemcitabine (●) (with gemcitabine content or equivalent content ranging from 0.0001 to 100 μg/ml), or poly-L-glutamic acid sodium salt (▲) (PGNa, carrier control, amount of PGNa used was equivalent to the amount of PG-G used for each treatment concentration in PG-G group) for 72 h. Each data point is expressed as the mean % ± SE ($n = 3$).

toxicity which clearly indicates that it is biocompatibility. On the other hand, gemcitabine and PG-G exhibited dose dependent cytotoxicity in all the cell lines tested, and generally the following order of decreasing cytotoxicity was observed: 4T1 [IC_{50}, 0.003 μg/ml (gemcitabine), 0.004 μg/ml (PG-G)] > MCF-7 [IC_{50}, 0.061 μg/ml (gemcitabine), 0.104 μg/ml (PG-G)] > MDA-MB-231 [IC_{50}, 0.143 μg/ml (gemcitabine), 0.216 μg/ml (PG-G)] > HDF [IC_{50}, 0.843 μg/ml (gemcitabine) and 2.735 μg/ml (PG-G)]. Gemcitabine is a nucleotide analogue of deoxycytidine; it exerts its cytotoxic effects via incorporation of its triphosphate derivative (dFdCTP) into the replicating DNA strands, inducing masked chain termination, which in turn leads to failure in DNA strand elongation and eventually cell death (Plunkett et al., 1996). Therefore, it is expected that cells with rapid doubling time such as 4T1 (doubling time = 19 h) (Li et al., 2000) will be more susceptible to gemcitabine compared to cells with a lower replication rate such as HDF (doubling time = 7–10 days) (Kwak et al., 2004).

PG-G displayed lower cytotoxicity compared to that of gemcitabine in MCF-7, MDA-MB-231 and HDF cells. Only in 4T1 cells was the IC_{50} for both PG-G and gemcitabine similar. These findings on MCF-7, MDA-MB-231 and HDF cells are consistent with previous studies that showed a reduction of *in vitro* cytotoxicity of anticancer drugs upon their conjugation to poly-L-glutamic acid carriers. These include PG-doxorubicin (Hoes et al., 1986, 1993; Zunino et al., 1989), PG-daunorubicin (Hurwitz et al., 1980), PG-Ara-C (Kato et al., 1984), PG-uracil (Mochizuki et al., 1985), PG-cyclophosphamide (Batz et al., 1974), PG-melphalan (Morimoto et al., 1984), PG-paclitaxel (Zou et al., 2001) and PG-camptothecin (Singer et al., 2000, 2001). Rapid, free diffusion of low molecular weight compounds into the cultured cells, as compared to the slow uptake of polymer conjugates into cells by endocytosis, was suggested to account for the relative ineffectiveness *in vitro* of the polymer-drug conjugates in intoxicating cancer cells (Duncan et al., 2005).

Pulse-chase *in vitro* cytotoxicity studies on the effects of exposure time, indicate that MCF-7 cells were less sensitive to PG-G compared to gemcitabine for the initial 8 h of exposure (2-way ANOVA; $P < 0.05$). Between 12 and 72 h, the toxicities of both PG-G and gemcitabine were similar ($P > 0.05$) (Fig. 5A). This result suggests that gemcitabine was gradually released from PG-G extracellularly and taken up into the cells rather than active uptake of macromolecular PG-G. The same mechanism is likely to occur for both the MDA-MB-231 and HDF cells tested here. This hypothesis is also supported by the hydrolytic release of gemcitabine from PG-G in our hydrolytic study discussed above and the findings reported on the uptake pattern of poly-L-glutamic acid-paclitaxel (PG-TXL) by MDA-MB 453 cells (Oldham et al., 2000).

Pulse-chase time exposure studies on 4T1 indicate that both free gemcitabine and PG-G exhibited similar patterns of cytotoxicity from 0 to 72 h (2-way ANOVA; $P > 0.05$) (Fig. 5B). The maximum cytotoxicity was observed after 0.5 h of exposure to either gemcitabine or PG-G. The hydrolytic release of gemcitabine from PG-G is relatively low or negligible for the first 3 h (Fig. 2B), and therefore it is unlikely that the amount of gemcitabine released from PG-G extracellularly is the same as the free gemcitabine at 0.5 h exposure (Fig. 5B). Moreover, the IC_{50} values of PG-G (0.004 μg/ml) and gemcitabine (0.003 μg/ml) on 4T1 cells after 72 h exposure were similar (Fig. 4A). These results suggest that macromolecular PG-G can be actively taken up by 4T1 cells, and probably occurs via endocytosis followed by intracellular hydrolytic release of gemcitabine from the backbone of PG-G, and may account for the short exposure time required to exhibit maximum cytotoxicity.

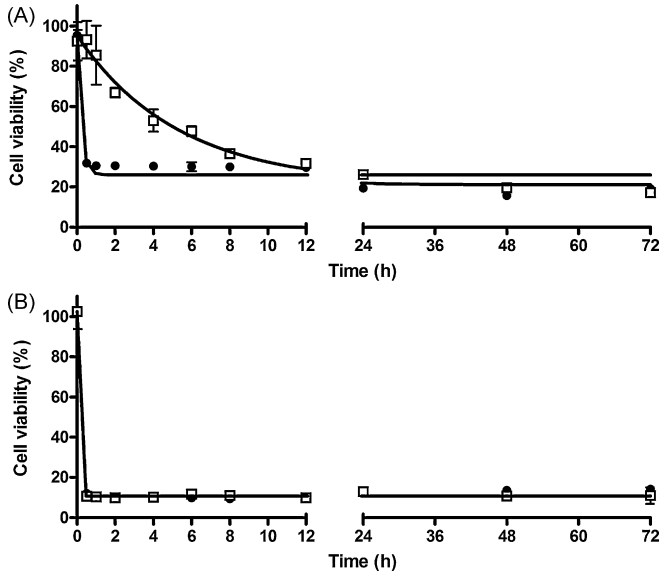


Fig. 5. The *in vitro* pulse-chase (time course exposure) study profiles of poly-L-glutamic acid-gemcitabine (PG-G) (□) and gemcitabine (●) in MCF-7 (A) and 4T1 (B) cell lines. The *y*-axis represents % of cell viability relative to the negative control and the *x*-axis is the exposure time (h) to either PG-G or gemcitabine. The profiles were generated from the results of MTT assays performed on the MCF-7 and 4T1 cells that had been first exposed to DMEM-GM containing either PG-G or gemcitabine at appropriate gemcitabine or equivalent concentration of 10 μg/ml for different exposure times (0, 0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72 h), and then further incubated in drug free DMEM-GM until the entire duration of 72 h was reached. Each data point is expressed as the mean % ± SE ($n = 3$).

6. Conclusion

We report preliminary findings on the use of poly-L-glutamic acid as an alternative polymeric carrier for gemcitabine. The synthesis of PG-G can be carried out through simple conjugation chemistry. The drug conjugate demonstrated improved drug loading, *in vitro* hydrolytic stability and plasma stability as compared to other reported polymeric gemcitabine conjugates (Cavallaro et al., 2006). The cytotoxicity studies suggest that PG-G possesses lower non-specific tissue toxicity compared to free gemcitabine. Furthermore, tumour annihilation may be facilitated by improved drug targeting via accumulation at the tumour sites through the enhanced permeability and retention (EPR) effect (Maeda & Matsumura, 1989; Reynolds, 1995). The extended sustained release profile of the PG-G conjugate may render it a potential gemcitabine anticancer prodrug.

Acknowledgements

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