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Injectable self-assembled block copolymers for sustained gene and drug co-delivery: An *in vitro* study

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ABSTRACT

A temperature-responsive pentablock copolymer (PB) was designed to deliver DNA *and* provide prolonged gene expression by forming a thermogelling release depot after subcutaneous or intratumoral injection. A synthetic barrier gel was developed based on poly(ethylene glycol) diacrylate to enable the released vectors to instantly and continuously transfect cultured cells. The aim of this setup was to provide a simple and controlled *in vitro* system to mimic tumor matrix to optimize the release system and to study the influence of the continuous and sustained release of the polyplexes on the *in vitro* transfection. The porosity of the barrier gel was adjusted by addition and removal of Pluronic F127 (PL), and the properties of the gel were characterized by visual inspection and scanning electron microscopy (SEM). Concentrated PB-based vector was administered to the barrier gel and allowed to be released to the buffer. We found that most of feed vector could be released from the barrier gel without unpacking the polymer–DNA complexes. Based on the specific construction of the PB-based vectors, an anti-cancer drug paclitaxel (PTX) was further loaded into the same vector designed for DNA delivery, thus formulating a gene and drug co-delivery system. This system was tested for sustained delivery to human ovarian carcinoma cells SKOV3 using the barrier gel *in vitro* as a tumor mimic. Transfection efficiency was found to be significantly enhanced by co-delivering PTX, while PTX also showed its effect as an anti-cancer drug to induce cell death. Yet both of the two effects of PTX shown in SKOV3 cells turned out to be dramatically weaker in ARPE-19 cells, a human non-cancerous cell line, which might be related to the nature of the vector itself. Instant release of vectors showed the ability to maintain transfection up to the fourth day, making the gene expression stable at least for the first 5 days. Further study is still needed to improve the duration of effective release.

1. Introduction

Sustained gene delivery using polyplexes has attracted much attention over the past decade because of its potential to achieve a long-term therapeutic effect that otherwise must rely on repeated injections because of transient transfection (Agarwal and Mallapragada, 2008). One consideration in designing such a system lies in the release ability, which requires a reasonably high and steady release rate of vectors. Next, the released vector should maintain its function and transfect cells. To test this aspect, studies are usually done by collecting the vector released within a certain time course in an acellular environment and comparing its transfection efficacy with the control vectors without simulating the release process (Agarwal et al., 2008; Chun et al., 2005; Quick and Anseth, 2004). This comparison is convincing in terms of vector function preservation; however, it cannot reflect the real transfection ability of the sustained release system, with sustained release and transfection being treated as two separate processes. Thus, a more realistic testing setup is needed to investigate the transfection efficacy of a specific sustained release system. In this study, we report an experimental setup that allows the vector to be released directly to cells in a continuous fashion, by using a polyethylene glycol diacrylate (PEG-DA) barrier gel to mimic the role of tumor extracellular matrix in providing a diffusion barrier for the therapeutic agents to reach tumor cells. The PEG-DA gel is a highly simplified mimic, but provides a well-controlled environment *in vitro* to alter mechanical and transport properties by changing gel fabrication conditions, and to study the influence of these parameters on sustained gene delivery. With this simple setup, the released vector can then directly transfect cells using the injectable thermogelling poly(diethylaminoethyl methacrylate) (PDEAEM)-Pluronic F127-PDEAEM pentablock copolymer (PB) vector system (Agarwal et al., 2008) and can also be used to co-deliver drugs. Without the PEG-DA gel acting as a transport barrier, the PB vector thermogelling

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system would dissolve quickly in an excess of media *in vitro* and not allow for the investigation of sustained and continuous release to cells.

The self-assembled PB was previously synthesized via atom transfer radical polymerization (ATRP) (Determan et al., 2005), with Pluronic F127 (PL) to exhibit thermoreversible gelation and PDEAEM blocks to facilitate DNA condensation. This vector can be injected intratumorally to undergo sol–gel transition at physiological temperatures, providing vector release from the gels in a sustained manner. This vector has shown several advantages including promoting selective transfection in cancer cells (Zhang et al., 2009). Upon copolymerization, PB blocks exhibit enhanced mechanical property as compared to the Pluronic F127 alone (Agarwal et al., 2008), which is favorable for *in vivo* sustained release, because stronger gels can provide longer sustained delivery and can maintain their mechanical integrity over longer time periods. Self-assembled injectable hydrogels and polyplex gels eventually dissolved to release polyplexes (Agarwal et al., 2008) and are clinically superior to other chemically cross-linked hydrogels that involve harsh crosslinking environments (Lee et al., 2007), or scaffolds (Cohen-Sacks et al., 2004) that need to be surgically implanted. In order to improve the stability of PB vectors in a physiological environment, free PL was subsequently added to the PB/DNA complex to form a shield on the excess charges by self-assembly, making the PB-PL type of vector less liable to aggregate with serum proteins (Agarwal et al., 2007b). Along with the benefit, PL shield also provides the possibility of encapsulating hydrophobic anti-cancer drugs due to its amphiphilic nature (Kabanov et al., 2002), enabling the whole vector to deliver genes and drug simultaneously. Moreover, PL alone has been reported to have a chemosensitizing effect in multidrug-resistant (MDR) cells by inhibiting the P-glycoprotein related drug efflux (Kabanov et al., 2003).

The advantage of co-delivery of gene and drugs has been shown to be that expression of specific genes can help render drug-resistant cells sensitized back to the drug (Janat-Amsbury et al., 2004; Wang et al., 2006), and uptake of drug into cells can enhance the level of gene expression (Nair et al., 2002; Wang et al., 2006; Wiradharma et al., 2010). Thus, a synergistic effect can be expected with a combination of gene therapy and drug treatment. Regarding this combination, it could be achieved by combining two separate treatments (gene and drug) physically (Janat-Amsbury et al., 2004), or by combining gene and drug in the same carrier and using it as a single treatment (Qiu and Bae, 2007; Wang et al., 2006). The latter is more advantageous, for it can ensure delivery of gene and drug into the same cell, thus maximizing the synergistic effect. Yet it is also more complicated, for it involves synthesis of a new carrier and possible interferences between the two payloads. Here we report an easily implemented method for synergistic delivery of gene and drug with PB-PL type of carriers. The anti-cancer drug paclitaxel (PTX) was incorporated in the PL shield and self-assembled into the PB/DNA complex later. By loading PTX and DNA separately, the potential interference can be reduced dramatically. More importantly, the two anti-cancer agents can be delivered in a simultaneous and sustained manner.

2. Experimental

2.1. Materials

Poly(ethylene glycol) diacrylate (PEG-DA) MW = 4000 was purchased from Polysciences Inc. (Warrington, PA). Pluronic F127 [$(PEO)_{100}$-b-$(PPO)_{65}$-b-$(PEO)_{100}$], (where PEO represents poly(ethylene oxide) and PPO represents poly(propylene oxide)) and photoinitiator 4-(2-hydroxyethoxy)phenyl-(2-hydroxy-2-propyl)ketone (Irgacure 2959) was obtained from BASF (Florham Park, NJ) and used without further modification. Lactate dehydrogenase (LDH) assay kit, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay kit and heparin sodium salt was obtained from Sigma–Aldrich (St Louis, MO). Luciferase assay system and passive lysis buffer were purchased from Promega (Madison, WI). Cell culture reagents: Dulbecco's Modified Eagle Medium (DMEM), heat inactivated fetal bovine serum (FBS), 0.25% trypsin-EDTA and Hank's buffered salt saline (HBSS) were purchased from Invitrogen (Carlsbad, CA), Dulbecco's MEM: Ham's Nutrient Mixture F-12, 1:1 Mix (DMEM/F-12) was purchased from ATCC (Manassas, VA). ExGen 500 was purchased from Fermentas Life Sciences (Hanover, MD). HiSpeed Plasmid Maxi Kit was obtained from Qiagen (Valencia, CA). 6.7 kb pGWIZ-luc (GeneTherapy Systems Inc., CA) plasmid encoding the luciferase reporter gene and 4.7 kb EGFP-N1 (ClonTech, CA) plasmid encoding GFP reporter gene were purified with Qiagen HiSpeed Maxi Kit. Polyester membrane with diameter of 24 mm and pore size of 3.0 μm were purchased from Corning (Lowell, MA). PicoGreen *ds*DNA Reagent was purchased from Invitrogen.

2.2. Cell culture

The human ovarian carcinoma cell line SKOV3 and human retinal cell line ARPE-19 were obtained from ATCC™ (Manassas, VA). SKOV3 Cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS at 37 °C under a humidified atmosphere containing 5% CO_2. ARPE-19 cells were cultured the same way but with DMEM/F-12 media. Subculture was carried out every 2–3 days. To maintain a similar performance of cells in transfection, we used SKOV3 cells with passage number of 30–40 and ARPE cells with passage number of 10–15 for transfection.

2.3. Barrier gel formation

To simulate extracellular matrix environment for the *in vitro* studies, a hydrogel system was created. To each well in a 24-well plate, 75 mg of PEG-DA, 100 μl of photo initiator (0.2 wt.% solution in water), 200 μl of deionized water and appropriate amount of Pluronic F127 (PL) were added with the final PL concentration of 0, 7, 12, 15 and 17% by weight in different wells. The plate was then placed in a refrigerator overnight and gently vortexed before exposure to UV light. The solution was photo crosslinked under UV beam of 50 MW/cm^2 for 2 min. To avoid interference among nearby wells, a copper tube that fits into the well of interest was used along with a thick black paper covering other wells. After gel formation, they were carefully scraped out using a spatula and soaked in 50 ml of DI water for 3 days at −4 °C with water changed every day. To calculate the mass loss and swelling ratio, gels were dehydrated in an oven at 37 °C and rehydrated thereafter. By comparing the weight of dehydrated gel with the weight of all feed chemicals, the PL was found to have been completely removed by the third day of dissolution. The swelling ratio was determined by the ratio of the weight of rehydrated gel to that of dehydrated gel, which was found to be about 1200–1300% for a PEG-DA gel with 15% PL dissolved away. Throughout the manuscript the PEG-DA gelrefers to a PEG-DA gel with PL removed by dissolution in water. Gels formed in this way contain a concave top surface that allows sample loading.

For making gels with an indentation, the plate lid was molded with a glass tube (d = 10 mm). Instead of shining UV from above, the incident UV light was made to shine from below. After gel formation, the mold was removed from the gel with great care to avoid any damage to the boundary of indentation. The capacity of indentation depends on the length of mold (glass tube) and volume of gel solution. The complete process of making a

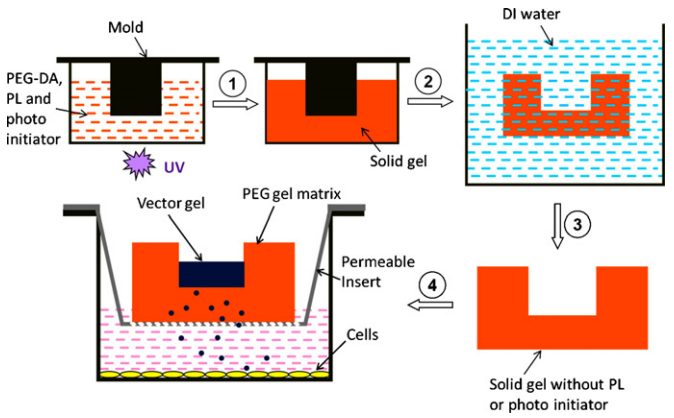

Fig. 1. Process of making PEG-DA gel matrix with an indentation for sustained gene delivery: (1) gel solution containing PEG-DA, Pluronic F127 (PL) and photo initiator was UV crosslinked in a well mold; (2) solid gel was removed from the mold and soaked in deionized water to dissolve away PL; (3) PEG-DA gel matrix was obtained and disinfected; (4) PEG-DA gel was set in a cell-culture well with a supporting permeable insert; vector gel solution was injected into the indentation and solidified in response to change of temperature, releasing the polyplex vector to the cells in a sustained fashion. Mold shown here was flat-bottomed, but a round-bottomed mold was also available depending on need.

molded PEG-DA gel for investigating sustained release is depicted in Fig. 1.

2.4. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was employed to investigate the morphological structure of PEG-DA gels. The hydrated gels were rapidly frozen in liquid nitrogen bath and immediately placed in a lyophilizer for 72 h. The resulting cryo-fractured samples were sputter-coated with gold and observed with a FEI quanta 250 SEM instrument.

2.5. Dynamic mechanical analysis

A native stress-controlled oscillatory rheometer (TA, AR2000EX) was used to investigate the mechanical properties of PEG-DA gels with strain sweep tests. A parallel-plates type of geometry was used, with plate diameter 25 mm. The swollen gels were placed on the lower plate, and the upper plate was lowered to contact the samples until reaching 1 N normal force. The measurement was started instantly at a constant frequency (6.283 rad/s) with the temperature set to 25 °C.

2.6. Polyplex formation

Pentablock copolymers (PB) used in this study were synthesized by ATRP as reported previously (Determan et al., 2005). All polymer solutions were prepared in 0.5× HBS buffer, pH 7.0 unless stated otherwise. To form the polyplex with desired N/P (nitrogen/phosphate) ratios, various amounts of pentablock copolymer (2 mg/ml) were added to the fixed amount of DNA at equal volume. The mixture was then gently vortexed and allowed to stand at room temperature for 20 min. In an effort to improve the stability of polyplex in the presence of serum, free Pluronic F127 (PL) (10 mg/ml) was further added at the same volume to give a weight ratio of 5:1 with regard to the corresponding PB. Upon this, PL self-assembles with PB on the surface of PB/DNA polyplexes and form a shield layer through the hydrophobic interaction (Agarwal et al., 2007b). Polyplex formation, including the formation of the polyplex containing DNA and drug to be discussed later, is illustrated in Fig. 2. The cytotoxicity and stability of resultant PB-PL/DNA polyplex has been thoroughly investigated in our previous work (Agarwal et al., 2007a,b).

2.7. In vitro polyplex release

Appropriate amount of PL was added to the polyplex solution at low temperatures (e.g. 4 °C), to make the final PL concentration around 20 wt.%. At this concentration, the vector solution can be injected as liquid and can form a solid gel in response to the change in surrounding temperature. The pre-warmed PEG-DA gels (soaked in warmed PBS buffer or cell growth medium) were placed in the Transwell inserts equipped on the 6-well plates. Each well contained 1.5 ml of PBS or growth medium. The prepared vector solution was injected into the concave surface of non-molded gels or the indentation of molded gels. Gelation occurred instantly due to temperature change. The plate was then placed on an incubator shaker and shaken with 100 rpm at 37 °C for a week. All solution in the well was collected every day and replaced with 1.5 ml of fresh PBS or growth medium. 150 μl of each collected sample was treated with 2 μl of heparin (200 μg/μl) for 40 min to separate the DNA from pentablock copolymer. The concentration of DNA was then measured using a Picogreen assay on a dual monochromator spectrofluorimeter (Fluoromax-4, Horiba Jobin Yvon) with excitation at 480 nm and slit widths of 3 nm (excitation and emission). Data were recorded at the emission peak (520 nm) for DNA-bound picogreen and analyzed using a standard curve.

2.8. In vitro transfection

Cells were seeded into 96-well or 6-well plates 1 day prior to transfection with initial numbers of ~1.2×10^4 or ~1.0×10^5 cells per well, respectively. After 24 h growth, cells reached a ~70% confluence when the old medium was replaced with fresh growth medium containing 10% FBS. Transfection was then carried out by adding polyplexes of various formulations to the medium with 0.6 μg pGIZ-luc per well for 96-well or 3 μg EGFP-N1 DNA per well for 6-well plate, respectively. Cells were allowed to incubate with polyplexes for 3 h, followed by medium changing to remove the polyplexes. After an additional 45 h post-transfection, cells in the 96-well plates were lysed and analyzed for luciferase activity with luciferase assay kit on an automated Veritas™ Microplate Luminometer. The luminescence was measured in arbitrary Relative Luminescence Units (RLU). Cells in 6-well plate were examined for GFP expression with fluorescence microscope (Nikon, Eclipse-Ti). Each transfection was done in triplicate. ExGen 500, a sterile solution of linear 22 kDa polyethylenimine (PEI), was used as positive control at an N/P ratio of 6 according to the manufacturer's protocol.

2.9. Cytotoxicity

The cytotoxicity of polyplexes was examined with two assay kits: LDH assay, based on the amount of cytoplasmic LDH released into the medium following cell membrane rupture; MTT assay, based on the activity of mitochondrial dehydrogenases of viable cells. Samples were collected at the end of post transfection. Blank cells were used as a negative control to provide 0% cytotoxicity and Triton-X was used as a positive control to provide 100% cytotoxicity. LDH-based cell viability was determined as follows:

$$\text{Cell viability}\,\% = 100 - \frac{\text{Abs(sample)} - \text{Abs(blank cells)}}{\text{Abs(Triton-X)} - \text{Abs(blank cells)}} \times 100$$

MTT-based:

$$\text{Cell viability}\,\% = \frac{\text{Abs(sample)}}{\text{Abs(blank cells)}} \times 100$$

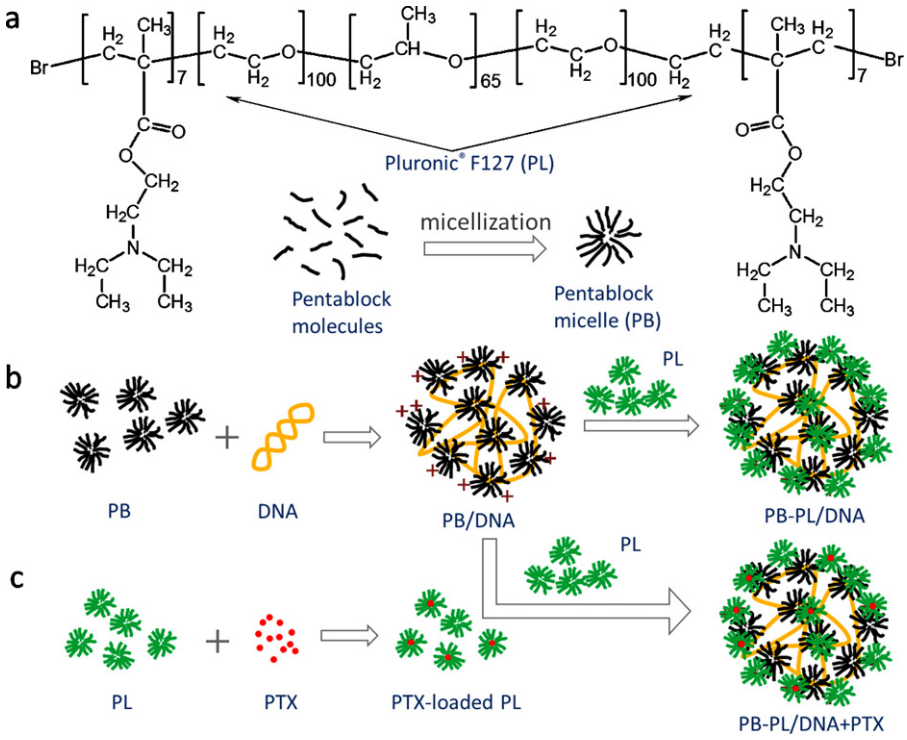

Fig. 2. Schematic illustration of formation of various polyplexes with pentablock copolymer micelle (PB) and Pluronic F127 (PL). (a) The pentablock copolymer molecules form micelles in aqueous solution via self-assembly in the same way as Pluronic F127. (b) The pentablock micelles condense plasmid DNA into polyplexes of PB/DNA via ionic interactions. Excess positive charges on the surface of PB/DNA were shielded by further addition of free PL micellar solution via self-assembly. (c) Paclitaxel (PTX) was encapsulated in PL and further mixed with plain PL to self-assemble into PB/DNA, thus producing a vector containing both DNA and PTX payloads, PB-PL/DNA + PTX.

2.10. Drug encapsulation

Paclitaxel (PTX) was encapsulated in Pluronic F127 (PL) micelles by solvent evaporation method. Briefly, 2 mg of PTX and 100 mg of PL were co-dissolved in 10 ml of acetonitrile in a 50 ml flask. The solvent was slowly evaporated under reduced pressure at 50 °C and a transparent solid layer was formed around the flask which was then placed in a vacuum oven at room temperature overnight to remove the residual solvent. The solid layer was reheated in the same flask and re-dissolved in 10 ml warmed water with medium stirring. The resultant PTX loaded PL micelle solution was filtered into a 0.22 μm filter to remove the undissolved PTX and other impurities. The filtrate was then lyophilized and stored at 4 °C for later use. The amount of PTX was determined using a standard curve of absorbance at 227 nm. The drug loading efficiency was expressed as the ratio of the weight of feed drug to the weight of encapsulated drug, which was found to be about 73%.

2.11. Gene and drug co-delivery

Polyplexes were formed in a similar way as described above, but instead of using free Pluronic F127 (PL) alone to provide the additional shield, desired amount of PTX-loaded PL was mixed in at this step (Fig. 2c). In this way, PTX and DNA could be delivered using a single carrier at the same time. Transfection followed the procedure stated above.

2.12. Sustained delivery performed with PEG-DA barrier gel

Cells of interest were seeded on 6-well plates 1 day before transfection was performed. Following the procedure of measuring polyplex release, concentrated polyplex gel solution was injected into the indentation of PEG-DA barrier gels. As a result, polyplex containing DNA and PTX was gradually released into cells grown on bottom. The sustained release was allowed to proceed for 24 h when the whole release setup was switched to another well of fresh cells for a new daily release. Transfected cells were collected and analyzed for luciferase activity after additional 24 h post-transfection.

3. Results and discussion

3.1. Property of synthetic PEG-DA gels serving as tumor mimics

In the development of a barrier gel that can mimic tumor extracellular matrix to hinder delivery of macromolecules such as polyplex vectors, the goal was to attain reasonable diffusion rates that can be readily adjusted. Co-dissolving Pluronic F127 (PL) with PEG-DA turned out to be a simple and inexpensive method. Gels were first formed by photo crosslinking in the presence of various amounts of PL – all gels looked identically clear (data not shown). After rinsing off PL, the gels exhibited different transparencies (Fig. 3A). The plain PEG-DA gel maintained a transparent appearance in the whole process, whereas gels with addition and removal of PL experienced a change from transparent to opaque at different extents. The development of opaqueness, which should result from phase separation and/or increased number of pores/channels (Zawko and Schmidt, 2010), showed a positive correlation with the amount of PL. Thus the involvement of PL in gel formation led to a more loosely tied network morphology. This assumption was proved by SEM microscopy. Compared to the plain PEG-DA gel showing a spherical porous morphology, the gel treated by 17% PL was found to have larger pores with irregular shapes (Fig. 3B). The PL induced change in gel morphology meant that addition and removal of PL could reduce the internal diffusion resistance of gel matrix, which in turn would increase the diffusional transport of macromolecules through the gel. The stress–strain shown in Fig. 3C provided additional evidence that, compared to plain PEG-DA gel,

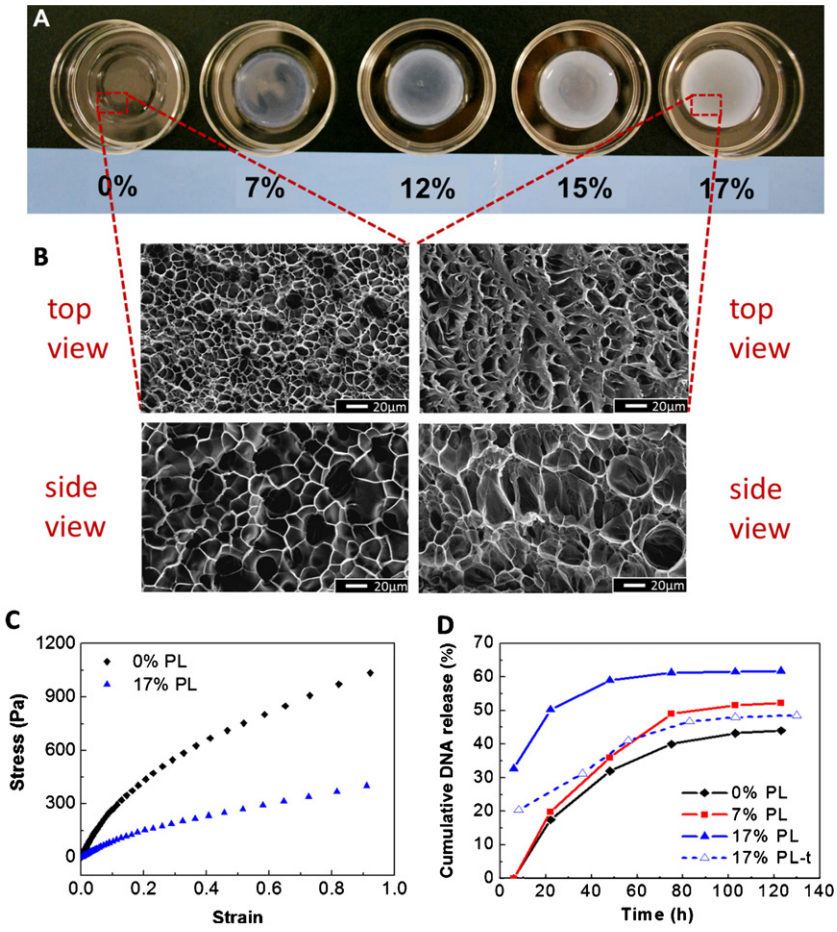

Fig. 3. (A) Appearance of PEG-DA gels after washing away all Pluronic F127 (PL) added to the gel solution at various concentrations before crosslinking. All gels were crosslinked at the same condition and appeared identically transparent before the PL was washed away. (B) Representative SEM images of 0% (left) and 17% (right) PEG-DA gels from top and side view. (C) Stress–strain curve of 0% and 17% PEG-DA gels. (D) Release of DNA from PEG-DA gels that were made out of 300 μl gel solution except 17% PL-t where 600 μl was used. Vector gel solution containing 4 μg DNA was loaded to the concave surface of each PEG-DA gel where it solidified as a vector gel at 37 °C.

a less condensed network was produced in the gel incorporating PL that was then removed. Responding to the same stress over the entire test range, the 17% PL gel displayed a significant larger strain than the 0% PL gel did, proving that it was formed as a softer gel with larger pore size and possibly crosslinked to a lesser degree. The diffusion test also showed data in agreement with these results. As shown in Fig. 3D, a total 62% of DNA payload was released from 17% PL gel and 52% from 7% PL gel, as compared to 44% from the plain gel in 120 h, demonstrating that PB-PL/DNA polyplexes were released faster through a PL treated gel. When the thickness of the gel was doubled (refer to 17% PL-t), the release of DNA showed a dramatic decrease with more DNA being sequestered in the gel matrix, probably due to the increased volume of small and/or non-interconnected pores. It has been reported that the mesh size of PEG-DA based gel fluctuated with the thickness of the gel as well as the depth of cross-section, and the center plane exhibited significantly smaller pores than top and bottom planes (Wu et al., 2010). However, the initial burst release only occurred with the two 17% PL gels as recorded at $t = 6$ h, implying removal of PL at this concentration might generate similar micro-channels to allow fast transport across the gel.

In addition to a desired release rate, the DNA also needs to be safely packaged in the released sample to allow effective transfection. The integrity of released polyplex was examined by a dye exclusion assay using Picogreen, an ultrasensitive double-stranded DNA dye that exhibits significant fluorescence enhancement upon intercalating DNA. The inaccessibility of DNA characterized by dye exclusion indicates the affinity between DNA and the pentablock copolymers. As shown in Table 1, all released polyplexes showed an average dye exclusion of about 80%, similar to the case of polyplex formulated in solution. Therefore, the PB-PL/DNA polyplex maintained the complexed structure during transport across the gel even after 4-day release and is thus expected to have the similar transfection ability as that seen with the control polyplex.

3.2. Release of DNA from molded PEG-DA gels

To make a PEG-DA gel with greater capacity for vector gel loading, we developed a well mold that can produce an indentation of desired shape and size on the top of gel (Fig. 1). Four factors were investigated for their influence on DNA release: *V* – the volume of gel solution (thickness of gel), UV – UV exposure time, DNA – amount of DNA in the vector gel and the shape of indentation. Fig. 4A shows release profiles of DNA from three 17% PL gels with flat-bottomed (FB) indentation. The duration of UV exposure

Table 1
Average dye exclusion of polyplexes released by various samples within first 4 days.

	Polyplex in buffer	Polyplex in released samples (first 4 days)		
		0% PL	7% PL	17% PL
Dye exclusion (%)	87 ± 2	76 ± 5	79 ± 5	84 ± 4

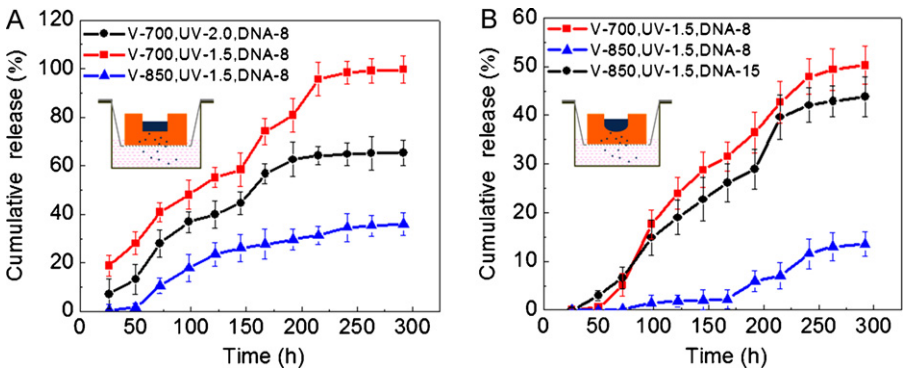

Fig. 4. DNA release from the PB-PL vectors through PEG-DA gels (17% PL) made out of flat bottomed mold (A) and round bottomed mold (B) in various conditions including: *V* – volume of PEG-DA gel solution in μl, UV – UV exposure time in min and DNA – amount of loaded DNA in μg. For instance, V-700, UV-2.0, DNA-8 implies that the gel was formed out of 700 μl PEG-DA solution by exposure to UV for 2 min, and subsequently loaded with vector gel containing 8 μg DNA. All samples were released to PBS buffer. For samples in (A), 8 μg DNA was added initially; for samples in (B), 1.5 min UV exposure was used for crosslinking.

plays an important role in gel permeability, as demonstrated by 100% DNA released from UV-1.5 gel (red-square curve) versus 65% released from UV-2 gel (black-circle curve). Longer exposure to UV light resulted in a higher crosslinking degree, and a more crosslinked network. Again, thicker gels showed greater resistance to vector diffusion, and this effect seemed to be remarkably significant in the molded gel. With all the other conditions being the same, V-850 gel (blue-triangle curve) led to only about 35% release of DNA as compared to 100% release from V-700 gel (red-square curve). Similar trends also occurred in gels with round-bottomed (RB) indentation (Fig. 4B). But interestingly, release from RB type showed an overall slower rate than the case of FB counterpart. V-850 gel only allowed release of 50% DNA (Fig. 4B, red-square curve) and V-700 only showed 14% release (Fig. 4B, blue-triangle curve). One possible reason for the thickness effect might relate to the additional sequestration of vector molecules by the side wall around the indentation. Besides diffusing straight down through the gel, the vector may also diffuse outward to the side wall and probably get entrapped somewhere during the subsequent transport. The loss of vector by sequestration might be greater in RB gel type, as its average thickness of the gel below indentation was larger and there was more area of side wall in contact with the vector gel. That is why a noticeably slower release is seen in RB gels as compared to FB gels. From this, we can believe that the real tumor shape may exert complex effects on release rate of polyplexes, which is quite difficult to predict accurately by *in vitro* studies. When loading twice the amount of vector on V-850 gel (Fig. 4B, black-circle curve), the release was increased from 14% to 44%, implying that the entrapped vectors did not block the effective pores to allow additional vectors to pass through. In addition to release into the buffer, samples were also allowed to release into cell growth medium through medium-soaked PEG-DA gels, where similar release profiles were found as seen with the release into buffer (data not shown).

Thus far, we have examined the influence of various factors on the release of vector from PEG-DA gels and found that the V-700, UV-1.5, FB gels can provide a relatively fast and steady release within 9 days, which make it appropriate for *in vitro* gene release study. The extracellular matrix (ECM) of the tumor acts as a potent barrier against the transport of biopharmaceuticals such as polyplex vectors and therapeutic proteins. There are numerous influencing factors limiting the diffusion of macromolecules through ECM, such as tumor type, the content of collagen (Pluen et al., 2001), and the amount of glycosaminoglycan (GAG) (Davies et al., 2002; Netti et al., 2000). Meanwhile, the dynamic nature of ECM and vasculature (Monsky et al., 1999) makes it extremely difficult to predict or mimic the real transport in the tumor matrix using synthetic gels. What we have presented here is an attempt to render a more practical experimental setup for *in vitro* sustained gene release with an inexpensive and easily implemented barrier gel.

3.3. *In vitro co-delivery of DNA and paclitaxel*

Besides gene delivery, we have also incorporated the anti-cancer drug paclitaxel (PTX) in the vector to make it able to deliver gene and drug simultaneously. Since PTX was encapsulated in the Pluronic F127 (PL) shield rather than the DNA condensing pentablock copolymers (PB), the drug loading was assumed not to induce any change in the interaction between DNA and PB. This assumption was tested by gel electrophoresis as shown in Fig. 5; DNA was compacted identically tightly in PTX loaded and PTX-free polyplexes. One of the advantages of combined delivery of gene and drug is the positive synergistic effect of drug on gene expression (Nair et al., 2002; Wang et al., 2006). Fig. 6 shows the influence of PTX on luciferase gene expression in cancerous SKOV3 and non-cancerous ARPE cells. As expected, the presence of PTX enhanced transfection efficiency, but to a different degree for each cell type with little dependence on the concentration of PTX studied. When 50% of PL shield was replaced with PTX loaded PL, SKOV3 showed up to 12-fold increase in luciferase levels as opposed to 3-fold increase seen in APRE-19 cells at N/P of 20. Considering the overall low level of gene expression in ARPE-19, especially for the control vector at N/P of 20 which showed only one order of magnitude higher than blank cells (data now shown), the effect of PTX in enhancing transfection was very limited in this cell type. We have previously reported that the PB-PL/DNA vector presents selective transfection in cancerous over non-cancerous cells due to greater lysosomal sequestration of DNA in the latter case because of differences in

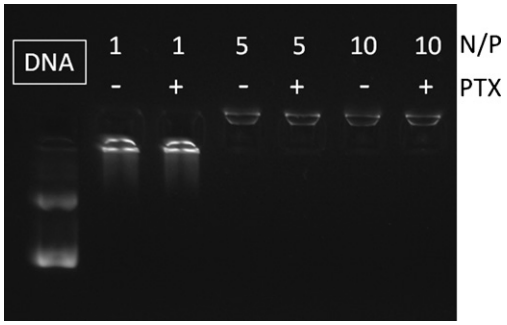

Fig. 5. Effect of loading PTX on the interaction of PB with DNA by agarose electrophoresis assay.

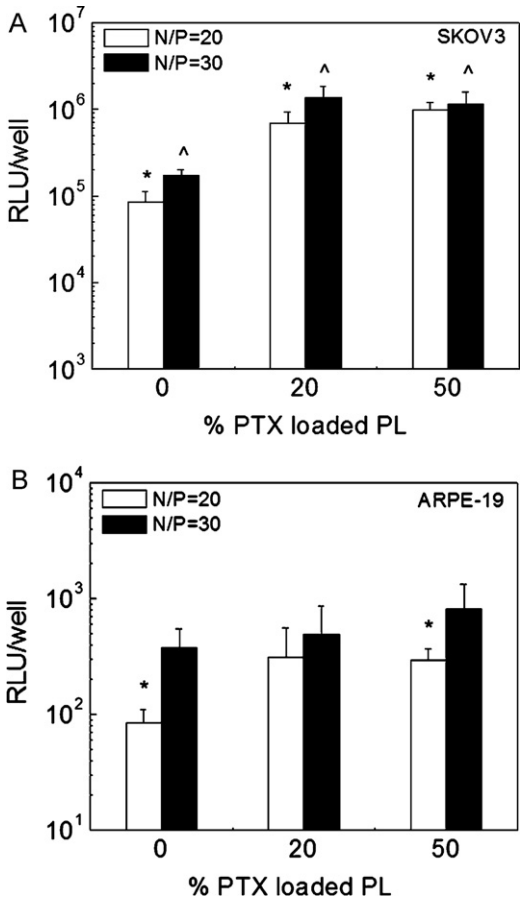

Fig. 6. Effect of PTX on luciferase based transfection efficiency of SKOV3 (A) and ARPE (B) cells mediated by PB-PL/DNA polyplex containing various percent of PTX loaded PL in the free PL shield; $n = 3$, $p < 0.01$. Significant differences were relative to conditions with no PTX added.

lysosomal pH values in cancer and non-cancer cells (Zhang et al., 2009; Zhang and Mallapragada, 2011a,b). Addition of chloroquine (CLQ), a transfection enhancer, resulted in significant higher fold-increase in APRE-19 cells when compared to SKOV3 cells (Zhang and Mallapragada, 2011b), which is contrary to what was seen here with PTX. CLQ restored the gene expression in ARPE-19 cells probably by overcoming the lysosomal barrier that served as a primary cause of low transfection, whereas PTX failed to so. The most direct reason should be the fact that PTX uses a different mechanism to improve gene expression, which is possibly related to its anti-mitotic function (Nair et al., 2002). Since PTX must get to microtubules in the cytosol to function through binding to the tubulin, entrapment in the endo/lysosomal vesicles would completely inhibit the function of PTX. Previous intracellular trafficking results indicate that there were a lot more polyplexes getting entrapped in the more acidic vesicles in non-cancerous cells relative to in the less acidic vesicles in cancerous cells (Zhang and Mallapragada, 2011b). Therefore, lack of enhanced gene expression in PTX treated APRE-19 cells might result from the endo/lysosomal entrapment. The entrapped particles could then be exported out of the cell by exocytosis as what has been reported about colloidal silica nanoparaticles (Park et al., 2006). Besides, the non-cancerous ARPE-19 cells could be more resistant to PTX in themselves compared to cancerous cells. A study on K858, an anti-mitotic agent that can induce similar mitotic arrest as PTX, showed that ARPE-19 cells were slightly affected by this agent relative to other cancerous cells (Nakai et al., 2009). In this sense, even if PTX could be released to the cytoplasm of ARPE-19 cells, its ability to enhance gene expression by arresting mitosis might be quite weak. In contrast, the SKOV3 cells, which have been found PTX sensitive (Duan et al., 1999), could benefit from PTX easily to gain an enhancement in gene expression. The similar effect of PTX on SKOV3 cells was also observed with transfection by DNA encoding EGFP reporter gene as shown in Fig. 7.

Cytotoxicity measurement further confirmed the above analysis about the selective enhancement of gene expression by PTX in SKOV3 and ARPE-19 cells. As shown in Fig. 8, both LDH and MTT methods showed PTX-induced additional cell death compared to the condition without PTX, indicating that PTX was released from polyplexes as a functional anti-cancer drug. Yet this effect of PTX was found to be more significant in cancerous SKOV3 cells as opposed to non-cancerous APRE-19 cells. As a result, the PTX induced enhancement on gene expression showed similar selectivity. Taken together, co-delivery of DNA with PTX clearly generated a synergistic effect demonstrated by the increased gene expression, and the original selective transfection presented in the PB-PL type of vector was retained and further enhanced in the co-delivery method.

3.4. In vitro sustained delivery of DNA and PTX to cells

With the above discussion about barrier gel and synergistic effect of drug and gene co-delivery, DNA and PTX were loaded in the PB-PL vectors simultaneously and transferred to PEG-DA gel for sustained release to SKOV3 cells. To examine the transfection ability of newly released polyplexes, the release setup was moved to fresh cells after every 24 h of incubation. In this way, we can investigate how the transfection efficacy of daily released vectors varies with time. As shown in Fig. 9, polyplexes released through PEG-DA barrier gel were still able to transfect cells as efficiently as the control vector that experienced no diffusion process, but with transfection efficiency decreasing over time. This might be due to formation of large aggregates of polyplexes during the prolonged incubation with cells in serum containing medium. From the release profile shown in Fig. 4A, the amount of DNA released to cells were found to be around 1.4 μg, 0.8 μg, 1.0 μg and 0.6 μg, respectively, for the first 4 days. The first day's release was the largest amount of DNA with the expected smallest vector size, which should together lead to the highest gene expression as seen in Fig. 9. The transfection efficiency for the released

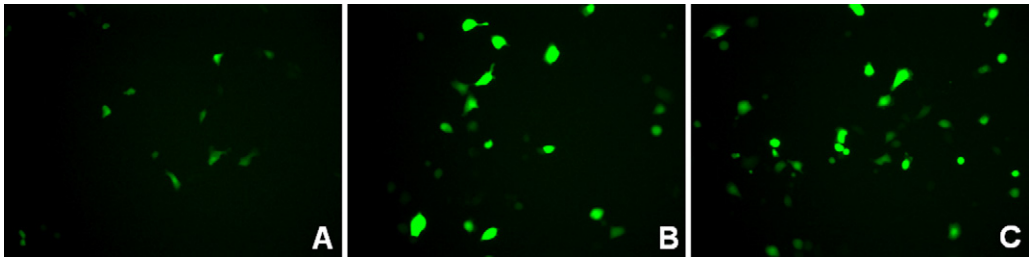

Fig. 7. EGFP expression in SKOV3 cells transfected with PB-PL/DNA at N/P = 20, with free PL shield composed of 0% (A), 20% (B), 50% (C) PTX-loaded PL.

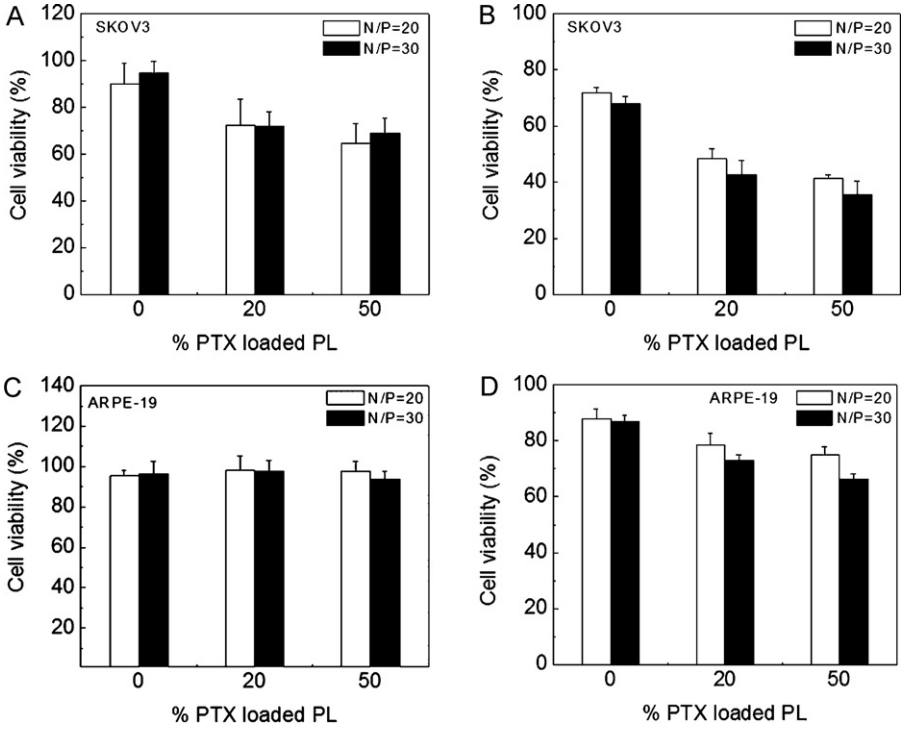

Fig. 8. LDH-based (A and C) and MTT-based (B and D) cell viability of PB-PL/DNA polyplex formulated with various percentages of PTX loaded F127 in the free Pluronic F127 (PL) shield on SKOV3 (A and B) and ARPE-19 (C and D) cells; $n = 3$.

vector from days 2 to 4 showing a decreasing trend that might be dominantly affected by vector size rather than the total amount released. With co-packaging of PTX, however, transfection efficiency was significantly enhanced along the release time course as opposed to single delivery of DNA. Though the high level of gene expression did not persist for a very long time, the enhancing effect of PTX on transfection and the toxicity of PTX as an anticancer drug were found to have been well maintained after release through the barrier gel. Thus, this sustained synergistic effect of co-delivery of PTX and DNA can be expected for similar *in vivo* studies. Release conditions need to be further optimized to achieve a longer duration of high transfection efficiency. Together with the potential selectivity of this system for cancerous over non-cancerous cells, the self-assembling pentablock copolymers hold a promise as an injectable vector for controlled drug and gene co-delivery to tumors.

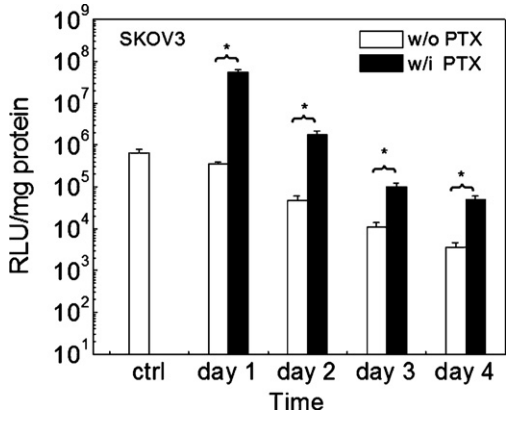

Fig. 9. Transfection in SKOV3 cells mediated by PB-PL/DNA polyplexes containing PTX-loaded or PTX-free PL shield on PEG-DA barrier gels; $n = 3$, $p < 0.01$.

4. Conclusions

PL-treated PEG-DA gels were developed as a simple and controlled transport barrier to simulate the tumor matrix in an *in vitro* release study on injectable PB based vectors. Various influencing factors for vector release rate were investigated and optimized. It was found that nearly 100% of the vector could be released through the optimal PEG-DA gel without losing the polyplex complexation. Paclitaxel (PTX) was co-packaged in the PB-PL type of vectors together with DNA to construct a dual delivery system. Sustained co-delivery of PTX and DNA to SKOV3 cells showed significantly enhanced gene expression as compared to just the DNA delivery. Daily released vectors maintained function after diffusion through the PEG-DA gel, but subject to a decreasing transfection efficacy within 4 days at current conditions. Further efforts will be made to improve the duration of high gene expression.

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