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Preparation and characterization of gemcitabine liposome injections

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Gemcitabine liposome injection (stealth liposomes) has facilitated the targeting of gemcitabine for cancer treatment. We systemically review liposome-based drug-delivery systems, which can improve pharmacokinetics, reduce side effects and potentially increase tumor uptake, for pancreatic cancer therapy. A novel liposomal formulation, which allows for higher tumor targeting efficiencies and can be used in current clinical trials to treat this challenging disease, has gained great popularity and attention. In this study, since extrusion technology was used to make sterile preparation of liposomes, the process included aseptic production process and sterile filtration. During the preparation, it has been found that the lipid concentration, emulsification speed and time, the homogenization times and pattern, the lipid solution temperature are all critical parameters for the character of the gemcitabine liposome injection. The particle size method and zeta potential method to characterize a PEGylated liposomal drug formulation of the anti-cancer agent gemcitabine was developed. The methods are specific, precise, reproducible and sensitive, therefore they are suitable for the determination of particle size and zeta potential of gemcitabine liposome injection. Negative staining technology of transmission electron microscopy revealed that gemcitabine liposome injection has a typical morphology, which enables liposomal surfaces could be seen so additional visual information on the stealth liposome can be routinely obtained in a fast and reliable manner. Moreover, the above three methods are simple, fast and would be used for continuous quality control of gemcitabine liposome injection when it moves to cGMP production scale.

1. Introduction

Despite of rapid advances in cancer diagnosis and treatment, pancreatic cancer remains one of the most difficult human malignancies to be treated, with a mortality rate nearly equal to its incidence. Although gemcitabine (GEM) has been established as the standard first-line treatment for advanced pancreatic cancer (Stathis and Moore 2010; Yu et al. 2010; Toschi et al. 2005), GEM-based combination chemotherapy showed either marginal or no improvement in survival. GEM is a new fluorinated nucleoside analogue (2′,2′-difluorodeoxycytidine). When effective anti-tumor doses of this compound were given, hematological toxicity and other side effects occurred (although they are lower than those of other antineoplastic drugs) (Fossella et al. 1997; Dasanu 2008). Following systemic administration, GEM is rapidly converted into the inactive metabolite 2′-deoxy-2′,2′-difluorouridine by cytidine deaminase. This metabolite is rapidly excreted in the urine (Abbruzzese et al. 1991; Storniolo et al. 1997). To reduce these therapeutic complications and improve the drug biopharmaceutical features, such as plasmatic half-life, several strategies have been proposed (Reddy and Couvreur 2008), i) the encapsulation of the drug in the aqueous liposomal compartment after a pH gradient generation (Celano et al. 2004), ii) its entrapment in vesicle bilayers or polymeric nanoparticles as lipophilic derivates, iii) the inclusion in polyaspartylhydrazide copolymer-based supramolecular vesicular aggregates, iv) stealth liposomes can improve its tumor localization. In particular, liposomal device offers the possibility to easily modulate the biopharmaceutical properties of the active compound without use of drug chemical structural modification such as covalent bonds as a consequence of the opportunity to modify the technological characteristics of the carrier.

Developments in liposomal delivery systems (stealth liposomes) have facilitated the targeting of specific agents for cancer treatment. Such systems could be developed as platforms for future multi-functional nano devices tailor-made for the combined detection of early cancer and functional drug delivery. We systemically review liposome based drug-delivery systems, which can provide improved pharmacokinetics, eliminate side effects and potentially enhance tumor uptake, for pancreatic cancer therapy. Novel liposomal formulations allowing for higher tumor targeting efficiencies and used in current clinical trials to treat this challenging disease are emphasized.

2. Investigations, results and discussion

The gemcitabine liposome injection was prepared by an extrusion method. The extrusion method was designed to generate homogeneous populations of unilamellar vesicles (liposomes) without the need for solvents or detergents. The extrusion method was quick and it is one-step procedure that produces liposomes by forcing aqueous suspensions of lipid through polycarbonate filters with defined pore size. It is relatively gentle,

Table 1: Results of precision and intermediate precision study of particle size for gemcitabine liposome injection determination

Sample	*d10* %R.S.D. [a], day-1	*d50* %R.S.D. [a], day-1	*d90* %R.S.D. [a], day-1	*d10* %R.S.D. [a], day-2	*d50* %R.S.D. [a], day-2	*d90* %R.S.D. [a], day-2
Size-1	1.47	2.61	3.98	1.73	2.88	3.88
Size-2	1.73	2.00	3.71	1.34	2.65	4.76
Size-3	1.14	2.69	4.59	1.65	2.48	4.88
Size-4	1.39	2.75	4.18	1.56	2.29	4.65
Size-5	1.51	2.73	4.56	1.43	2.77	4.41
Size-6	1.74	2.69	4.81	1.48	2.86	4.46
Overall %R.S.D. (18 injections)	1.77	2.46	4.37	1.51	2.46	4.69

[a] Each sample was analyzed in triplicate at 25 °C

exposing the formulation to moderate pressures (100∼500 psi) at controlled temperatures, at the same time it offered a number of advantages: i) extruders were reliable, had no moving parts, and were made of high grade stainless steel, ii) particle size was homogeneous, and readily controlled, iii) high trapping efficiencies were achieved, iv) operating costs were low and equipment was easy to use, v) bridging the gap from research prototypes to batches prepared for production under cGMP, vi) the extrusion method was also equipped with a cold storage, back-up generator, purified water distribution loop, WFI reservoirs, glycol heating system, HVAC, clean compressed air or bottled gases.
In this work a Zetasizer and transmission electron microscopy were used to characterize a PEGylated liposomal formulation of the anti-cancer agent GEM. The current particle size method and zeta potential method operated simply and rapidly. The method validation results indicate that the methods are specific, precise, reproducible and sensitive those are suitable for determination of particle size and zeta potential in GEM liposome injection. Negative staining technology of transmission electron microscopy revealed a typical morphology, which allowed it to be able to detect the nanostructure of GEM liposome injection on the molecular level in a fast and efficient manner.

2.1. Particle size

Particle sizes of gemcitabine liposome injection are shown in Fig. 1. The precision of the current method was evaluated by repeatability (intra-day) and intermediate precision (inter-day). The repeatability was done by analyzing six aliquots of GEM liposome injection from a single container, each sample was diluted 100-fold with ultra-pure water and analyzed in triplicate at 25 °C. The same process was repeated on a second day to assess intermediate precision using six freshly prepared aliquots of GEM liposome injection from the same container. The precision was measured by the %R.S.D. of the triplicate injections for each of the six samples. It was found that the precision were *d*10 1.77%, *d*50 2.46% *d* 90 4.37% on day-1 and *d*10 1.51%, *d*50 2.46% *d* 90 4.69% on day-2 (Table 1). These data demonstrated the acceptable precision of the method.

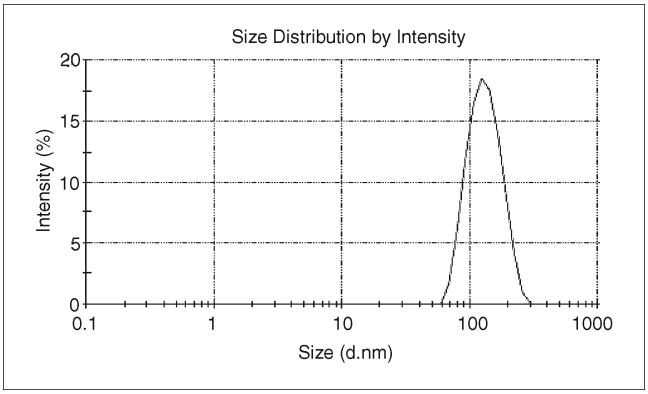

Fig. 1: Size distribution by intensity of gemcitabine liposome injection

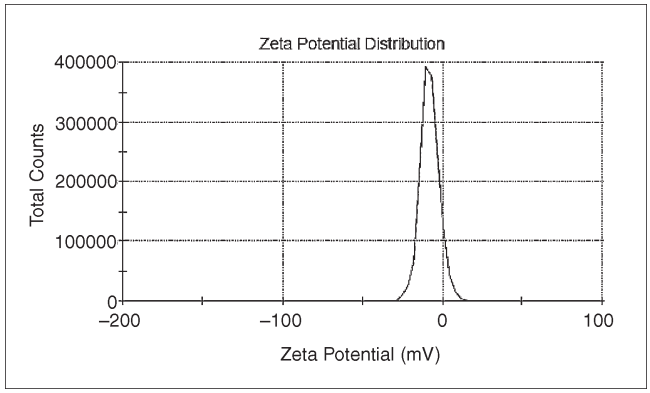

Fig. 2: Zeta potential distribution of gemcitabine liposome injection

2.2. Zeta potential

The zeta potential of gemcitabine liposome injection is given in Fig. 2. The values are the arithmetic mean of 5 measurements taken after 5, 15, 30, 60 min and 2 h, for each sample (Table 2). System reproducibility of the zeta potential method was validated.

2.3. Microscopic observation

Gemcitabine liposome injection was centrifuged at 60,000 revolutions per minute (300,000 × g) for 1 h with a Beckman Optima L-80XP super-speed centrifuge instrument. The portion of the supernatant was carefully transferred to a centrifuge tube. The other portion of the deposit dissolved in the ultrapure water. Because the surroundings of GEM liposome injection contain 0.3 mol/L glucose, the glucose could be interference Transmission electron microscopy detection. If glucose exists in the GEM liposome injection, the Transmission electron microscopy of liposome figures could not be seen except for a block of gray. The GEM liposome injection in the ultrapure water was instability (the size easy to increase). So when the figures of the

Table 2: Reproducibility of the zeta potential for gemcitabine liposome injection determination

Time	Zeta potential (mv)	Average (mv)	R.S.D(%)
5 min	−7.26		
15 min	−7.71		
30 min	−7.37	−7.40	−2.65%
60 min	−7.21		
2 h	−7.44		

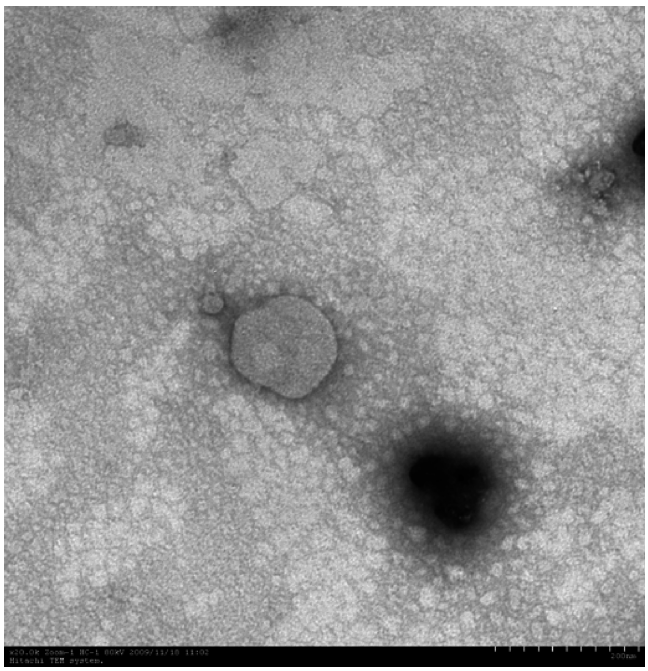

Fig. 3: Transmission electron micrograph (TEM) of GEM liposome injection. Magnification 20,000 × . Zoom-1. HC-1. 80kv. The bar corresponds to 200 nm

GEM liposome injection were investigational, the GEM liposome injection was immediately centrifuged.

As shown in Figs. 3–4 (Rossi et al. 2004), the morphological investigation using transmission electron microscopy revealed nanometric sized and quasi-spherical shaped liposomes. According to TEM micrographs, liposomes ranged in size from 120 to 160 nm correlating well with measurement obtained by PCS. Vesicle membranes were composed of HSPC bilayers. Cholesterol inserts the HSPC bilayers. DSPE-PEG2000 used as stealth liposome material. Structure chart is shown in Fig. 5.

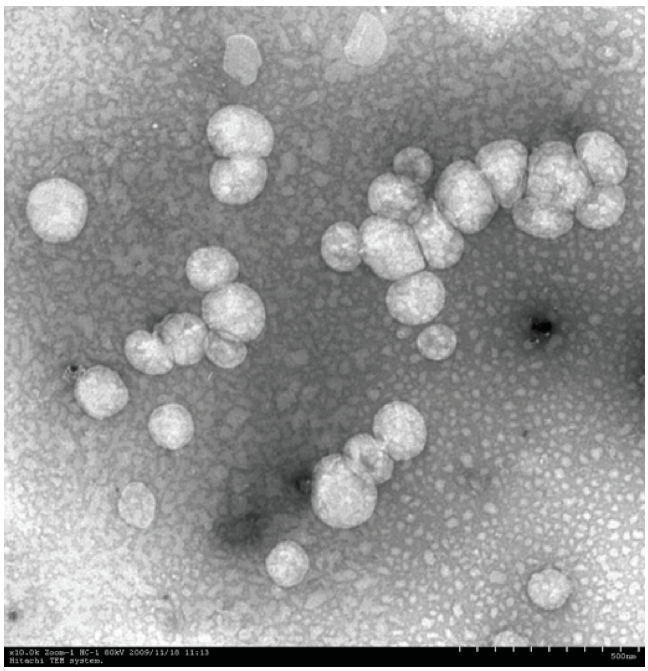

Fig. 4: Transmission electron micrograph (TEM) of GEM liposome injection. Magnification 10,000 × . Zoom-1. HC-1. 80 kv. The bar corresponds to 500 nm

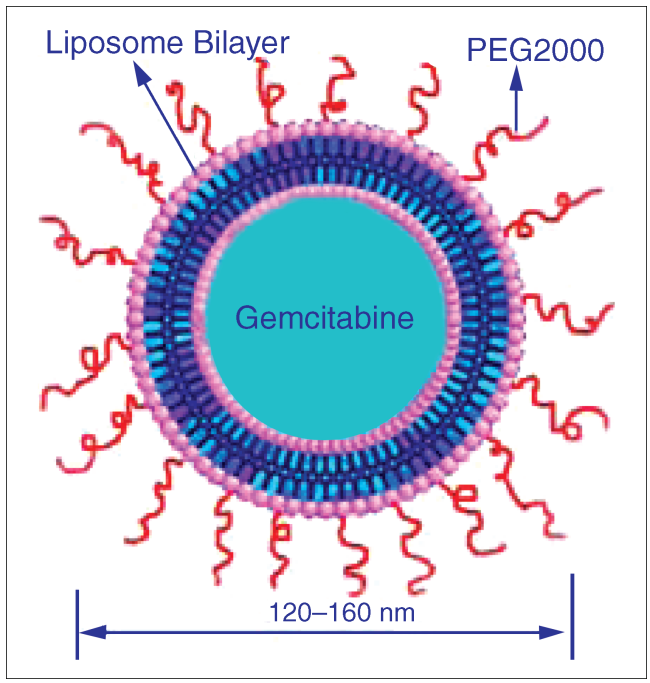

Fig. 5: Structure chart of GEM liposome injection

3. Experimental

3.1. Materials

Hydrogenated soy phosphatidylcholine (HSPC) and DSPE-PEG2000 were purchased from Lipoid, Ludwigshafen, Germany. The purity of both materials given by the manufacturer was > 99%. Cholesterol (Ch) was purchased from Avanti Polar Lipid (Alabaster city, AL, USA). Gemcitabine was supplied by Jiangsu Aosaikang Pharmaceutical Co., Ltd. The purity of gemcitabine was > 99.5%. Tertiary butyl alcohol was purchased from Sinopharm Chemical Reagent Co.,Ltd. The purity of Tertiary butyl alcohol given by the manufacturer was > 99%. All other reagents and solvents were of the highest purity available.

3.2. Liposome preparation

3.2.1. Preparation of lipid for hydration

The lipids (HSPC:Chol:DSPE-PEG2000 = 3:1:1,w/w) (Woodle et al. 1992; Ranson et al. 2001) must first be dissolved and mixed in tertiary butyl alcohol to assure a homogeneous mixture of lipids. The intent was to obtain a clear lipid solution for complete mixing of lipids. Typically lipid solutions were prepared at 10 mg lipid/ml tertiary butyl alcohol. Once the lipids were thoroughly mixed in tertiary butyl alcohol. The solvent was removed to yield a lipid film. The lipid solution was transferred to containers and frozen by placing the containers on a block of dry ice-alcohol bath. Cave should be taken when using the bath procedure that the container could withstand sudden temperature changes without cracking. After freezing completely, the frozen lipid cake was placed on a vacuum pump and lyophilized until dryness (1–3 days depending on volume). The thickness of the liquid cake should be no more than the diameter of the container being used for lyophilization. Dry lipid films or cakes could be removed from the vacuum pump. The container closed tightly and taped, and stored −20 °C until ready to hydrate (Lichtenberg and Barenholz 1988).

3.2.2. Hydration of lipid film/cake

Hydration of the dry lipid film/cake was accomplished simply by adding gemcitabine (GEM) solution (5% GS adjust to osmotic pressure of the human body, 290 mOsmol/kg) to the container of dry lipid and agitating. The temperature of the hydrating medium should be above the gel-liquid crystal transition temperature (Tc or Tm) of the lipid with the highest Tc before adding to the dry lipid. The gel-to-liquid crystalline phase transition temperature, Tm, of HSPC, 53 °C (Lichtenberg and Barenholz 1988). So we selected the temperature of the hydrating medium as 55 °C. Spinning the round bottom flask in the warm water bath maintained at a temperature of 55 °C allowed the lipid to hydrate in its fluid phase with adequate agitation. Hydration time was 20–30 min. Once a stable, hydrated large multilamellar vesicle (LMV) suspension had been produced, the particles could be downsized by extrusion.

3.2.3. Sizing of liquid suspension

Extrusion (T.800 LIPEXTM Extruder for preparing lipsomes, Northern Lipids INC, Canada): Lipid extrusion was a technique in which a lipid suspension was forced through a polycarbonate filter with a defined pore size to yield particles having a diameter near the pore size of the filter used. Prior to extrusion through the final pore size, LMV suspensions were disrupted by prefiltering the suspension through a larger pore size (typically 0.4 μm). This method helped prevent the membranes from fouling and improves the homogeneity of the size distribution of the final suspension. Then LMV suspensions were disrupted by filtering through 200 nm, 100 nm polycarbonate filter. As with all procedures for downsizing LMV dispersions, the extrusion should be done at a temperature above the Tc of the lipid. We selected a extrusion temperature of 55 °C. Because the extrusion temperature below the Tc would be unsuccessful as the membrane had fouled with rigid membranes which could not pass through the pores. Extrusion through filters with 100 nm pores typically yielded large, unilamellar vesicles (LUV) with a mean diameter of 120–160 nm.

3.3. Hollow fiber module

The Flexstand Benchtop system was purchased from GE (GE Healthcare, America). This module contained UFP-500-C-4X2MA polypropylene hollow fibers distributed in a uniform way around a central tube, so as to allow the use of the total membrane surface. The MasteFlex I/P pump was used. The hollow fiber module dimensions were as follows: an inner diameter of 0.5 mm, a pore size estimated at 500,000 NMWC. The fiber length was 60 cm and the total active membrane surface was 0.14 m^2 (1.5 ft^2).
The polypropylene hollow fibers were naturally hydrophobic. Hence, they must be treated before their first use as follows: a water/ethanol (50:50, v/v) solution was maintained in circulation through the module by applying a low pressure (0.2 bar). After 10 min, water droplets appeared on the filtrate side instead of the outlet side of the membrane device. The module lost its hydrophobic character and was then be rinsed with ultra-pure water. This method to treat the membrane module was suggested by the manufacturer [13].

3.4. Particle size and zeta potential analysis

The parameters used to measure zeta potential were: Malvern Instruments Nano-ZS, sample dispersant: water, sample temperature: 25 °C, sample viscosity: 0.8872 cP, sample RI: 1.330 sample equilibration time: 2 min, sample cell type: DTS0012-disposable size cuvette, measurement duration: automatic, number of measurements: 1. Dynamic light scattering (DLS), otherwise known as photon correlation spectroscopy (PCS), was extensively used in liposome size distribution analysis. In this study, a Malvern Zetasizer Nano-series (Malvern Instruments Nano-ZS, Malvern, UK) was used. Each sample was diluted 100-fold with ultra-pure water and analyzed in triplicate at 25 °C. The data on particle-size distribution were collected using the DTS (Nano) software (Version 5.03) provided with the instrument. The sizes mentioned correspond to the hydrodynamic diameter of these particles. In addition, the polydispersity index (PI) was calculated in terms of span factor defined as span = (d90–d10)/d50 where d10, d50 and d90 were the particle diameters at 10%, 50% and 90% of the cumulative liposome number, respectively. The PI was an indicator of the width of particle size distribution of a sample. It was ranging from 0 (monodispersed) to 0.5 (relatively broad distribution).
These data (particle size and PI) were expressed as the mean ± standard deviation (S.D). Prior to measuring size and zeta potential, no calibration was needed. The DLS method presented the advantage to be simple and fast. Measurements of zeta potential were commonly used to predict the colloidal system stability. The samples were directly measured without dilution. All the measurements were performed at least three times. The zeta potential was calculated from the electrophoretic mobility by the Helmholtz–Smoluchowski equation.

3.5. Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) images were taken using a H-7650 Transmission electron microscope (Hitachi, Japan) operating at an accelerating voltage of 80 kV. The sample preparation was performed according to previous study (Jaafar-Maalej et al., 2010). A drop of liposome suspension was placed onto a carbon-coated copper grid; the suspension excess was removed with a filter paper leaving a thin liquid film stretched over the holes. Negative staining using a 2% phosphotungstic acid solution (w/w), pH 7.1, was directly made on the deposit during 15–30 min. Finally, the excess of phosphotungstic solution was removed with a filter paper and stained samples were observed.

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References

Abbruzzese JL, Grunewald R, Weeks EA, Gravel D, Adams T, Nowak B, Mineishi S, Tarassoff P, Satterlee W, Raberet MN (1991) A phase I clinical, plasma, and cellular pharmacology study of gemcitabine. J Clin Oncol 9: 491–498.

Celano M, Calvagno MG, Bulotta S, Paolino D, Arturi F, Rotiroti D, Filetti S, Fresta M, Russo D (2004) Cytotoxic effects of gemcitabine-loaded liposomes in human anaplastic thyroid carcinoma cells. BMC Cancer 4: 63–70.

Dasanu CA (2008) Gemcitabine vascular toxicity and prothrombotic potential. Expert Opin Drug Saf 7: 703–716.

Fossella FV, Lippman SM, Shin DM, Tarassoff P, Calayag-Jung M, Perez-Soler R, Lee JS, Murphy WK, Glisson B, Rivera E, Hong WK (1997) Maximum-tolerated dose defined for single-agent gemcitabine. A phase I dose–escalation study in chemotherapy-naive patients with advanced non-small-cell lung cancer. J Clin Oncol 15: 310–316.

Laouini A, Jaafar-Maalej C, Sfar S, Charcosset C, Fessi H (2011) Liposome preparation using a hollow fiber membrance contactor – Application to spironolactone encapsulation. Int J Pharm 415: 53–61.

Lichtenberg D, Barenholz Y (1988) Liposomes: preparation, characterization, and preservation. Methods Biochem 33: 337–462.

Ranson MR, Cheeseman S, White S, Margison J (2001) Caelyx (stealth liposomal doxorubicin) in the treatment of advanced breast cancer. Crit Rev Oncol Hematol 37: 115–120.

Reddy LH, Couvreur P (2008) Novel approaches to deliver gemcitabine to cancers. Curr Pharm 14: 1124–1137.

Rossi C, Fardella G, Chiappini I, Perioli L, Vescovi C, Ricci M, Giovagnoli S, Scuota S (2004) UV spectroscopy and reverse-phase HPLC as novel methods to determine Capreomycin of liposomal formulations. J Pharm Biomed Anal 36: 249–255.

Stathis A, Moore ML (2010) Advanced pancreatic carcinoma: current treatment and future challenges. Nat Rev Clin Oncol **7**: 163–172.

Storniolo AM, Allerheiligen SRB, Pearce HL (1997). Preclinical, pharmacologic, and phase I studies of gemcitabine. Semin Oncol 242 suppl 7 2–7.

Toschi L, Finocchiaro G, Bartolini S, Gioia V, Cappuzzo F (2005) Role of gemcitabine in cancer therapy. Future Oncol 1: 7–17.

Woodle MC, Newman MS, Martin FJ (1992) Liposome, leakage and blood circulation: Comparison of adsorbed block copolymers with covalent attachment of PEG. Int J Pharm 88: 327–334.

Yu X, Zhang Y, Chen C, Yao Q, Li M (2010) Targeted drug delivery in pancreatic cancer. Biochim Biophys Acta 1805**:** 97–104.