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Poly(ethylene glycol) induces cell toxicity in melanoma cells by producing a hyperosmotic extracellular medium

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Abstract

Poly(ethylene glycol) is a polymer that is widely used as a biomaterial and has been approved in a host of applications. While generally viewed as inert, recent studies with poly(ethylene glycol) suggest that it may have some effects on cells and tissues, making it potentially attractive as a therapeutic agent. In this study, the effect of poly(ethylene glycol) on the cell viability, membrane transport and apoptotic markers of metastatic melanoma cells was examined. The data were combined with observed effects of the polymer on the cell media, including osmolality and viscosity, in order to elucidate any structure-function relationship between the polymer and cells. It was observed that poly(ethylene glycol) reduced the cellular viability of A375 cells, and that the effect was dependent on poly(ethylene glycol) molecular weight and concentration. The mechanism was highly correlated with changes in the osmolality of the cell medium, which is determined by the inherent structure of poly(ethylene glycol), and in particular the ethylene oxide units. This mechanism was specific to poly(ethylene glycol) and was not observed with the similar linear, hydrophilic polymer poly(vinyl pyrrolidone). Overall, the data suggest that poly(ethylene glycol) and poly(ethylene glycol)-like compounds have a distinct effect on cellular activity, presumably mediated in part by their osmotic effects, supporting the further investigation of these polymers as pharmaceutically active compounds.

Keywords

Poly(ethylene glycol), melanoma, toxicity, biological activity, viability

Introduction

Polymeric biomaterials, long used as implants and in other applications, are emerging as novel therapies in a variety of applications.[1,2] In fact, over the past decade, there have been a number of polymeric compounds that have been approved for clinical use due to their therapeutic effects. The main application for these materials has been in the sequestering and/or removal of excess ions and small molecules from the gastrointestinal system. For example, polymers have been indicated as a treatment for high levels of phosphate (hyperphosphatemia),[3] potassium (hyperkalemia)[4] and cholesterol (hyperlipidemia).[5] There is also some preclinical evidence that polymers have efficacy in the inactivation of bacteria and toxins such as Clostridium difficile and anthrax,[6,7] in entrapping viruses such as influenza,[8] producing sensitised multi-drug resistant cells[9] and as a potential treatment for autoimmune diseases.[10] However, there have been no polymers approved as therapeutics in any of these applications to-date.

Poly(ethylene glycol) (PEG) is a polymer that is widely used in biomedical applications, although it is generally regarded as inert.[11–14] There is recent evidence to suggest that PEG and PEG-like polymers, specifically poloxamer and Triton X-100, may have biological activity in certain applications. Poloxamers, composed of blocks of PEG and poly(propylene oxide), are widely used in medical applications, largely as drug carriers.[15] However, they have also been shown to enhance the transport of small molecules across cell membranes, including the membranes of multi-drugresistant cells and the blood-brain barrier.[15] Possible mechanisms for this transport include microviscosity modification of the cell membrane, inhibition of drug efflux transporters such as P-glycoprotein and enhancement of pro-apoptotic signalling pathways. Triton X-100 is a non-ionic surfactant composed of a PEG chain and an aromatic, lipophilic group. Due to its polar head group, it is widely used in biology to disrupt cell membranes, allowing for permeabilization or protein extraction.[16] However, surprisingly, it has also been shown to produce the hallmarks of apoptosis in human carcinoma cell lines within 60 min of exposure.[17] PEG alone has been shown to exhibit these properties as well, specifically inhibiting P-glycoprotein in intestinal cells, and causing apoptosis of human colon cancer cells.[18–24]

The goal of this study was to examine the effects of PEG on a human melanoma cell line. While an extremely simple polymer from a structural perspective, in solution PEG is able to produce strong changes in its environment. Specifically, it is able to hydrogen bond 2–3 water molecules per ethylene oxide unit, effectively creating a surrounding ‘cage’ of water.[25–27] This property has been exploited in PEG-modified surfaces or PEG-drug conjugates with enhanced solubility or increased residence time.[28] PEG has also been shown to exhibit amphiphilic character, secondary structures and molecular-weight-dependent properties.[29,30] The basis for the hypothesis that PEG in particular may have biological activity thus stems from these unique PEG properties and the knowledge that

cells are highly sensitive to their extracellular environment, which is composed mainly of water.[31]

Despite this, there remains a major gap in the current literature in terms of understanding the relationship between the structure of PEG and any observed biological effects. Therefore, this study was performed to better understand the interactions between PEG and cells and how these interactions result in cellular changes by specifically examining the changes in cellular activity, morphology, membrane integrity and transport as well as apoptosis markers, that are affected by the presence of PEG and the changes to the cell culture medium that are the result of PEG.

Since it has been suggested that PEG exerts effects on important membrane proteins such as P-glycoprotein and may induce apoptotic effects, studies were performed on an in vitro culture of A375 metastatic melanoma cells, which are known to express P-glycoprotein.[32] There is significant clinical need for improved treatments for metastatic melanoma; cutaneous malignant melanoma represents only 2% of skin cancers, but the survival rate of patients is 10–15% over 10 years.[33] PEG-specific effects on A375 cells were compared with two other cell types – a 3T3 murine fibroblast cell line and a human corneal epithelial cell line – to determine whether any effects observed were cell-specific. Furthermore, the specificity of the effects of PEG was investigated by comparing with poly(vinyl pyrrolidone) (PVP), a non-ionic, linear, hydrophilic, perceived as 'inert', medically approved polymer. However, in contrast with PEG, the structure of PVP contains a bulky, 5-membered lactam ring. The results of this work provide insight into the nature of the interactions between hydrophilic synthetic polymers and cells, and the potential of synthetic polymers as pharmacologically active compounds.

Materials

A375 (ATCC^{R}CRL-1619^{TM}) metastatic melanoma cells and 3T3 (ATCC^{R}CRL-1658^{TM}) mouse fibroblast cell line were obtained from ATCC via CedarLane (Burlington, ON). Human corneal epithelial cells were the generous donation of Dr May Griffith. Keratinocyte serum-free media with growth factors, Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS) (pH7.4),TrypLE,PrestoBlue,Click-iT^{R} TUNEL Alexa Fluor^{R} 594 Imaging Assay and CellEvent Caspase-3/7 Green Detection Reagent were obtained from ThermoFisher Scientific (Burlington, ON). T-75 culture flasks and black, clear-bottom, 96-well plates were obtained from Corning (Corning, NY). PEG with molecular weights of 200 (M_r 190–200), 2000 (M_r 1900–2200), 8000 (M_r 7000–9000) and 20,000 (M_r 16,000–24,000) were obtained from SigmaAldrich as BioUltra grade (St. Louis, MO). PVP with a molecular weight of 8000 was obtained from Alfa Aesar (Ward Hill, MA). Daunorubicin hydrochloride and Annexin V apoptosis kit were obtained fromSigma-

Aldrich(St.Louis,MO).Apo-ONE^{R} Homogenous Caspase 3/7 Assay was obtained from Promega (Fitchburg, WI). Sodium dodecyl sulfate was obtained from Bio Rad (Hercules, USA); 0.22 lM syringe filters were obtained from Millipore (Etobicoke, ON).

Methods

Polymers

PEG and PVP were weighed and dissolved at room temperature (~22– C) in DMEM þ FBS to a final stock concentration of 20% w/v. For cell culture studies, solutions were filtered using a 0.22-lM filter. Daunorubicin hydrochloride and sodium chloride were solubilized by the addition of sterile DMEM þ FBS. All solutions were warmed to 37– C prior to use.

Viability assay

A375 metastatic melanoma cells (A375 cells) were cultured in DMEM containing 10% FBS in T-75 filter-top flasks for at least 2 weeks and were subcultured at least three times after removal from cryogenic storage. After this point the cells were microscopically seen to grow at a consistent rate. Cells were kept in an incubator at 37–C with 5% CO_2. A375 cells were seeded at a density of 1000 cells/well into black, clear-bottom, 96-well plates with 200 mL of DMEM + FBS. Because A375 cells were found to divide very rapidly, this density was used to ensure subconfluence at the measurement time of 96 h after seeding. To ensure a constant temperature, cells were seeded only in the interior wells and only DMEM + FBS was placed in the exterior wells.

Seeded 96-well plates were incubated for 24 h to allow cells to adhere and begin normal proliferation after trypsinization. Test compounds were then added and plates were incubated for 72 h. Each condition was plated in triplicate on a single 96-well plate and each test was repeated in three independent experiments. After incubation, cells were washed three times with PBS and a PrestoBlue^{R} assay was performed according to the manufacturer's protocol. Briefly, 90 lL of prewarmed (37–C) cell media and 10 lL of PrestoBlue^{R} cell viability reagent were added to each well, and plates were incubated at 37–C for 10 min. PrestoBlue, a non-toxic resazurin-based reagent, is modified by the reducing environment in living cells, which alters its fluorescence, allowing for quantification of cellular viability in comparison to control. PrestoBlue^{R} fluorescence was measured at excitation/emission wavelengths set to 560/590 nm.

3T3 cells were grown and assayed under the same conditions. HCEC cells utilized Keratinocyte serum free media and were plated at 2500 cells/well, as lower seeding density provided optimal timing of log growth.

Controls

Cells incubated in cell media only were used as blank and negative controls, indicating the optimal cell viability (100%) at the endpoint. Sodium dodecyl sulfate, a surfactant, was used as a positive control for cell toxicity. PVP was chosen as the polymer-specific control. While saline is traditionally used as a control in many preclinical and clinical trials, the use of another polymer (PVP) as a control was chosen in these in vitro assays as it represents a better direct comparator of polymer-specific activity. Daunorubicin (DNR) was included as a comparator for cell viability, as it acts by intercalation, inhibiting macromolecule biosynthesis and leading to cell death by caspase-mediated apoptosis.[34] It passively permeates through the cellular membrane and is also a P-glycoprotein efflux substrate,[35] allowing for observations of membrane transport changes and observations of possible membrane efflux protein inhibition. DNR is not commonly used for treating cutaneous melanoma clinically, as more targeted therapeutics with less systemic side effects are the standard of care.[36] However, its availability, mode of action and drug properties allowed for design of experiments that can provide a broad variety of information on possible interactions between PEG and cellular membranes. Doxorubicin is of the same class and properties as DNR and was used for the TUNEL assay based on availability.

Viscosity

Polymer solutions in DMEM þ 10% FBS were warmed to 37–C in a water bath for 30 min prior to measuring viscosity. Viscosity was measured using a Malvern SV10 viscometer (Malvern, UK). The mean recording temperature was approximately 34–C, due to noninsulated instrumentation. Two or three concentrations of each test compound were measured; these PEG concentrations represented a range of cell-toxicity values.

Osmolality

Polymer solutions were prepared as described by dissolution into DMEMþ10% FBS. The osmolality of the solutions was measured using a 3320 MicroOsmometer (Advanced Instruments, Inc. Norwood, MA), which measures the freezing point to determine solute concentration.

Membrane transport

The foundation of this assay is that daunorubicin hydrochloride (DNR) is able to enter cells via diffusion through the cell membrane and into the nucleus, and it can be effluxed by non-specific membrane proteins such as P-glycoprotein.[35] By monitoring DNR transport, changes to the membrane permeability or membrane protein activity can be detected. DNR transport was tracked via its inherent fluorescence (emission/excitation wavelength of 490/595 nm). Specifically, daunorubicin hydrochloride is fluorescent in

solution, but loses fluorescence upon binding to DNA. The fluorescence of DNR was monitored every 5 min for 3 h at 37–C, as adapted from an assay developed by Regev et al.[37]

Caspase 3/7 apoptosis assay

Apoptosis was measured by detecting the activity of apoptosis executioner caspases 3 and 7 at various time points using a commercially available kit (PROMEGA). Cells were cultured as described in the viability assay, but measurements were taken at 2, 4 or 6 h because caspases are early apoptotic markers, and the initiation of apoptosis is unique to each cell type and compound combination. The complete caspase assay medium was prepared according to the manufacturer's protocol. The complete assay medium was then added in a 1:1 ratio to wells and cells were incubated at room temperature for 16 h. Fluorescence was measured at an excitation/emission wavelength of 499/521 nm.

Caspase 3/7 activity was also measured using the ThermoFisher detection agent at 24 h (due to availability), according to manufacturer's directions. Briefly, cells were cultured as described for the viability assay, then washed with PBS before the addition of the caspase-detecting agent for 30 min. Fluorescence was measured at 503/530 nm.

DNA fragmentation assay

The Click-iT^{R} TUNEL Alexa Fluor^{R} 594 Imaging Assay was obtained from ThermoFisher Scientific and used according to manufacturer's instructions. Briefly, cells were fixed using 4% paraformaldehyde in PBS and permeabilized using 0.25% Triton^{R} X-100. Terminal deoxyonucletidyl transferase (Tdt) was then applied with modified deoxyuridine triphosphates (dUTPs). The Click-iT^{R} reaction cocktail containing the Alexa Fluor^{R} 594 antibody was then applied to cells. Imaging was performed with an inverted confocal microscope using a Texas Red filter.

Results

Cell-specific assays

PEG exposure induced morphological changes to A375 cells.

The morphology of A375 cells was examined after 72 h of exposure to PEG (Figure 1(a)). Control cells, with no polymer, exhibited a morphology that is typical for A375 epithelial cells in culture,[38] as did cells exposed to 1% w/v of PEG_{200}, PEG_{2000} or $PEG_{20,000}$. Cells exposed to 10% w/v of PEG of all molecular weights were significantly fewer in number and smaller in size. These cells adopted a spherical shape and appeared condensed. The morphological changes were detected visibly as early as 24 h after PEG exposure (data not shown).

Supplemental Figure 1 demonstrates the morphology of cells exposed to the surfactant sodium dodecyl sulfate (SDS) for comparison with cells exposed to PEG. SDS-exposed cells appear elongated at lower concentrations and highly fragmented at high concentrations. In contrast, PEG-exposed cells appear shrunken but not elongated or fragmented (Figure 1).

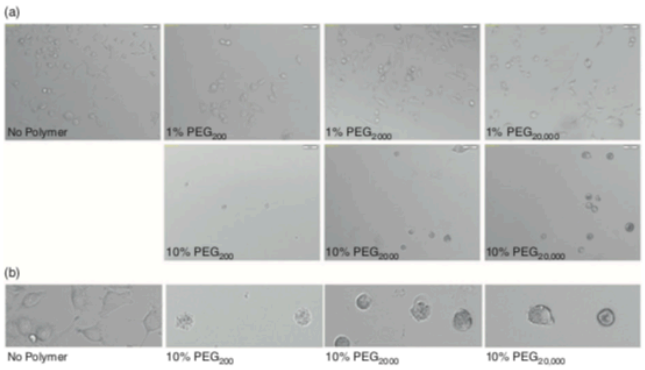

Figure 1. Comparative morphology of untreated and PEG-treated A375 metastatic melanoma cells show morphological changes indicative of low cell viability at higher PEG concentration. (a): Bright-field images at ×20 magnification. Scale bars represent 50 μM. (b) Unscaled close-up of cells from (a) images.
PEG: poly(ethylene glycol).

In order to determine whether the effects noted were PEG-specific, cells were also exposed to PVP, another hydrophilic polymer that does not have the same water-binding and amphiphilic properties. A molecular weight of 8000 was selected for comparing PEG and PVP. At high concentrations of PEG_{8000} (Supplemental Figure 2), the morphology of A375 cells appeared more spherical and condensed/ shrunken, which is similar to the morphology of cells exposed to PEG compounds; however, they appear to be even smaller in size than cells exposed to PEG.

PEG caused a decrease in cell viability as measured using a resazurin reduction assay. The reduction of the resazurinbased compound PrestoBlue^{R} was used to measure the viability of A375 cells after 72 h of PEG exposure. The effects of PEG concentration were observed by plotting cell viability with increasing ethylene oxide content (Figure 2). Supplemental Table 1 lists the estimated number of moles of each PEG compound at ~100% and ~40% viability, and the corresponding number of EO units for each compound.

Table 1. The slope of daunorubicin hydrochloride (DNR) uptake and quenching in the absence and presence of poly (ethylene glyol) (PEG).

		Daunorubicin hydrochloride +		
	DNR alone	1% PEG 200	1% PEG 2000	1% PEG 20,000
0.055 μM DNR				
Slope	−70.39	−63.92	−75.82	−69.46
R^2	0.993	0.968	0.999	0.955
0.01 μM DNR				
Slope	−41.50	−40.93	−45.19	−41.24
R^2	0.995	0.9717	0.994	0.993

In Figure 2, it can be seen that across all PEG compounds tested, a PEG concentration between 0.01 and 1% w/v produced no discernable difference in cell viability from cells not exposed to PEG. Changes to cell viability were first observed with PEG_{200} (~50% reduction at 3% w/v), followed by a more gradual reduction in viability with higher molecular weight PEGs. At 15% w/v PEG, cell viability was approximately equal (45%) across all PEG compounds. These data indicate an inverse correlation between PEG concentration and cell viability. Specifically, as the number of ethylene oxide units increases, cell viability decreases.

A very different trend was observed with PVP (Figure 2 inset), with a sharp decrease in the cell viability being observed at concentrations greater than approximately 1%, beyond which the level of cell viability was less than 50%. Therefore, it appears that the mechanism by which these polymers induce cellular changes is very different.

Overall, Figure 2 demonstrates an inverse correlation between PEG concentration and cell viability for each PEG that was tested. The data also suggest PEG chain length influences cell viability, as observed by the distinct variation in the class-trend in cells exposed to PEG_{200}.

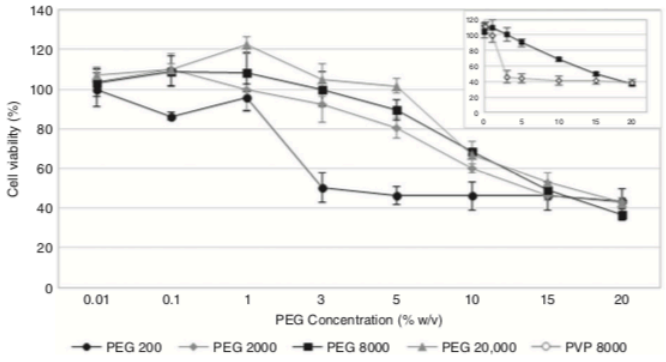

Figure 2. Cell viability of A375 melanoma cells is influenced by the presence of PEG. (a) An inverse relationship is observed between PEG concentration (ethylene oxide content) and cell viability. The inset shows cell viability in the presence of PVP_{8000} versus PEG_{8000}. (b) An inverse relationship between PEG molarity (number of chains) and viability of cells is seen. Error bars denote standard error ($n = 3$, triplicate samples, repeated three times).
PEG: poly(ethylene glycol).

Low PEG concentration did not alter rates of daunorubicin hydrochloride membrane transport. PEG has a linear structure with a very high degree of conformational freedom, making both hydrophobic and hydrophilic conformations as well as surfactant-like properties possible.[39,40] Thus, given the potential for PEG to have surfactant-like properties,[29] membrane permeabilization and changes in membrane transport were examined using the small, hydrophobic intercalating agent daunorubicin hydrochloride (DNR).[34] DNR toxicity over a large concentration range is shown in Supplemental Figure

1, confirming that DNR was transported into the nucleus of A375 metastatic melanoma cells, inducing cell death.

Note that surfactants can permeabilize cells at low concentrations without inducing toxicity.[16] Specifically, they are able to break hydrogen bonds between phospholipid head groups, which changes the membrane integrity.[16] This experiment was designed to examine whether a similar effect would be observed with PEG. In this case, cells were exposed to DNR alone or 1% w/v PEG with DNR. A non-toxic concentration of PEG was chosen to prevent artifacts due to cell death or membrane disintegration, but still allow for the potential observation of early changes to the membrane due to PEG. Two DNR concentrations were chosen for tracking as shown in Figure 3, with 0.055 lM DNR on the left vertical axis and 0.01 lM on the right vertical axis. Daunorubicin transport was measured for approximately 3 h. The transport was measured in the presence and absence of PEG of three molecular weights (200, 2000 and 20,000).

As expected, the higher DNR concentration produced greater fluorescence values, while the lower DNR concentration produced lower values. There were no statistically significant changes in DNR fluorescence at each timepoint for DNR alone versus DNR in the presence of PEG of any molecular weight.

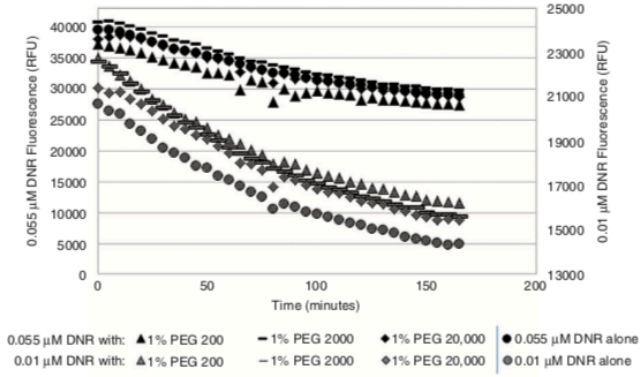

Figure 3. Daunorubicin hydrochloride (DNR) transport into cells, as measured by fluorescence quenching upon nucleic acid binding. Error bars ($n = 3$) were omitted from the figure for better visualization of the data points.

Given the apparently linear decrease in DNR fluorescence observed in Figure 4, the slope was determined using linear regression of the data. Table 1 depicts the change in DNR concentration over time. A more gradual slope in the presence of PEG would indicate less fluorescence quenching – i.e. a reduction in the transport/binding of DNR to nucleic acids. A steeper slope would indicate more fluorescence quenching – i.e. an increase in the transport/binding of DNR to nucleic acids, presumably as a result of the presence of the PEG. However, results indicate that there was no statistically significant variation/difference between the slopes and thus no statistically significant changes in the transport of DNR as observed using this assay.

PEG increases the presence of apoptosis markers: Caspases 3 and 7. Caspase activity was measured at multiple timepoints (after 2, 4 and 6 h of exposure to polymer), because the initiation of apoptosis is not predictable.[41] The caspase 3/7 substrate fluorescence was normalized to the baseline values (cells with no test compound added), thus, the value '1' on the vertical axis represents the baseline, and increases above this value indicate increases in caspase 3/7 activity.

The results after 6 h are shown in Figure 4(a). A statistically significant ($p<0.05$) increase in caspase 3/7 activity was detected with 15% w/v PEG_{200}. PEG_{2000} and $PEG_{20,000}$ did not demonstrate statistically significant increases in caspase 3/7 activation at 6 h; 1 lM DNR showed a statistically significant increase in caspase 3/7 activation after 6 h of exposure. At 2 and 4h, 15% w/v PEG_{200} also demonstrated statistically significant ($p<0.05$) increase in caspase 3/7 activity, while PEG_{2000}, $PEG_{20,000}$ and DNR did not (data not shown).

Caspase 3/7 activation was further tested after 24 h of exposure to PEG or control (Figure 4(b)). At this timepoint, exposure to PEG_{2000} showed an over _8 increase in caspase activity versus control, which was statistically significantly different. Doxorubicin control also demonstrated a statistically significant increase (~_2) in caspase activity at this timepoint. These data further support the case for induction of apoptosis by PEGs through the detection of early apoptotic activation of caspases 3 and 7. Of note, PEG_{200} cells may still have caspase activity at 24 h; however, due to the lower number of cells (as observed microscopically), the fluorescent signal was not higher than that observed with the untreated cells.

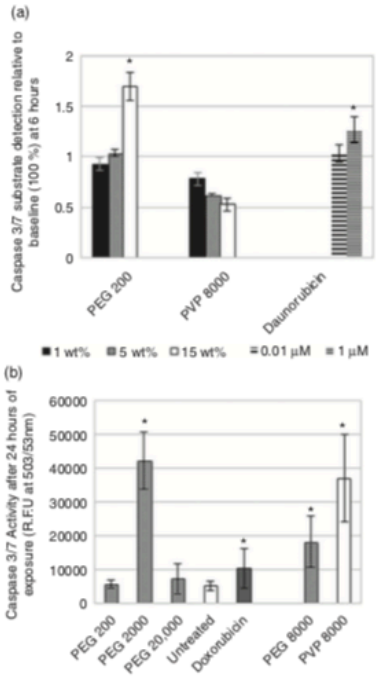

Figure 4. Caspase 3/7 levels after exposure to polymers or controls. Error bars represent standard deviation ($n = 3$). Asterisk represents statistically significant ($p < 0.05$) change from baseline. (a) Caspase 3/7 activation relative to baseline (1) after 6 h of exposure to PEG or daunorubicin-HCl. (b) Caspase 3/7 activation after 24 h of exposure to PEG or control. PEG: poly(ethylene glycol).

PEG increases the presence of apoptosis markers: TUNEL signal. TUNEL staining was used to determine whether DNA fragmentation (a late apoptosis marker) can be detected in cells exposed to PEG for 24 h (Figure 5).

The DNAse I control showed both strong staining of the nucleus and some visible staining of DNA fragments. Doxorubicin-HCl-exposed cells show more staining of DNA fragments within the A375 cells versus DNAse exposed cells. This difference in staining across controls is expected, given the short time the cells were exposed to DNAse versus the longer exposure to doxorubicin. Cells exposed to PEG_{200} were visibly fewer in number, with some staining of fragmented DNA. Cells exposed to $PEG_{20,000}$ showed visible condensation in the brightfield image, and minimal DNA fragmentation, indicating minimal detection of late apoptosis. Cells exposed to PEG_{8000} showed DNA fragmentation across all cells visualized. Thus, 24 h after polymer exposure, the staining of this late apoptosis marker complements the data seen with the activation of caspases 3/7 (early apoptosis makers) in Figure 4, as well as the toxicity trends seen in Figure 2. Further, in contrast to PEG_{8000}, PVP_{8000}-exposed cells showed strong nuclear staining, but with residual cell fragments seen in the background of the fluorescent image, indicating an alternative timing of apoptotic events.

Taken together, the apoptosis markers (caspases and TUNEL staining) and morphological analysis demonstrate apoptosis induction by PEGs. The data show apoptosis occurring at different timepoints depending on the type of PEG to which the cells were exposed.

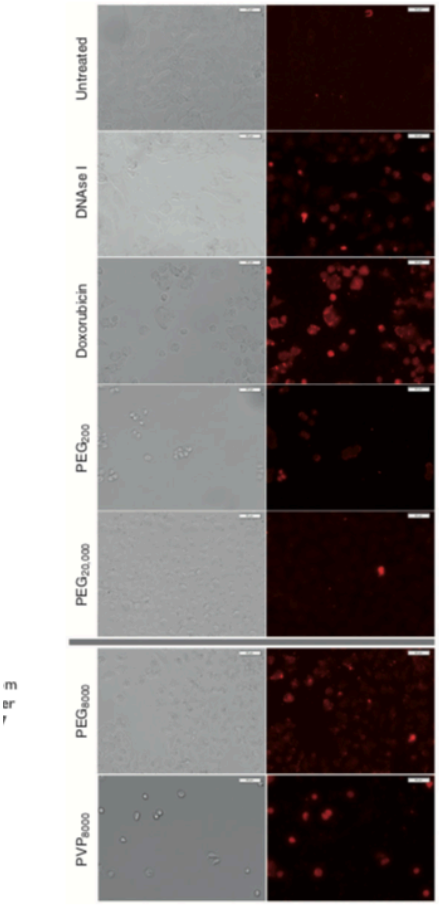

Figure 5. TUNEL staining of A375 cells after 24 h of exposure to PEG, PVP or controls.
PEG: poly(ethylene glycol); PVP: poly(vinyl pyrrolidone).

Comparison of PEG effects with other cell types. The impacts on cell viability were examined for cell-specificity by performing the PrestoBlue® viability assay with a mouse cell line and with non-cancerous human cells (Figure 6).

In Figure 6(a), a 3T3 mouse fibroblast cell line was exposed to PEG_{200}, PEG_{2000} or $PEG_{20,000}$ for 72 h. At 0.5 and 1% w/v, there was no discernible reduction in cell viability for all PEG molecular weights. However, by 10% w/v, PEG_{200} and $PEG_{20,000}$ reduced cell viability to ~60%, and at 20% w/v, viability was approximately 50%. In Figure 6(b), human corneal epithelial cells were exposed to $PEG_{200,}$ PEG_{2000}, or $PEG_{20,000}$ for 72 h. From 0.01 to 0.1% w/v, there was no discernible reduction in cell viability for all PEG

molecular weights. At 1% w/v, PEG 200 demonstrated a reduction in cell viability to ~70%, which further decreased to ~40% at 3% w/v. Thus, the cellular effects of PEG on A375 cells appear to be maintained across species and cell types.

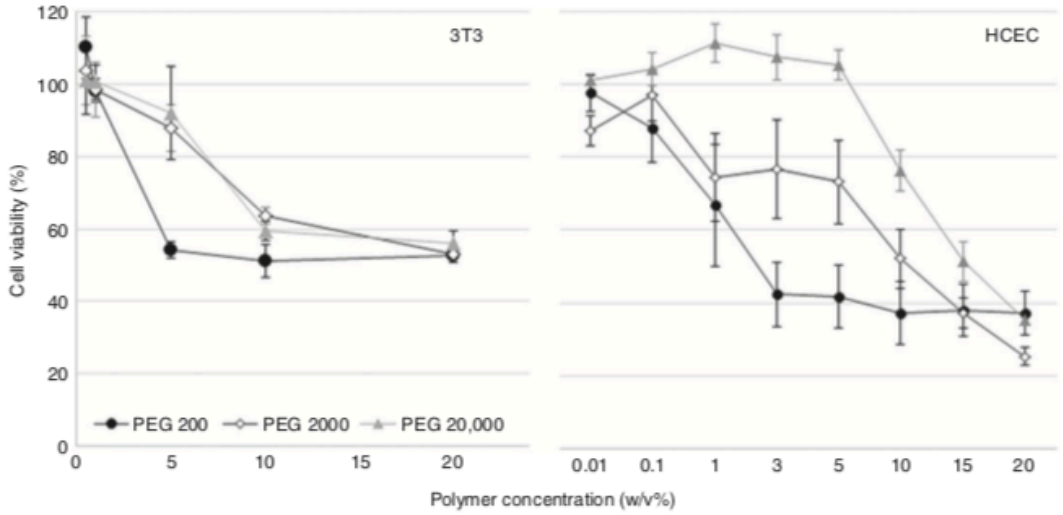

Figure 6. Influences of PEG_{200}, PEG_{2000} and $PEG_{20,000}$ on murine 3T3 fibroblast cells (a) and human corneal epithelial cells (b). Error bars denote standard deviation ($n = 3$).
PEG: poly(ethylene glycol).

Assays of the cellular environment

PEG effects on media viscosity

To assess whether the impact of PEG is not necessarily biological but rather the result of changes to media surrounding the cells, the physical properties of the PEG-containing media were examined. The viscosity of PEG-containing medium at various PEG molecular weights and concentrations is shown in Figure 7. The viscosity increased with increasing PEG concentration for all PEG molecular weights, although the magnitude of increase in viscosity differed. For example, a solution of 15% PEG_{2000} was approximately 2.6_ more viscous than 5% PEG_{2000}. At the same time, the viscosity of 15% $PEG_{20,000}$ was approximately 6.2_ higher than 5% $PEG_{20,000}$.

The magnitude of change in viscosity did not correlate directly with the magnitude of change seen in cell viability (Figure 2), although there was a general trend toward decreased cell viability with increased medium viscosity. For example, ~50% cell viability was seen with a solution viscosity of 1.09 mPas with PEG_{200}. At approximately the same viscosity, cells exposed to media with PEG_{2000} were 75% viable and cells exposed to $PEG_{20,000}$ were 100% viable. PEG_{200} was an exception, as there was no real difference in viscosity (1% vs. 3%) but yet there was a large drop in viability. Therefore, it can likely be concluded that the changes to the viability of the cells are not the direct

result of alterations to the viscosity of the medium.

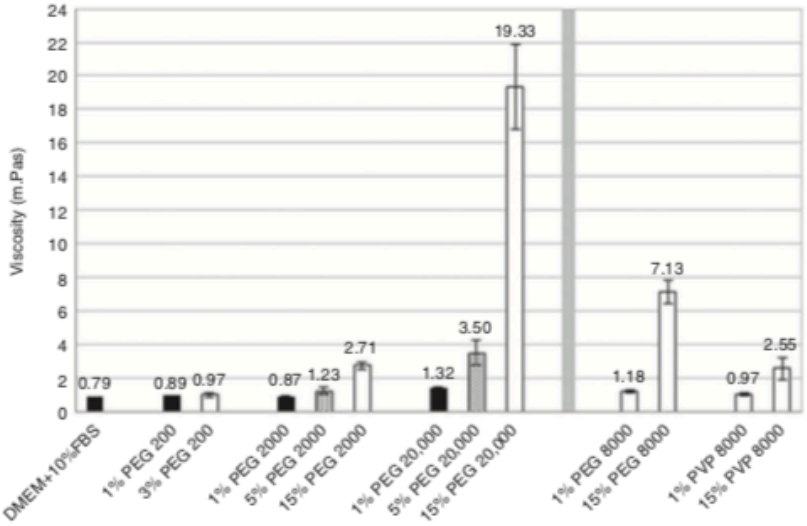

Figure 7. Viscosity of cell media with varying polymer molecular weights and concentrations. Precise average viscosity values are stated above each bar for ease of comparison. Error bars denote standard deviation ($n = 3$).

PEG effects on media osmolality

Cells require a plasma osmolality of approximately 280–295 mOsm/kg for adequate cellular activity.[42] Hypertonic solutions are well known to cause biological changes.[31,43–45] Given that the dissolution of PEG in cell media alters the osmolarity, it was investigated whether PEG may exert toxicity towards A375 cells through the alteration of the media osmolality, specifically by producing hyperosmotic cell media (Figure 8). Note, the viability values in this Figure represent viability after 72 h of exposure to test compounds.

Cell viability was observed to decrease with increasing osmolality of the cell media consistently across all PEG molecular weights. Reduction in cell viability began at a media osmolality of ~400 mOsm/kg (DMEM þ FBS alone was ~360 mOsm/kg). This negative correlation continued to ~40% viability in the presence of media with an osmolality of ~900 mOsm/kg. The correlation was independent of PEG molecular weight. Cells exposed to PEG_{200} experienced stronger reductions in cell viability at lower media osmolality than when exposed to PEG_{200}, PEG_{8000} or $PEG_{20,000}$ (seen at ~3% w/v PEG_{200}). Looking specifically at PVP_{8000} alone, there does not appear to be an inverse correlation between osmotic effects and cell viability. This is observed in particular between PVP_{8000} concentrations of 1 and 3% w/v, where cell viability rapidly decreased, while osmolality increased only slightly. Thus, the data indicate that there is a direct correlation between cellular viability and media osmolality in the presence of PEG.

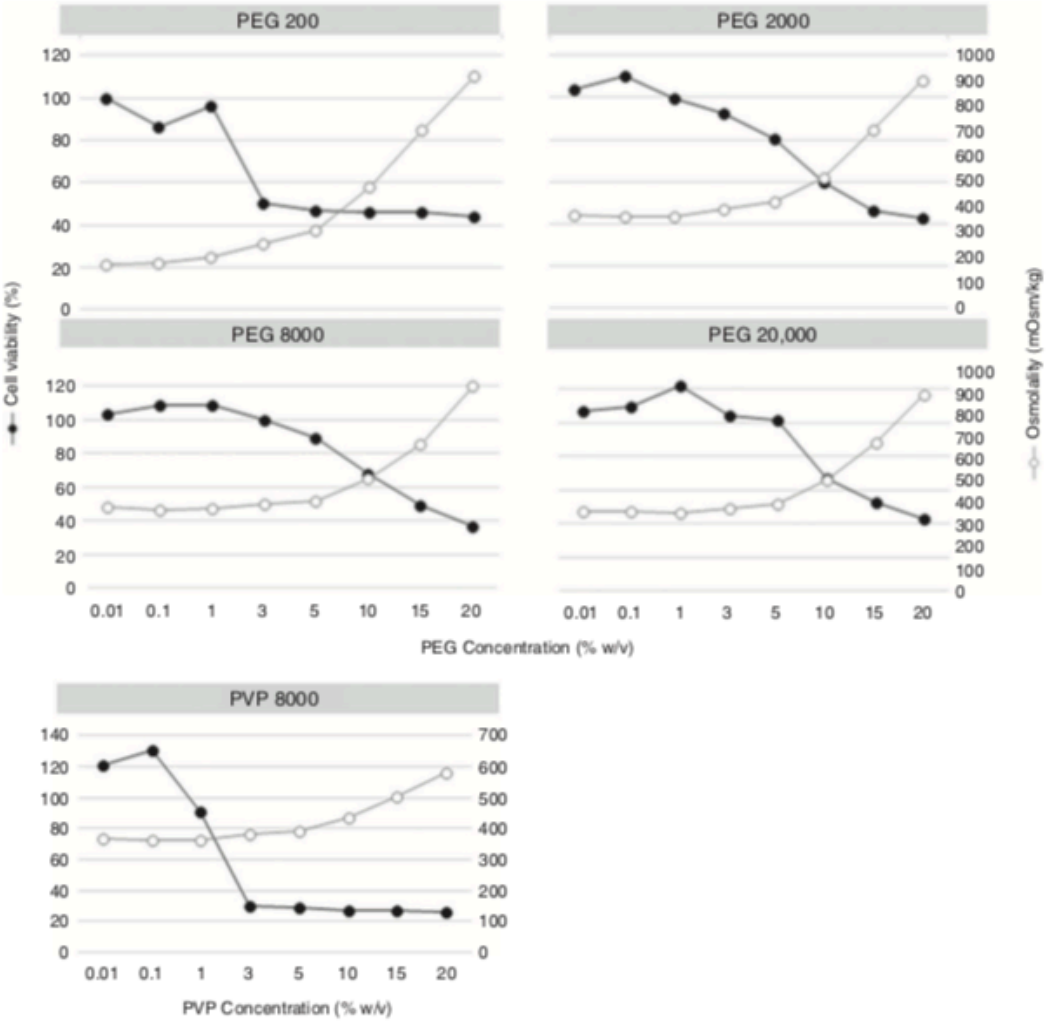

Figure 8. The effect PEG and PVP on media osmolality and cellular viability. PEG: poly(ethylene glycol); PVP: poly(vinyl pyrrolidone).

Discussion

PEG effects on A375 cells

PEG of three different molecular weights (200, 2000 and 20,000) was investigated for its biological activity using A375 metastatic melanoma cells, based on literature that indicates PEG may have some inherent biological activity. Both changes to the cells and the effect of PEG on the cell medium were examined to determine the cause of any biological effects. The most significant finding was that all the PEG compounds tested demonstrated toxicity towards A375 cells. This toxicity was dependent on PEG concentration/structure as seen in Figure 2.

Despite literature reports which suggest that PEG may have an effect on P-glycoprotein, the results of this work suggest that the mechanism of toxicity is more physical than biological. In fact, as shown in Figure 8, the effect of PEG is thought to be primarily related to the change in the osmolality of the medium and there was a direct inverse correlation between osmolality of the PEG solution and the viability of the cells. While for most compounds osmolality is only dependent on the concentration of the solute, PEG does not act as an ideal solute[46] and, therefore, the measured osmolality represents an interaction between the concentration of PEG in a solution and its molecular weight (i.e. the number of EO units present). Taken together, these data suggest that the ethylene oxide subunits alter the osmolality of the cell media, and the ensuing hyperosmolality then induces cell death.

It is important to note that there exists a good understanding of the relationship between media osmolarity and cell toxicity. Upon the addition of solute into the extracellular environment, a hyperosmotic condition is created where the extracellular concentration of solute is greater than the intracellular concentration of solute. The spontaneous, natural phenomena of osmosis then occurs, which results in the passive diffusion of water from inside the cell to the outside. This movement results in a net water loss for the cell, and leads to cell shrinkage.[47] Cell shrinkage (or 'apoptotic volume decrease' (AVD)) contributes to the signaling of apoptosis, and is an early marker of apoptosis, sometimes occurring even prior to the activation of executioner caspase 3.[48] In some cell types, cell shrinkage has also been shown to be essential for apoptosis to occur.[49]

Based on this knowledge, it was expected and indeed observed that the morphology of A375 cells is 'shrunken' after being exposed to PEG. The cells exposed to higher concentrations of PEG (correlating to low cell viability and high osmolality) were spherical in shape and appeared highly condensed (based on the darker pigmentation inside the cells). In contrast, cells exposed to lower concentrations of PEG (correlating to high viability and osmolality similar to control) appeared similar in morphology to control cells.

The activity of apoptotic executioner caspases 3 and 7 were also measured. PEG_{200} demonstrated statistically significant increases in caspase 3/7 activation as early as 2 h after PEG exposure and PEG_{2000} and $PEG_{20,000}$ generally demonstrated increases in mean caspase 3/7 activation with increasing PEG concentration although later (Figure 4). The initiation of apoptosis is known to vary between different compounds and thus additional later timepoints should be tested to obtain a full spectrum of induction times.

The resazurin viability results, osmolarity measurements, cell morphology, viability threshold of ethylene oxide units, caspase 3/7 activation and TUNEL staining results strongly support the hypothesis that PEG has biological activity; specifically, the activity is reduced cellular viability due to altered osmolality of the cell medium, which is related to the PEG structure (hydrogen bonding via its ethylene oxide subunits).

Specificity of results

The observations of PEG toxicity towards A375 cells were unique when compared with another linear, nonionic, hydrophilic polymer: PVP. Overall, the data demonstrated that PVP reduced cellular viability. However, there is likely an alternative mechanism of action for PVP, because all PVP data showed a distinctly different pattern than was observed with the PEG-exposed samples of the same molecular weight. For example, PVP was shown to activate caspase 3/7 dependent apoptosis after 24 h of exposure, but it also significantly reduced caspase activity after up to 6 h of exposure. This suggests that early triggering of cell death may be via a different pathway than PEG. Further investigation of viscosity effects may be warranted to better understand the PVP mechanism of toxicity.

The cell-specificity of the PEG effects was consistent across multiple cell-types and they were not shown to be exclusive to A375 metastatic melanoma cells. PEG produced very similar reductions in cell-viability with a murine 3T3 fibroblast cell line and human corneal epithelial cells (Figure 6). In both cases, the viability was also concentration and molecular-weight dependent. These data support the osmotic mechanism of action of PEG, as hyperosmotic conditions are expected to produce similar effects in all cell types.

An early hypothesis for the viability effects of PEG on A375 cells was that PEG could act as a surfactant on the cellular membrane. This was hypothesized to be possible due to the amphiphilic character of PEG that has been reported in the literature.[29] Morphology data (Figure 1) suggested that this was not the case, as cells exposed to the surfactant were not shrunken, but were rather visibly elongated (at lower surfactant concentration) or fragmented (at higher surfactant concentration) (Supplemental Figure 1) in contrast to the PEG-exposed cells. In addition, the transport of DNR was not affected. These data suggest PEG does not act as a surfactant on A375 cell membranes.

Additional insights and potential future studies

While viscosity may be a factor with lower-molecular weight PEG, somewhat surprisingly, similar viscosity-related effects were not observed with higher PEG molecular weights of 2000 and 20,000. Thus, it is likely that viscosity is not directly correlated with cell viability changes, however it is theoretically possible it may have some influence at the highest measured value seen with $PEG_{20,000}$. At high viscosity, a hydrated gel may form due to the ability of PEG to form hydrogen bonds with water, thereby limiting diffusion in these gels. Viscosity-dependent cell effects have been previously observed with alginate solutions.[50] The unique effects of PEG_{200} may alternatively be due to the possibility that PEG_{200} is more readily broken down into the toxic ethylene oxide than the higher-molecular weight PEGs.

Taken together, the data strongly suggest a structure-function relationship mediated by

osmotic effects. It may also be possible that PEG also acts on the cell membrane, a protein, a cellular cascade or in the production of toxic metabolites. The current membrane transport data (Figure 4) suggests that there are no changes to the membrane with PEG exposure. However, some studies using intestinal cells have shown that PEG increases the rigidity of the phospholipid head groups,[20] which could prevent the function of membrane proteins, or lead to initiation of the apoptotic pathway. Further studies on membrane and protein effects are needed to better understand the downstream effects of PEG on cells and it is important that future experiments elucidate the process in more detail, as cancer cells can become drug-resistant over time by modulating ion channels and transporters to prevent cell apoptosis.[51]

Potential applications of PEG

Given the mechanism of action by osmotic effects, it is proposed that any potential application will require targeted administration of PEG in order to produce and maintain osmotic effects. Potential applications could include PEG as a topical cream for dermal cancers or gastrointestinal cancers (given that PEG > 400 MW is generally not absorbed and is passed through the GI tract). In fact, the work by Roy et al.[18] has already demonstrated that PEG_{3350} (a strong laxative administered prior to colonoscopy) produces apoptosis in HT-29 colon cancer cells. A limitation of this work was that upstream events leading to cell death were unclear and this current study may provide a possible explanation for their observed results.

Another mechanism by which localized, targeted PEG effects may be achieved is by exploring the therapeutic potential of PEG when conjugated to a targeted therapeutic or as a drug-delivery vehicle. PEG is the first-line polymer for improving drug solubility and increasing residence time by being directly conjugated to the therapeutic.[52] In this case, this established system may provide a unique and simple mechanism for exploring the chemotherapeutic potential of PEG. PEG is also frequently investigated as a drug-delivery vehicle or part of a drug-delivery vehicle, and with this knowledge it may be possible to improve the delivery system by capitalizing on the biological activity of PEG against cancer cells. PEG can also be extensively modified from a structural perspective, and with appropriate modification, there is the potential for targeted, localized delivery of PEG alone to disease sites as part of this approach as well.

Conclusion

Overall, this study indicates for the first time that PEG has inherent biological activity in human and non-human cells that is linked to its structure and properties. The results specifically indicate that the mechanism of toxicity is due to changes in extracellular osmolality, which can be correlated with the number of ethylene oxide units present in the extra-cellular space. It is important to note that these data do not suggest new clinical concern for the safety of PEG in pharmaceutical, cosmetic or food applications,

especially given the long-term evidence of PEG safety in these applications. Rather, this study highlights the untapped potential of PEG, and possibly other polymers, as therapeutic agents. Given that the structure and properties of polymers, particularly synthetic polymers, can be very finely tuned, there is limitless potential for polymers (and PEG specifically) to become part of modern medical treatments, rather than being solely used as a pharmaceutical filler or as therapeutic carriers.

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References

1. Dhal PK, Holmes-Farley SR, Huval CC, et al. Polymers as drugs. Adv Polym Sci 2006; 192: 9–58.
2. Li J, Yu F, Chen Y, et al. Polymeric drugs: advances in the development of pharmacologically active polymers. J Control Release 2015; 10: 369–382.
3. Sanofi Aventis. "Renvela (sevelamer carbonate) Product Monograph," 2017.
4. Sanofi Aventis. "Sodium Polystyrene Sulfonate Product Monograph,"2014.
5. Valeant. "Colesevelam hydrochloride Product Mono- graph." 2014.
6. Mulvey GL, Marcato P, Kitov PI, et al. Assessment in mice of the therapeutic potential of tailored, multivalent shiga toxin carbohydrate ligands. J Infect Dis 2003; 187: 640–649.
7. Joshi A, Saraph A, Poon V, et al. Synthesis of potent inhibitors of anthrax toxin based on poly- l -glutamic acid. Bioconjug Chem 2006; 17: 1265–1269.
8. Sigal GB, Mammen M, Dahmann AG, et al. Polyacrylamides bearing pendant a-sialoside groups strongly inhibit agglutination of erythrocytes by influenza virus: the strong inhibition reflects enhanced binding through cooperative polyvalent interactions. J Am Chem Soc 1996; 118: 3789–3800.
9. Alakhova DY and Kabanov AV. Pluronics and MDR reversal: an update. Mol Pharm 2014; 11: 2566–2578.
10. Koenig P-A, Spooner E, Kawamoto N, et al. Amino acid copolymers that alleviate experimental autoimmune encephalomyelitis in vivo interact with heparan sulfates and glycoprotein 96 in APCs. J Immunol 2013; 191: 208–216.
11. Alcantar NA, Aydil ES and Israelachvili JN. Polyethylene glycol-coated biocompatible surfaces. J Biomed Mater Res 2000; 51: 343–351.
12. Turecek PL, Bossard MJ, Schoetens F, et al. PEGylation of biopharmaceuticals: a review of chemistry and non- clinical safety information of approved drugs. J Pharm Sci 2016; 105: 460–475., no.
13. Zhu J. Bioactive modification of poly(ethylenglykol) hydrogels for tissue engineering. Biomaterials 2010; 31: 4639–4656.
14. D'souza AA and Shegokar R. Polyethylene glycol (PEG): a versatile polymer for pharmaceutical applications. Expert Opin Drug Deliv 2016; 13: 1257–1275. no.
15. Batrakova EV and Kabanov AV. Pluronic block copoly- mers: Evolution of drug delivery concept from inert nanocarriers to biological response modifiers. J Control Release 2008; 130: 98–106. no.
16. Koley D and Bard AJ. Triton X-100 concentration effects on membrane permeability of a single HeLa cell by scanning electrochemical microscopy (SECM). PNAS 2010; 107: 16783–16787. no.
17. Borner MM, Schneider E, Pirnia F, et al. The detergent Triton X-100 induces a death pattern in human carcino- ma cell lines that resembles cytotoxic lymphocyte- induced apoptosis. FEBS Lett 1994; 353: 129–132. no.
18. Roy HK, DiBaise JK, Black J, et al. Polyethylene glycol induces apoptosis in HT-29 cells: potential mechanism for chemoprevention of colon cancer. FEBS Lett 2001; 496: 143–146.
19. Wali RK, Kunte DP, Koetsier JL, et al. Polyethylene glycol-mediated colorectal cancer chemoprevention: roles of epidermal growth factor receptor and Snail. Mol Cancer Ther 2008; 7: 3103–3111.
20. Hugger ED, Audus KL and Borchardt RT. Effects of poly(ethylene glycol) on efflux transporter activity in Caco-2 cell monolayers. J Pharm Sci 2002; 91: 1980–1990.

21. Hugger ED, Cole CJ, Raub TJ, et al. Automated analysis of polyethylene glycol-induced inhibition of P-glycopro- tein activity in vitro. J Pharm Sci 2003; 92: 21–26.
22. Johnson BM, Charman WN and Porter CJH. An in vitro examination of the impact of polyethylene glycol 400, Pluronic P85, and vitamin E d-alpha-tocopheryl polyeth- ylene glycol 1000 succinate on P-glycoprotein efflux and enterocyte-based metabolism in excised rat intestine. AAPS PharmSci 2002; 4: E40.
23. Nerurkar MM, Burton PS and Borchardt RT. The use of surfactants to enhance the permeability of peptides through Caco-2 cells by inhibition of an apically polar- ized efflux system. Pharm Res 1996; 13: 528–534.
24. Mountfield RJ, Senepin S, Schleimer M, et al. Potential inhibitory effects of formulation ingredients on intestinal cytochrome P450. Int J Pharm 2000; 211: 89–92.
25. Liu K-J and Parsons JL. Solvent effects on the preferred conformation of poly(ethylene glycols). Macromolecules 1969; 2: 529–533.
26. Maxfield J and Shepherd IW. Conformation of poly(eth- ylene oxide) in the solid state, melt and solution measured by Raman scattering. Polymer (Guildf) 1975; 16: 505–509.
27. Duncan R. The dawning era of polymer therapeutics. Nat Rev Drug Discov 2003; 2: 347–360.
28. Veronese FM and Pasut G. PEGylation, successful approach to drug delivery. Drug Discov Today 2005; 10: 1451–1458.
29. Israelachvili J. The different faces of poly(ethylene glycol). Proc Natl Acad Sci USA 1997; 94: 8378–8379.
30. Milton Harris J and Zalipsky S. Poly(ethylene glycol) chemistry and biological applications. Vol.680. Washington, DC: American Chemical Society, 1997.
31. Verbalis JG. Disorders of body water homeostasis. Best Pract Res Clin Endocrinol Metab 2003; 17: 471–503.
32. Wouters J, Stas M, Gremeaux L, et al. The human mel-
anoma side population displays molecular and functional characteristics of enriched chemoresistance and tumori- genesis. PLoS One 2013; 8: e76550.
33. Dickson PV and Gershenwald JE. Staging and prognosis of cutaneous melanoma. Surg Oncol Clin N Am 2011; 20: 1–17.
34. Turnbull KJ, Brown BL and Dobson PRM. Caspase-3-like activity is necessary but not sufficient for daunorubicin-induced apoptosis in Jurkat human lymphoblastic leukemia cells. Leukemia 1999; 13: 1056–1061.
35. Spoelstra EC, Westerhoff HV, Dekker H, et al. Kinetics of daunorubicin transport by P-glycoprotein of intact cancer cells. Eur J Biochem 1992; 207: 567–579.
36. National Cancer Institute. “Drugs Approved for Melanoma,” [Online], https://www.cancer.gov/about- cancer/treatment/drugs/melanoma (2018, accessed 16 September 2018).
37. Regev R, Katzir H, Yeheskely-Hayon D, et al. Modulation of P-glycoprotein-mediated multidrug resistance by acceleration of passive drug permeation across the plasma membrane. Febs J 2007; 274: 6204–6214.
38. ATCC. “A-375 ATCC-CRL-1619^{TM} Homo sapiens skin malignant melanoma cells. Manassas, Virginia, USA.”
39. Bjoerling M, Karlstroem G and Linse P. Conformational adaption of poly(ethylene oxide): a carbon-13 NMR study. J Phys Chem 1991; 95: 6706–6709.
40. Ahlnaes T, Karlstroem G and Lindman B. Dynamics and order of nonionic surfactants in

neat liquid and micellar solution from multifield carbon-13 NMR relaxation and carbon-13 NMR chemical shifts. J Phys Chem 1987; 91: 4030–4036.
41. Sundquist T, Moravec R, Niles A, et al. Timing your apoptosis assays. Cell Notes 2006; 16: 18–21.
42. Ahmed N. Clinical biochemistry. Oxford University Press, 2016; 281.
43. Shukla A, Hashiguchi N, Chen Y, et al. Osmotic regula- tion of cell function and possible clinical applications. DNA Repair (Amst) 2004; 21: 391–400.
44. Argyropoulos C, Rondon-Berrios H, Raj DS, et al. Hypertonicity: pathophysiologic concept and experimen- tal studies. Cureus 2016; 8: e596.
45. Thompson GJ, Langlais C, Cain K, et al. Elevated extracellular [Kþ] inhibits death-receptor- and chemical-mediated apoptosis prior to caspase activation and cytochrome c release. Biochem J 2001; 357: 137–145.
6. Schiller LR, Emmett M, Santa Ana CA, et al. Osmotic effects of polyethylene glycol. Gastroenterology. 1988; 94: 933–941.
47. Lang F and Hoffmann EK. Role of ion transport in con- trol of apoptotic cell death. Compr Physiol 2012; 2: 2037–2061.
48. Maeno E, Takahashi N and Okada Y. Dysfunction of regulatory volume increase is a key component of apo- ptosis. FEBS Lett 2006; 580: 6513–6517.
49. Ernest NJ, Habela CW and Sontheimer H. Cytoplasmic condensation is both necessary and sufficient to induce apoptotic cell death. J Cell Sci 2008; 121: 290–297. 0. Vlak JM. Insect cell cultures: fundamental and applied aspects. Kluwer, 1996; 207.
51. Hoffmann EK and Lambert IH. Ion channels and trans- porters in the development of drug resistance in cancer cells. Philos Trans R Soc Lond B Biol Sci 2014; 369: 20130109
52. Knop K, Hoogenboom R, Fischer D, et al. Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives. Angew Chem Int Ed Engl 2010; 49: 6288–6308.