Pharm Res (2012) 29:1908–1918
DOI 10.1007/s11095-012-0716-6

RESEARCH PAPER

# Relationship between the Affinity of PEO-PPO-PEO Block Copolymers for Biological Membranes and Their Cellular Effects

Martin Redhead • Giuseppe Mantovani • Selina Nawaz • Paola Carbone • Dariusz C. Gorecki • Cameron Alexander • Cynthia Bosquillon

Received: 10 January 2012 / Accepted: 20 February 2012 / Published online: 3 March 2012


## ABSTRACT

***Purpose*** The interactions of poly(ethylene oxide)-co-poly(propylene oxide) tri-block copolymers (PEO-PPO-PEO block copolymers, Pluronics®, Synperonics®, Poloxamers) of differing chemical composition with cell membranes were systematically investigated in order to clarify the mechanisms behind their previously reported various cellular responses.

***Methods*** Relationships between the structural components of a defined series of PEO-PPO-PEO block copolymers and i) their interactions with biological membranes; ii) their cytotoxic potential were probed using a combination of haemolysis studies and cytotoxicity assays in the Caco-2 and HMEC-1 cell lines.

***Results*** The length of the PPO block as well as the PEO/PPO ratio were determinants of their membrane binding constant and cytotoxicity endpoints measured in the MTS and LDH assays. Similar 2D parabolic relationships were found between polymer composition and their affinity for membranes or their cytotoxicity potential. Cytotoxicity was related to the ability of the copolymers to form ion transversable pores within the cell membrane.

***Conclusions*** The data suggest a link between the affinity of certain Pluronics for biological membranes and their cellular adverse effects. This first cell-based investigation of the interactions of Pluronics with biological membranes is an important step towards unravelling the complex mechanisms which govern the biological effects of widely used amphiphilic materials.

**KEY WORDS** biocompatibility · drug delivery · haemolysis · membrane · pluronics

## INTRODUCTION

PEO-PPO-PEO block copolymers (synonyms: Pluronics®, Synperonics®, Poloxamers) are ABA triblock copolymers, with a central hydrophobic poly(propylene oxide) (PPO) block flanked by two hydrophilic poly(ethylene oxide) (PEO) blocks. In solution, PEO-PPO-PEO block copolymers can assemble into micelles, with the PPO blocks forming a hydrophobic core and the PEO blocks the surrounding hydrophilic corona (1).The hydrophobic core of these micelles can be used to incorporate poorly water soluble drugs, and thus improve their pharmacokinetics and biodistribution (2,3).

Although PEO-PPO-PEO block copolymers are widely used and many are essentially inert to biological substrates, materials within the overall class have recently been reported to elicit marked biological responses, both *in vitro* and *in vivo*. Copolymers of differing chemical composition have displayed varying cytotoxicity profiles, as well as therapeutically desirable effects (4,5). For instance, they have the

**Electronic supplementary material** The online version of this article (doi:10.1007/s11095-012-0716-6) contains supplementary material, which is available to authorized users.

M. Redhead · G. Mantovani · C. Alexander · C. Bosquillon
Division of Drug Delivery and Tissue Engineering, School of Pharmacy
University of Nottingham
University Park, Nottingham NG7 2RD, UK

S. Nawaz · P. Carbone
School of Chemical Engineering and Analytical Science
The University of Manchester
Oxford Road, M13 PL9, Manchester, UK

D. C. Gorecki
School of Pharmacy and Biomedical Sciences
University of Portsmouth
White Swan Road, Portsmouth PO1 2DT, UK

C. Bosquillon (✉)
School of Pharmacy, Centre for Biomolecular Sciences
University of Nottingham
University Park, Nottingham NG7 2RD, UK
e-mail: cynthia.bosquillon@nottingham.ac.uk

capacity to circumvent multi drug resistance in cells over-expressing the antiapoptotic protein Bcl-2 (6), or to inhibit the P-glycoprotein pump (P-gp) which, when overexpressed leads to the rapid efflux of cytotoxic drugs out of tumoral cells (7). Formulations of Pluronics and doxorubicin have reached clinical trials in cancer therapy and have been shown to be more efficacious than the drug alone (8,9). Other reported biological effects of PEO-PPO-PEO block co-polymers include the depletion of glutathione and ATP, NF κB activation and enhanced expression of transfected genes (4,10,11), although this latter effect may be promoter dependent (12).

It has been shown both the PEO and PPO blocks of the copolymers impact on the intensity of the biological effects measured, with moderately lipophilic copolymers being the most potent (4,5). Nevertheless, detailed relationships between polymer composition and biological effects remain to be defined. Similarly, it is unclear whether cellular responses to amphiphilic co-polymer exposure result from their interactions with biological membranes. For example, P-gp inhibition has been ascribed to their effect on membrane fluidity (7,13). In contrast, the mechanism by which they induce NF κB activation remains speculative, although it has been suggested this may involved a previously undisclosed signalling pathway (14).

One of the possible mechanisms by which amphiphilic polymers such as PEO-PPO-PEO block copolymers cause cytotoxicity might be via cell membrane solubilisation. Indeed, a high hydrophobic content in PEO-PPO-PEO block copolymers has been shown to increase their surfactant activity in dipalmitoylphosphatidylcholine (DPPC) monolayers and to reduce the surface pressure required to penetrate the lipid layers (15). For polymers with long PEO blocks, penetration into monolayers was enhanced by the presence of cholesterol, reportedly due to hydrogen bonding between PEO chains and cholesterol (16).

While several studies have explored the mechanisms of PEO-PPO-PEO block copolymer insertion into artificial membranes (15–18), their interactions with cellular membranes have not been extensively investigated, which has, so far, prevented polymer adverse effects on cells to be related to their affinity for biomembranes. Accordingly, a systematic investigation of Pluronic cytotoxic potential is required to assist in the selection of safe and efficient carriers for specific biomedical applications.

In this study, a combination of cytotoxicity and haemolysis assays were employed to probe relationships between the structural features of PEO-PPO-PEO block copolymers and their interactions with biomembranes. Cytotoxic endpoints in the intestinal epithelial Caco-2 cell line and in Human Microvascular Endothelial Cells HMEC-1 were compared to the copolymer membrane binding constant. Correlations between the polymer chemical constituents and the biological responses measured were investigated to gain a better understanding of the mechanisms behind the cellular effects of PEO-PPO-PEO block copolymers.

## MATERIALS AND METHODS

### Materials

Caco-2 and HMEC-1 cells were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). PEO-PPO-PEO block copolymers L31, F38, P85, L62 and L122 were purchased from BASF (Cheadle, UK), the MTS Reagent and CytotoxONE™ LDH assay kit were obtained from Promega (Southampton, UK) and the cell culture medium RPMI 1640 from Lonza (Slough, UK). 96-well plates were obtained from Corning, (Birmingham, UK). All other reagents were purchased from Sigma-Aldrich (Poole, UK) and used as received. The PPO *vs.* PEO content of the PEO-PPO-PEO block copolymers was determined by $^1$H NMR in $dCHCl_3$ by comparing the integral of the methyl group of the propylene oxide repeating unit (3H) at 1.2 ppm to those of the CH and $CH_2$ of the PPO (3H) and PEO (4H) units, respectively, at 3.3–3.8 ppm.

### Cell Culture

Caco-2 cells (passage 55–70) were cultured in Dulbecco's Modified Eagle Medium supplemented with 2 mM L-glutamine, 1000 IUmL penicillin, 10 μg/mL streptomycin and 10% foetal bovine serum (FBS). HMEC-1 cells (passage 1) were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 1000 IU/mL penicillin, 10 μg/mL streptomycin and 10% FBS. Cells were maintained at 37°C and 5% $CO_2$ in a humidified atmosphere.

### MTS Metabolism Assay

Caco-2 cells were plated at a density of 15,000 cells per well in 96-well plates and allowed to attach overnight. They were treated with a series of PEO-PPO-PEO block copolymers (Table I) over a concentration range of 0.0005-1% w/v for 5 h. Untreated cells (100% MTS metabolism) and cells treated with 2% sodium dodecylsulfate (SDS) (0% MTS metabolism) were included as controls. The cells were then washed with PBS, and fresh media containing the MTS reagent was added. After 2 h of incubation, the absorbance was read at 490 nm using a colorimetric plate reader (TECAN, Männedorf, Switzerland). The assay was performed on two separate occasions ($n$=6 replicates per plate)

Dose response curves were generated for each polymer, showing MTS metabolism relative to the controls *versus* polymer concentration. Integrals were calculated for each

**Table I** Polymer Composition and Measured Cytotoxicity Endpoints

| Polymer Name | $M_n$ PPO | % PEO | | Integrated MTS score (Caco-2) | Integrated LDH score | | Haemolysis | | | JC-1 Green : Red Fluorescence ratio |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Quoted | $^1$H NMR | | Caco-2 | HMEC-1 | $R_e$ ($\mu$M) | $D_w$ ($\mu$M) | $K_b$ ($M^{-3}$) | |
| L31 | 900 | 10 | 14 | 0.3039 | 0.06597 | 0.20532 | 955.7 | 7639 | 0.13 | 2.44 |
| F38 | 900 | 80 | 86 | 1.10646 | – | 0.08561 | – | – | – | – |
| L61 | 1800 | 10 | 13 | −0.00829 | 0.4366 | 0.61353 | 77.9 | 1018 | 0.97 | – |
| L62 | 1800 | 20 | 29 | −0.04603 | 0.28918 | – | – | – | – | 1.48 |
| L64 | 1800 | 40 | 39 | 0.20236 | – | 0.31583 | 336.9 | 599.5 | 1.66 | – |
| F68 | 1800 | 80 | 81 | 0.86001 | −0.13604 | −0.03245 | 0* | 0* | 0* | 3.32 |
| L81 | 2400 | 10 | 11 | – | 0.55 | 0.77279 | – | – | – | – |
| P85 | 2400 | 50 | 50 | 0.85686 | 0.03591 | – | – | – | – | 0.98 |
| P105 | 3000 | 50 | 51 | 0.70631 | – | – | – | – | – | 1.08 |
| L121 | 3600 | 10 | 15 | 0.97248 | 0.017958 | −0.01397 | 0* | 0* | 0* | – |
| L122 | 3600 | 20 | 25 | 1.11403 | – | – | – | – | – | – |
| F127 | 3600 | 70 | 73 | 1.00599 | 0.04207 | −0.11096 | 0* | 0* | 0* | – |

- indicates the Pluronic was not tested in the assay

* values set at 0 as polymer did not induce haemolysis

dose response curve in order to compare cytotoxic effects in lieu of LD50 values. LD50 values were not obtained for all polymers due to concerns that cytotoxicity would be caused at higher polymer concentrations by mechanisms other than membrane disruption, e.g. hypertonicity.

The integrals were fitted to a matrix against $M_n$ PPO and % PEO using the Renka-Kline algorithm, and a 3D surface plot was overlaid on the data points using the Origin Pro 8.0 software (OriginLab, Northampton, UK).

## LDH Release Assay

Throughout the lactate deshydrogenase (LDH) assay, cells were incubated with serum in which any endogenous LDH present had been inactivated by incubation at 70°C in a water bath for 40 min. HMEC-1 and Caco-2 cells were plated at a density of 15,000 cells per well and allowed to attach overnight. They were treated with a series of PEO-PPO-PEO block co-polymers (Table I) over a concentration range of 0.0005-1% w/v for 5 h. Untreated cells (spontaneous LDH release), cells treated with 4% Triton X-100 (100% LDH release) and culture media containing heat inactivated serum without cells (endogenous background LDH activity) were included as controls. Following incubation, the plates were centrifuged at 250 g, 4°C for 4 min and 100 µL of media was added to each well. 100 µL of media was removed from each well, placed in a fresh plate and incubated with an equal volume of CytotoxONE™ - reagent, containing pyruvate, NAD+, ATP, rezazurin and diaphorase, for 15 min. 50 µL stop solution (Promega) was added to each well. The fluorescence was then read in a fluorometric plate reader at an excitation wavelength of 560 nm and emission wavelength of 590 nm. The assay was performed on 2 sets of 6 replicates. Dose response curves showing LDH activity normalised against controls *versus* polymer concentration were generated for each polymer. Integrals of each dose response curve were calculated and plotted against $M_n$ PPO and % PEO. A surface was fitted to the data points using Origin Pro 8.0 and surfaces generated for the two cell lines were directly compared.

## Determination of the Polymer Membrane Binding Constants

Ethical approval was obtained from the University of Nottingham Medical School Ethics Committee to collect blood samples from healthy adult male volunteers. The erythrocytes were separated by centrifugation and suspensions containing erythrocytes at hematocrits ranging from 0.03%–0.3% were prepared. These were added to 96-well plates containing various PEO-PPO-PEO block co-polymers of increasing concentrations over the range 0.0005–0.1 M. Untreated erythrocytes (spontaneous lysis), erythrocytes treated with 4% Triton X-100 (100% lysis) and solutions of each polymer without erythrocytes (background absorbance) were run simultaneously as controls. After 4 h of incubation, the plates were centrifuged, the supernatant containing released haemoglobin was extracted and placed in a fresh plate. The absorbance was read at 410 nm in a TECAN colorimetric plate reader (2 sets of 6 replicates). Dose response curves showing percentage haemolysis normalised against controls *versus* polymer concentration were drawn.

In order to determine membrane binding constant values ($K_b$) from the haemolysis assay, the analysis of Litchenberg *et al.* (19) was employed. The concentration at which the membrane is saturated with surfactant ($C^{sat}$) was obtained from the sigmoidal haemolysis dose response curves as the point where the exponential increase in haemolysis ended and further increases were linear (around 10% haemolysis). These $C^{sat}$ values were then plotted against the molar concentration of lipid (calculated according to Malheiros *et al.* (20)), which resulted in a straight line predicted by Eq. 1.

$$D_t = R_e\left[\frac{L+1}{K_b(R_e+1)}\right] \quad (1)$$

Where $D_t$ is the total surfactant, $R_e$ is the molar ratio of lipid to surfactant, L is the lipid concentration and $K_b$ is the membrane binding constant. The gradient of this line was used to derive $R_e$ and the intercept gave $D_w$, the free surfactant in water. This was used to derive $K_b$ values via Eq. 2.

$$R_e = \frac{K_b \cdot D_w}{(1 - K_b \cdot D_w)} \quad (2)$$

$K_b$ values were then fitted to a matrix against $M_n$ PPO and % PEO using Origin Pro 8.0.

## Erythrocyte Protection Assay

Erythrocytes collected as previously described were adjusted to a haematocrit of 0.5% in PBS. The erythrocytes were added to Pluronic solutions in PBS so that the final polymer concentration was 50 mM and the haematocrit was 0.25%. Solutions containing 4% w/v Triton X-100 (100% lysis), PBS (spontaneous lysis) and PBS containing polymer without erythrocytes (background absorbance) were used as controls. The polymer/erythrocyte preparations were incubated at 37°C for 5 min to allow the polymers to bind to the membrane and 150 μL were then added to an equal volume of $dH_2O$ or PBS (negative control). After 10 min of incubation, samples were centrifuged for 4 min at 600 g and the supernatant was transferred into a fresh 96 well plate. The absorbance was measured at 410 nm (3 replicates). The absorbance values were normalised against the controls and expressed as % haemolysis.

## JC-1 Mitochondrial Membrane Potential Assay

JC-1 is a lipophilic cationic dye which forms J-aggregates that fluoresce in the red at high mitochondrial membrane potential ($\Delta\psi m$) and switches to a monomeric form which fluoresces in the green at low $\Delta\psi m$ (21).

Caco-2 cells were plated at a density of 25,000 cells per well. After 48 h, cells were treated with a range of PEO-PPO-PEO block copolymers (Table I) at a concentration of 0.3 mM, which was the highest concentration that did not cause complete cytotoxicity in the MTS and LDH assays for any of the polymers. Untreated cells and cells incubated with 0.01 mg/mL valinomycin for 1 h were used as controls. The plates were then incubated for 3 h. The media was aspirated and cells were incubated for 15 min with the JC-1/bisbenzimide staining solution (Sigma-Aldrich). The JC-1/bisbenzimide staining solution contained 9.2 μM JC-1, 11.2 μM bisbenzimide in 5 mL JC-1 staining buffer and was made up to 10 mL with 5 mL Caco-2 culture media. JC-1 staining buffer contained 137 mM NaCl, 3.6 mM KCl, 0.5 mM, $MgCl_2$, 1.8 mM $CaCl_2$, 0.8 mM HEPES, 1 mg/mL D-glucose, 1% non-essential amino acids and was adjusted to pH 7.2 with NaOH. The plates were washed twice with the JC-1 staining buffer. The plates containing JC-1 staining buffer were placed on ice, and the wells were imaged with a Leica DM IRB microscope with a QICAM FAST1394 camera (QImaging, Surrey, Canada) on the red, green and blue fluorescence channels.

The images obtained from the red (JC-1 aggregates) and green (JC-1 monomers) fluorescence channels were each independently merged with the images from the blue channel (bisbenzimide nuclear stain) using paint.net (obtained from dotPDN llc). The images were then analysed using Origin Pro 8.0, and histograms were generated to show the relative levels of fluorescence per pixel in the red and blue channels, and green and blue channels, respectively (Fig. S1). These histograms were used to calculate the total fluorescence for each channel, and the red and green fluorescence were normalised to the blue fluorescence in each image. The pair of images for each well was then used to generate green to red fluorescence ratios. An increase in green to red fluorescence ratio was interpreted as a decrease in mitochondrial membrane potential.

## Molecular Modelling

Atomistic molecular dynamics simulations were used to investigate the molecular structure of a model biological membrane composed of 128 molecules of DPPC and hydrated with 3655 water molecules upon insertion of one PEO-PPO-PEO copolymer chain. The simulations were carried out at 325 K for 300 ns and at constant lateral pressure (1 atm) in order to simulate a tensionless bilayer. The software used was the GROMACS package version 4.5.2.

## Statistical Analysis

Data sets were compared by performing a 2 sample *F*-test for variance using Origin Pro 8.0.

## RESULTS

### MTS Metabolism Assay

The MTS assay was employed to determine the effects of the structural components of the polymers, namely the size of the PPO central block ($M_n$ PPO) and the weight percentage of hydrophilic PEO side blocks, on Caco-2 cell metabolism. Within the group of Pluronics tested (Table I), both cytotoxic and non-cytotoxic polymers were identified. Concentration-dependent effects were seen for the polymers that caused a decrease in MTS metabolism but with varying cytotoxic potency (Fig. 1a). It is noteworthy that no alteration in the trend of the dose response curve was observed above the critical micelle concentration (CMC) for any of the copolymers tested.

As it was not possible to generate LD50 values for the polymers which did not display pronounced cytotoxic effects, the integrals of the dose response curves were compared. For polymers containing 10% PEO (w/w), a relationship was found between the $M_n$ PPO and the integrated MTS metabolism values which fitted the general form of a quadratic (parabolic) function (Fig. 1b). Polymers with a $M_n$ PPO of around 1.8 kDa caused the greatest reduction in MTS metabolism, whereas polymers with a $M_n$ PPO of 3.6 kDa had no impact on MTS biotransformation. For polymers with a $M_n$ PPO of 1.8 kDa, increasing the percentage of PEO content linearly decreased the polymer ability to affect MTS metabolism. Polymers with 10% PEO were the most cytotoxic in the MTS assay, whereas polymers with 80% PEO did not affect the dye conversion (Fig. 1c). These two dimensional relationships were fitted into a matrix with $M_n$ PPO and % PEO against the integrals of the dose response curves for each polymer (Fig. 1d). It was found that a polymer consisting of $M_n$ PPO 1.8 kDa and 10% PEO, i.e., polymer L61, caused maximum reduction in MTS metabolism.

### LDH Release Assay

The LDH assay was performed in the cancerous cell line Caco-2 and in immortalized HMEC-1 cells to evaluate

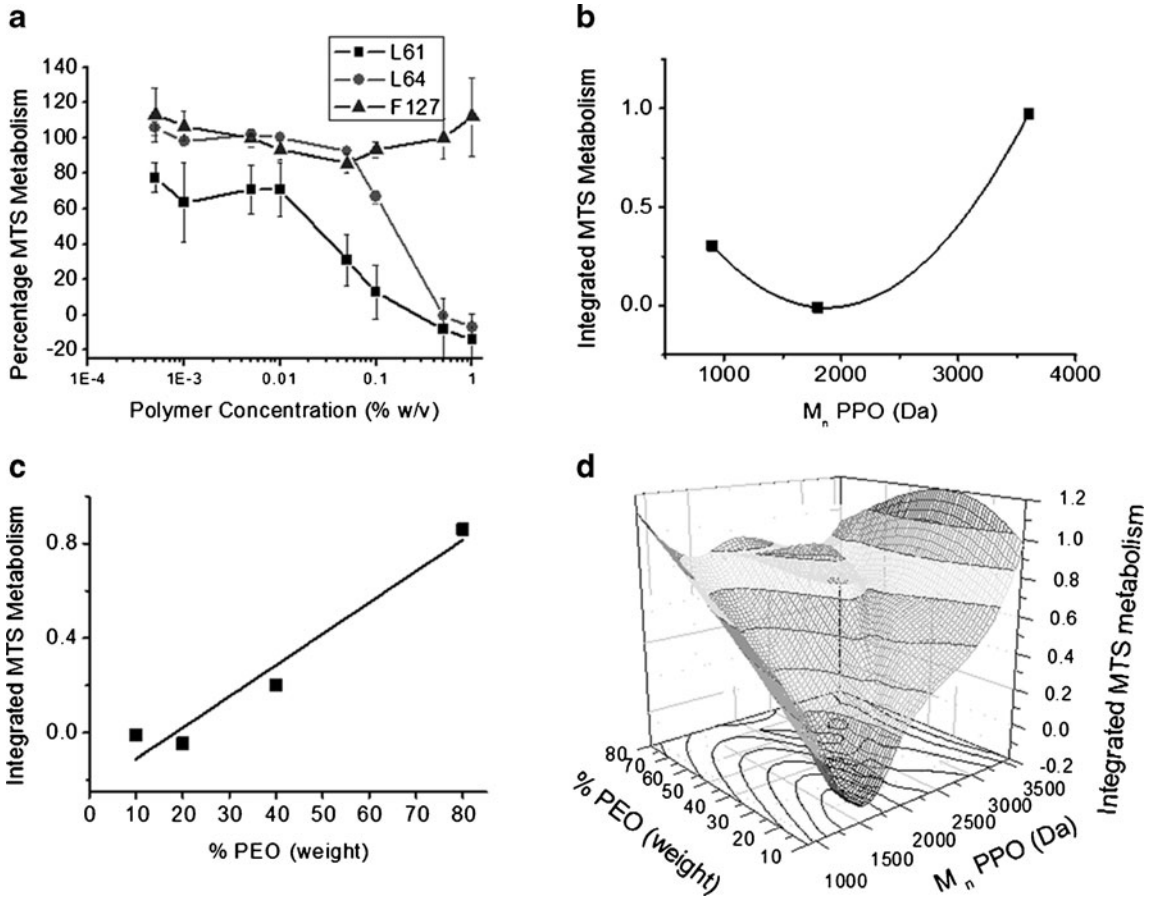


**Fig. 1** Effect of PEO-PPO-PEO block co-polymers on MTS metabolism in Caco-2 cells. (**a**) Examples of dose response curves for a non cytotoxic polymer (F127), a polymer with moderate cytotoxicity (L64) and a highly cytotoxic polymer (L61); data are mean ± SEM. (**b**) Parabolic relationship between $M_n$ PPO and MTS metabolism for polymers of equal % PEO (10%). (**c**) Linear relationship between % PEO and MTS metabolism for polymers of equal $M_n$ PPO (1800 Da). (**d**) 2D surface plot generated using the Renka-Kline Algorithm detailing the effects of both $M_n$ PPO and % PEO on integrated MTS metabolism.

polymer cytotoxicity via membrane disruption/solubilisation. As in the MTS assay, polymers which caused LDH release as well as polymers which did not disrupt cell membranes were highlighted without any impact of the CMC on the cytotoxicity profile. Again, the integrals of each dose response curve were compared in lieu of LD50 values.

In both the Caco-2 and HMEC-1 cell lines, for polymers exhibiting a PEO content of 10% (w/w), the molar mass ($M_n$) of PPO fitted with LDH release in terms of a parabolic function, with polymers of a $M_n$ PPO around 2.2 kDa causing the greatest increase in LDH release, and polymers with a $M_n$ PPO of 3.6 kDa having no effect on membrane integrity (Fig. 2a and d). For polymers with a similar $M_n$ PPO of 1.8 kDa, an inverse relationship was found between the percentage PEO and LDH release, with polymers composed of 80% PEO (w/w) being non membrane disruptive (Fig. 2b and e).

Two dimensional surfaces were fitted to the data sets against both $M_n$ PPO and percentage PEO. The resulting matrix was found to obey a 2D parabolic relationship (Fig. 2c and f). This empirical relationship can be described by Eq. 3.

$$LDH\ release = A \cdot exp\left[\frac{1}{2}\left(\frac{M_nPPO - 2200}{750}\right)^2 - \frac{1}{2}\left(\frac{\%PEO - 10}{20}\right)^2\right] \quad (3)$$

where $A$ is the maximum LDH release under the system, i.e. LDH release caused by a polymer which, according to our model, is predicted to have a $M_n$ PPO of 2.2 kDa and to contain 10% PEO (w/w). The chemical composition of this polymer is intermediate between that of Pluronic L61 and L81.

No significant difference was found when the data sets derived from each cell line were compared ($p$=0.7), indicating these were neither affected by the cancerous nature nor the tissue origin of the cells.

## Haemolysis Assay

Haemolysis assays were performed to quantify the affinity of the polymers for erythrocyte membranes.

Similarly to observations in the two cytotoxicity assays, different polymers had variable haemolysis capacity (Table I). Haemolysis dose response curves were generated for each polymer in order to calculate their $K_b$ (Fig. 3a).The polymer concentration at which the membrane was saturated ($C^{sat}$) was used for determination of $K_b$ values instead of the concentration at which complete lysis occurred ($C^{sol}$). This is due to the presence of mixed micelles of lipid and polymer, rather than intact erythrocytes at concentrations above $C^{sat}$. As predicted by Eq. 1, a linear relationship between the $C^{sat}$ and lipid

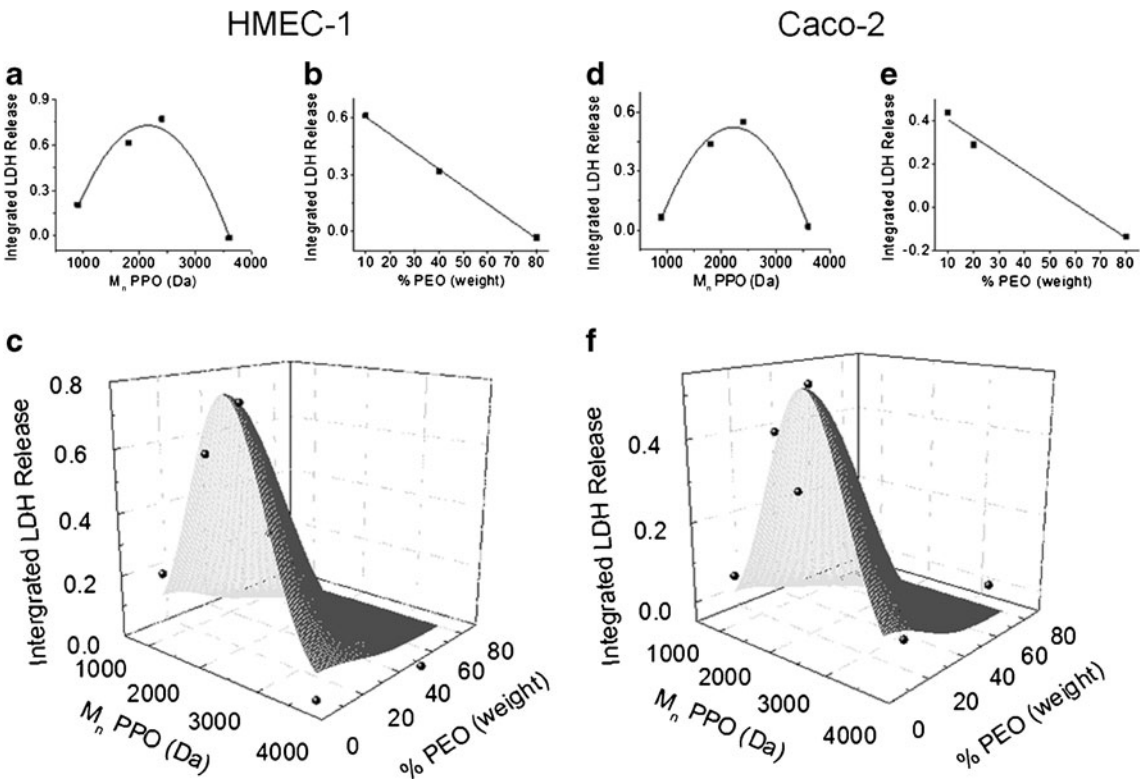


**Fig. 2** Effect of PEO-PPO-PEO block co-polymers on LDH release in HMEC-1 and Caco-2 cells. (**a**, **d**) Parabolic relationship between polymer $M_n$ PPO and LDH release for polymers of equal % PEO (10%) in HMEC-1 and Caco-2 cells, respectively. (**b**, **e**) Linear relationship between LDH release and % PEO for polymers of equal $M_n$ PPO (1800 Da) in HMEC-1and Caco-2 cells, respectively. (**c**, **f**) Surface fit models showing LDH release against $M_n$ PPO and % PEO in HMEC-1 and Caco-2 cells, respectively.

concentration was observed for the haemolytic polymers (Fig. 3b). These plots allowed the calculation of the ratio of lipid to surfactant ($R_c$) from the gradient, and the free surfactant in water ($D_w$). From this, $K_b$ values could be generated using Eq. 2.

By analogy with the LDH assay, $K_b$ values were compared to both $M_n$ PPO and percentage PEO and a 2D surface was fitted to the data (Fig. 3c). Because not all polymers displayed haemolytic properties, the $K_b$ values was set as 0 for non lytic polymers. The 2D parabolic relationship observed can be described by Eq. 4.

$$K_b = 1.5 \cdot \exp\left[\frac{1}{2}\left(\frac{M_nPPO - 2200}{750}\right)^2 - \frac{1}{2}\left(\frac{\% - 40}{20}\right)^2\right] \quad (4)$$

wherein a polymer with a $M_n$ PPO of 2.2 kDa and 40% PEO (w/w) is predicted to have the greatest affinity for erythrocyte membranes with a $K_b$ of 1.5 $M^{-3}$.

## Erythrocyte Protection Assay

In the erythrocyte protection assay, polymers capable of forming ion traversable pores within the cell membrane protect erythrocytes from lysis induced by hypotonic stress.

A reduction in the level of haemolysis was observed when erythrocytes were pre-treated with Pluronics L61, L64 and L81; i.e., polymers that were highly cytotoxic in the LDH assay or showed a high $K_b$ (Fig. 4). In contrast, polymers that did not interact with erythrocyte cell membrane (F38 and L121) or caused a moderate LDH release (P85) were unable to protect erythrocytes against hypotonic stress (Fig. 4).

## JC-1 Mitochondrial Membrane Potential Assay

The JC-1 assay detects any reduction in mitochondrial membrane potential ($\Delta\ \Psi_m$) via an increase in the ratio of green to red fluorescence of the JC-1 dye, relative to the blue fluorescent bisbenzimide nuclear stain (Fig. 5a).

Polymers with a $M_n$ PPO of 3.6 kDa, which had shown no activity in the MTS, LDH or haemolytic assays (Table I) did not affect $\Delta\ \Psi_m$ in Caco-2 cells (Fig. 5b). All other polymers tested were able to lower $\Delta\ \Psi_m$ to some extent, although the intensity of the effect appeared independent of the polymer composition (Fig. 5b).

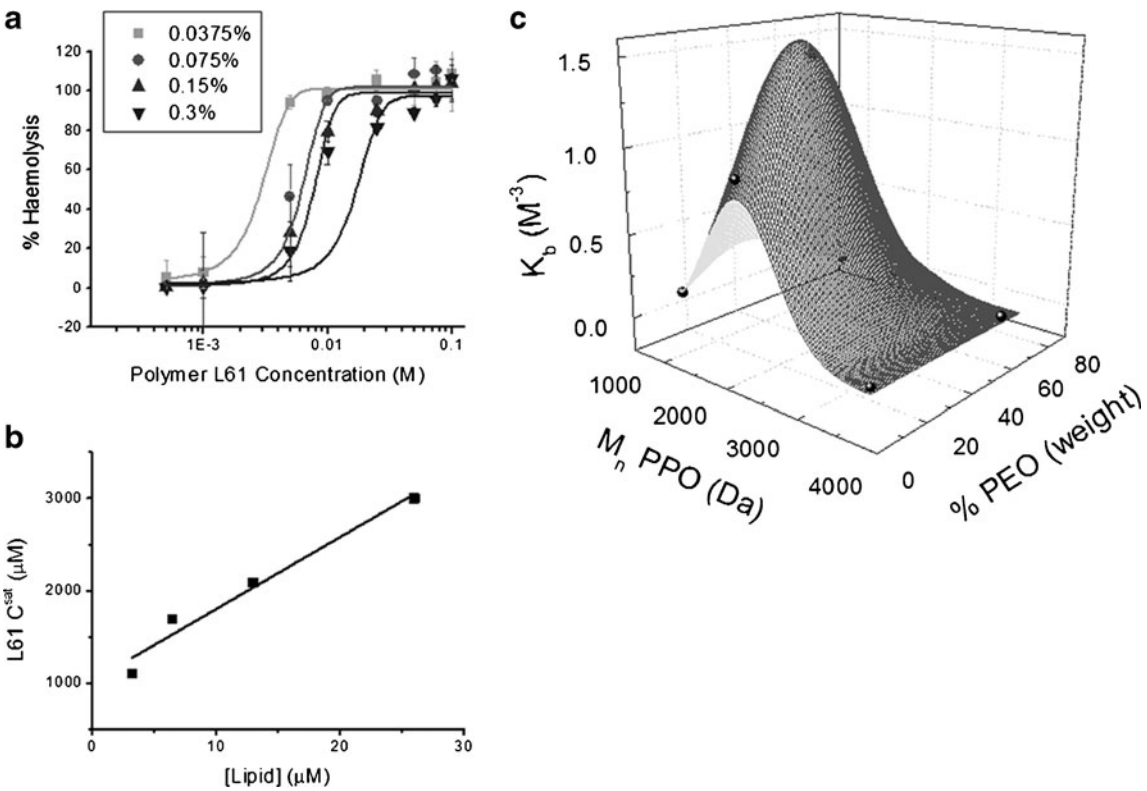


**Fig. 3** Affinity of PEO-PPO-PEO block co-polymers for erythrocyte membranes. (**a**) Example of haemolysis dose response curves over a range of haematocrits. (**b**) Example of linear relationship between $C_{sat}$ values derived from **a**, as predicted by Eq. 1. (**c**) Surface fit model showing $K_b$ values against polymer $M_n$ PPO and % PEO.

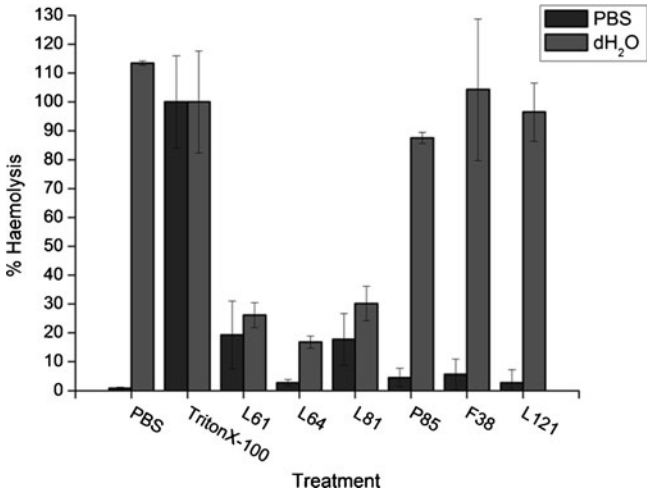


**Fig. 4** Effect of PEO-PPO-PEO block co-polymer on erythrocyte lysis induced by hypotonic stress. Exposure to PBS was used as a negative control. Data are presented as mean ± standard deviation ($n$=3 replicates).

## DISCUSSION

The interest in PEO-PPO-PEO block copolymers as bioactive excipients arose from the discovery that, when included in drug delivery systems, certain of these polymers were able to increase the efficacy of their therapeutic cargo. Their use has been shown to be advantageous in a number of different applications, including cancer therapy where circumvention of multi drug resistance (MDR) via P-gp inhibition (7) and ATP/glutathione depletion have been demonstrated (11). As, to date, studies have principally described their interactions with artificial membranes (15–18), the link between the cellular effects of the polymers and their affinity for biological membranes that has been previously suggested (5) has not yet been experimentally confirmed. Moreover, a more wide-ranging determination of the relationships between polymer structure and biological responses is required to assist in the selection of the most promising drug carriers.

In the present study, we followed a systematic approach based on a combination of cytotoxicity, haemolysis and apoptosis assays to investigate the interactions of PEO-PPO-PEO block copolymers with biomembranes and consequences on key cellular processes. We showed the chemical constituents of the copolymers are determinants of both their affinity for biological membranes and intensity of short-term cytotoxic effects. Our data strongly suggests a correlation between the two phenomena and confirms the importance of the polymer composition on their ability to trigger biological responses.

The acute cytotoxicity of a defined series of PEO-PPO-PEO block copolymers was evaluated in Caco-2 and HMEC-1 cells after 5 h of exposure. This was to capture cellular events that might have occurred as a result of membrane disruption by the polymers, independently of mechanisms of delayed toxicity. Concordant results were obtained in both the MTS and LDH assays with a general trend being that, when the same mass of each polymer was tested, the more hydrophobic the polymer, the higher its cytotoxicity, with polymers containing 10% PEO causing the greatest cytotoxic effect. Based on the empirical model obtained by interpolation of our experimental data, polymers with a $M_n$ PPO around 2 kDa were predicted to be the most cytotoxic. The haemolysis assays supported this finding as polymers with a $M_n$ PPO of around 2 kDa were predicted to give the highest membrane binding constant and hence, to be the most efficient at solubilising erythrocyte membranes. They also suggested that a polymer containing around 40% PEO would solubilise cell membranes most efficiently as a consequence of its high affinity for lipid

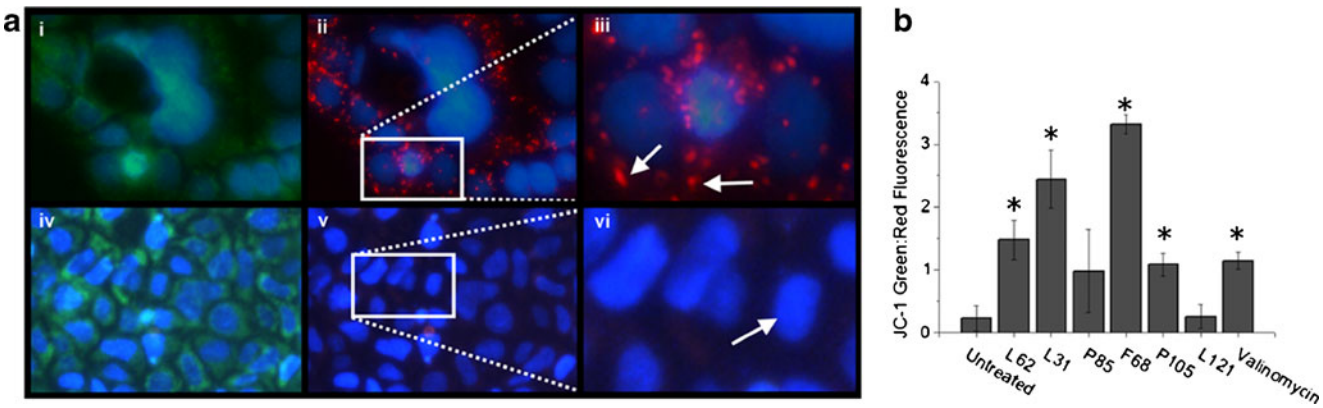


**Fig. 5** Effect of PEO-PPO-PEO block co-polymer treatment on mitochondrial membrane potential in Caco-2 cells. (**a**) Fluorescent microscopy images obtained from the JC-1 assay multiplexed with a blue bisbenzimide nuclear stain. Images i, ii, iii show untreated cells and images iv, v, vi cells following treatment with polymer L31. Images i & iv show green fluorescence due to monomeric JC-1 merged with blue fluorescence from the nuclear stain. Images ii, iii & v, vi show red fluorescence due to JC-1 aggregates merged with the blue nuclear stain. The arrows in iii point to healthy mitochondria with a high membrane potential, and in vi show the lack of red mitochondria. (**b**) Ratio of green to red JC-1 fluorescence normalised against blue bisbenzimide fluorescence after various treatments. Data are presented as mean ± standard deviation ($n$=3 replicates).

bilayers. This polymer displays a 30% increase in PEO content as compared to the most cytotoxic polymer identified in the two cytotoxicity assays. This apparent discrepancy could be explained by considering that dose response curves were obtained in the MTS and LDH assays utilising equal mass of each polymer, whereas an equal number of macromolecular chains (derived from concentrations expressed in moles) was employed in the haemolysis assays. The polymer with the highest $K_b$ values, i.e., L64, has a total $M_n$ of 3.0 kDa whereas the most cytotoxic polymer in the MTS and LDH assays, i.e., L61, has a total $M_n$ of 2.0 kDa (Table I). This results in a 50% increase in the number of polymer chains available for interaction with cell membranes at any given concentration based on polymer mass. It must be noted that when a plot similar to Fig. 2c was built using integrated LDH release values based on Pluronic molar concentrations, a polymer with a $M_n$ PPO of 2.0 kDa and 40% PEO was predicted to be the most membrane disruptive (Fig. S2). Although it has been reported that the toxicity of Pluronics was reduced at concentrations higher than their CMC due to the formation of micelles (1), this phenomenon was not observed in our study. Likewise, exposure of calcein-loaded liposomes to Pluronic L64 resulted in the leakage of the dye following membrane permeabilisation both below and above its CMC, suggesting a transfer of polymer molecules from the micelles to the lipid bilayer (22).

Both PPO and PEO blocks were shown to modulate polymer affinity for biological membranes and their cytotoxic effects. Similarly, the extent of P-gp inhibition by the Pluronics was affected by the length of the PPO block and their overall hydrophilicity (5). Moderately lipophilic copolymers with an intermediate number of PPO units were the most potent due to their ability to increase the fluidity of the cell membrane (5). It has been reported that the central hydrophobic PPO block of the polymers is able to anchor into the hydrophobic subspace of artificial biomembranes (17). However, polymers with a PPO block shorter than the bilayer thickness incorporated poorly and had limited effects on the fluidity of the biomembrane mimics (18). In our study, polymers with a large PPO central block ($M_n$ PPO of 3.6 kDa) were consistently found to be biologically inactive, which may be due to their hydrophobic section being too bulky to allow penetration within the cell membrane. For polymers that can insert into the membranes, the hydrophilic PEO blocks were shown to interact with the polar head groups of the phospholipids within the same leaflet or in the two opposite leaflets depending on the length of the PPO block (18). Our preliminary computational dynamic simulations of the interactions between Pluronic molecules and model DPPC membranes reveal that, in the latter case, the PEO blocks drag the polar head groups toward the centre of the bilayers, providing a potential mechanism underlying the formation of aqueous pores observed in the experimental erythrocyte protection assay (Fig. 6). Similarly, the ability of Pluronic L64 to create pores in artificial lipid bilayers has been described (23). Detailed simulations are ongoing to dissect the molecular interactions between the PEO blocks and membrane lipids with the aim to better understand the influence of chain length on membrane disruption. It has nevertheless been reported previously that very long PEO chains are able to project outside artificial membranes (18). In our study, Pluronics with a very high hydrophilic content were generally found to be non cytotoxic (Table I). This could be due to limited interaction sites between long PEO chains and phospholipid head groups within the membrane, which possibly minimises the disruptive potential of highly hydrophilic polymers.

The JC-1 assay revealed that exposure to certain polymers caused a drop in the mitochondrial membrane potential of Caco-2 cells, which is one of the defining steps in the apoptotic process (24). This might possibly be via formation of ion traversable pores within the mitochondria membranes as demonstrated in the external cell membrane (Fig. 4). PEO-PPO-PEO block copolymers are capable of circumventing anti-apoptotic strategies in Bcl-2 overexpressing MDR cancer cells when co-delivered with doxorubicin (6). Bcl-2 overexpression protects MDR cells through prevention of MAC (mitochondrial apoptosis induced channels) formation. The MAC allow the influx of $Ca^{2+}$ ions to the mitochondria, which lowers the mitochondrial membrane potential (24). It is therefore possible that

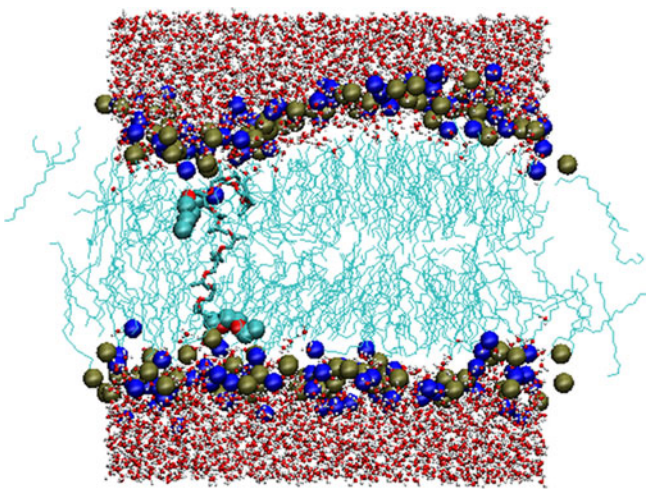

**Fig. 6** Snapshot of L31 absorbed in a dipalmitoyl phosphatidylcholine (DPPC) bilayer membrane taken 200 ns after the initiation of the computational simulation. The top and bottom layers of red and white beads represent water molecules in the system. The gold and blue beads correspond to, respectively, the phosphor and nitrogen atoms of the DPPC polar heads. The large aqua and red beads are the PEO blocks of the copolymer chain whereas the central PPO block is represented by thick lines. For clarity the hydrophobic carbonyl tails of the lipids are shown as thin aqua line.

Pluronics reverse MDR strategies due to creation of pores in the mitochondrial membrane independently of the MAC. Polymers with lower $K_b$ values showed a greater effect on the mitochondrial membrane potential than polymers with a high affinity for the erythrocyte membrane (Fig. 5).This may be ascribed to the tendency of polymers with a high $K_b$ value to remain in the outer cell membrane due to the strength of their lipophilic interactions. The ability of the PEO-PPO-PEO block co-polymer L31 to interact with the mitochondrial membrane preferentially to the external cell membrane can be illustrated by comparing its dose response curves in the MTS and LDH assays. Although this polymer appeared non destructive in the LDH assay, causing little LDH release above the baseline, it showed much greater efficacy in inhibiting MTS metabolism (Table I, Fig. S3). This ability of non-ionic surfactants to cause changes in the micro-fluidity of mitochondrial membranes and hence, disturb the cytochrome P450 family of enzymes has been previously described and suggested as a possible strategy for reducing drug metabolism in the intestinal barrier (25).

## CONCLUSIONS

A combination of cytotoxicity and haemolysis assays performed on a defined series of PEO-PPO-PEO block copolymers revealed similar 2D-parabolic relationships between the chemical constituents of the copolymers and their affinity for biological membranes or between polymer structure and their cytotoxicity potential, suggesting the biological effects of the copolymers are linked to their interactions with cell membranes. The chemical composition of the polymers was equally shown to affect their ability to reduce the mitochondrial membrane potential. We believe these findings may help to elucidate the mechanisms behind the biological effects of PEO-PPO-PEO block copolymers, as well as contribute to the design of safe drug formulations with optimal efficacy.

## ACKNOWLEDGMENTS & DISCLOSURES

This work was funded by the Biotechnology and Biological Science Research Council (BBSRC, UK) and by the Engineering and Physical Science Research Council (EPSRC, UK). DCG is supported by the Transchannel Neuroscience Network (TC2N), EU Interreg Program.

## REFERENCES

1. Batrakova EV, Kabanov AV. Pluronic block copolymers: evolution of drug delivery concept from inert nanocarriers to biological response modifiers. J Control Rel. 2008;130:98–106.
2. Kozlov MY, Melik-Nubarov NS, Batrakova EV, Kabanov AV. Relationship between pluronic block copolymer structure, critical micellization concentration and partitioning coefficients of low molecular mass solutes. Macromolecules. 2000;33:3305–13.
3. Kabanov AV, Batrakova EV, Miller DW. Pluronic((R)) block copolymers as modulators of drug efflux transporter activity in the blood-brain barrier. Adv Drug Deliv Rev. 2003;55:151–64.
4. Sriadibhatla S, Yang Z, Gebhart C, Alakhov VY, Kabanov AV. Transcriptional activation of gene expression by pluronic block copolymers in stably and transiently transfected cells. Mol Ther. 2006;13:804–13.
5. Batrakova EV, Li S, Alakhov VY, Miller DW, Kabanov AV. Optimal structure requirements for Pluronic block copolymers in modifying P-glycoprotein drug efflux transporter activity in bovine brain microvessel endothelial cells. J Pharmacol Exp Ther. 2003;304:845–54.
6. Minko T, Batrakova EV, Li S, Li YL, Pakunlu RI, Alakhov VY, *et al.* Pluronic block copolymers alter apoptotic signal transduction of doxorubicin in drug-resistant cancer cells. J Control Rel. 2005;105:269–78.
7. Batrakova EV, Li S, Li YL, Alakhov VY, Kabanov AV. Effect of pluronic p85 on ATPase activity of drug efflux transporters. Pharm Res. 2004;21:2226–33.
8. Danson S, Ferry D, Alakhov V, Margison J, Kerr D, Jowle D, *et al.* Phase I dose escalation and pharmacokinetic study of pluronic polymer-bound doxorubicin (SP 1049C) in patients with advanced cancer. Br J Cancer. 2004;90:2085–91.
9. Valle JW, Armstrong A, Newman C, Alakhov V, Pietrzynski G, Brewer J, *et al.* A phase 2 study of SP1049C, doxorubicin in P-glycoprotein-targeting pluronics, in patients with advanced adenocarcinoma of the esophagus and gastroesophageal junction. Invest New Drugs. 2011;29:1029–37.
10. Lemieux P, Guerin N, Paradis G, Proulx R, Chistyakova L, Kabanov A, *et al.* A combination of poloxamers increases gene expression of plasmid DNA in skeletal muscle. Gene Ther. 2000;7:986–91.
11. Alakhova DY, Rapoport NY, Batrakova EV, Timoshin AA, Li S, Nicholls D, *et al.* Differential metabolic responses to pluronic in MDR and non-MDR cells: A novel pathway for chemosensitization of drug resistant cancers. J Control Rel. 2010;142:89–100.
12. Lavigne MD, Pohlschmidt M, Novo JF, Higgins B, Alakhov V, Lochmuller H, *et al.* Promoter dependence of plasmid-pluronics targeted alpha galactosidase A expression in skeletal muscle of Fabry mice. Mol Ther. 2005;12:985–90.
13. Batrakova EV, Li S, Vinogradov SV, Alakhov VY, Miller DW, Kabanov AV. Mechanism of pluronic effect on P-glycoprotein efflux system in blood-brain barrier: Contributions of energy depletion and membrane fluidization. J Pharmacol Exp Ther. 2001;299:483–93.
14. Yates LL, Gorecki DC. The nuclear factor-kappaB (NF-kappa B): from a versatile transcription factor to a ubiquitous therapeutic target. Acta Biochim Pol. 2006;53:651–62.
15. Chang LC, Lin CY, Kuo MW, Gau CS. Interactions of Pluronics with phospholipid monolayers at the air-water interface. J Colloid Interface Sci. 2005;285:640–52.
16. Chang L-C, Chang Y-Y, Gau C-S. Interfacial properties of Pluronics and the interactions between Pluronics and cholesterol/DPPC mixed monolayers. J Colloid Interface Sci. 2008;322:263–73.
17. Firestone MA, Wolf AC, Seifert S. Small-angle x-ray scattering study of the interaction of poly(ethylene oxide)-b-poly(propylene oxide)-b-poly(ethylene oxide) triblock copolymers with lipid bilayers. Biomacromolecules. 2003;4:1539–49.
18. Lee B, Firestone MA. Electron density mapping of triblock copolymers associated with model biomembranes: Insights into conformational states and effect on bilayer structure. Biomacromolecules. 2008;9:1541–50.
19. Lichtenberg D. Characterization of the solubilization of lipid bilayers by surfactants. Biochim Biophys Acta. 1985;821:470–8.

20. Malheiros SVP, Meirelles NC, de Paula E. Pathways involved in trifluoperazine-, dibucaine- and praziquantel-induced hemolysis. Biophys Chem. 2000;83:89–100.
21. Reers M, Smith TW, Chen LB. J-aggegate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. Biochemistry. 1991;30:4480–6.
22. Pembouong G, Morellet N, Kral T, Hof M, Scherman D, Bureau MF, *et al.* A comprehensive study in triblock copolymer membrane interaction. J Control Release. 2011;151:57–64.
23. Gau-Racine J, Lal J, Zeghal M, Auvray L. PEO-PPO block copolymer vectors do not interact directly with DNA but with lipid membranes. J Phys Chem B. 2007;111:9900–7.
24. Ryu S-Y, Peixoto PM, Teijido O, Dejean LM, Kinnally KW. Role of mitochondrial ion channels in cell death. Biofactors. 2010;36:255–63.
25. Christiansen A, Backensfeld T, Denner K, Weitschies W. Effects of non-ionic surfactants on cytochrome P450-mediated metabolism *in vitro*. Eur J Pharm Biopharm. 2011;78:166–72.