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Abstract

Sustained vaginal delivery of siRNA has been precluded by the mucosal barrier lining the vaginal tract. In contrast to prior reports, we showed that conventional lipoplexes administered intravaginally are unable to reach vaginal epithelium under normal physiological conditions. Here we have developed a novel alginate scaffold system containing muco-inert PEGylated lipoplexes to provide a sustained vaginal presence of lipoplexes *in vivo* and to facilitate the delivery of siRNA/oligonucleotides into the vaginal epithelium. These PEGylated lipoplex-entrapped alginate scaffolds (PLAS) were fabricated using a freeze-drying method and the entrapment efficiency, release rate, and efficacy were characterized. We demonstrated that these PLAS system had an entrapment efficiency of ~50%, which released PEGylated lipoplexes gradually both *in vitro* and *in vivo*. While the presence of alginate diminished the cell uptake efficiency of PEGylated lipoplexes *in vitro*, as expected, we showed a six-fold increase their uptake into the vaginal epithelium compared to existing transfection systems following intravaginal administration in mice. A significant knockdown of Lamin A/C level was also observed in vaginal tissues using siLamin A/C-containing PLAS system *in vivo*. Overall, our results indicated the potential of the biodegradable PLAS system for the sustained delivery of siRNA/oligonucleotides to vaginal epithelium.

Key words: Vaginal delivery, gene, liposomes, alginate, scaffold

Abbreviations

A:L, Alginate:Lipid; Bp, Base pair; DNA, Deoxyribonucleic acid; DOTAP, Dioleoyl trimethylammonium propane; FACS, Fluorescence activated cell sorting; HIV, Human immunodeficiency virus; LP, Lipoplexes; MW, Molecular weight; N-9, Nonoxynol-9; Oligo, Oligonucleotides; PEG, Polyethylene glycol; PLAS, PEGylated lipoplex-entrapped alginate scaffold; RNA, Ribonucleic acid; SEM, Scanning electron microscope; SD, Standard deviation; SEM, Standard error of the mean; siRNA, Small interfering RNA.

1. Introduction

Vaginal application of nucleic acids holds great potential for the prevention and treatment of various viral infections responsible for diseases such as genital herpes, acquired immune deficiency syndrome (AIDS) and cervical cancer (Reviewed in [1]). Vaginal administration is non-invasive, bypasses first pass hepatic clearance and provides local delivery to the target tissue and has been described in several pre-clinical trials using plasmid DNA for vaccination [2] or small interfering RNA (siRNA) for prevention of herpes simplex virus (HSV) infections [3]. For DNA vaccination, plasmid DNA needs to be delivered to the immune cells in the mucus to induce the desired immune response [4]. In contrast, vaginal treatment of cancer or viral infections by gene silencing is more challenging as it requires the siRNA molecules to be delivered through the mucosal barrier, avoid rapid nuclease degradation, and be taken up by the cervicovaginal epithelium.

Mucus presents as a one of the biggest hurdles for efficient vaginal siRNA delivery. It serves as a protective barrier for underlying tissues and removes foreign particles efficiently [5]. Thus, sustained release at the mucosal site, though desirable, is challenging. In addition, the changes in the physical environment in the vaginal cavity throughout the estrous cycle could also dramatically affect the delivery efficiency [2]. To overcome these barriers, strategies such as the use of progesterone or mucus removal in the vaginal cavity prior to siRNA administration have been examined with success in the past by using liquid-based formulations such as cationic transfection reagents [3], cholesterol-conjugated siRNA [6], or biodegradable poly(lactic-co-glycolic acid) (PLGA) nanoparticles [7]. However, a solid vaginal siRNA delivery system which can be retained in the vaginal cavity following administration without prolonged anaesthesia is yet to be developed. Such a system could significantly improve clinical outcome. Thus, in this present study, we aimed to develop a more clinically applicable vaginal siRNA delivery platform which cannot only be retained in the vaginal cavity following administration but also delivers siRNA efficiently to vaginal tissues under normal physiological conditions.

In this report, we described a novel PEGylated Lipoplex-entrapped Alginate Scaffold (PLAS) system which brings together a muco-inert non-viral gene delivery vector, to avoid trapping and clearance by the mucus barrier, and a biodegradable alginate tissue-engineered scaffold which provides continuous presence of the siRNA in the vaginal cavity. Alginate is a naturally–occurring polysaccharide which is readily cross-linked by the use of divalent cations such as calcium, into a solid matrix without the use of organic solvents [8, 9]. Alginate scaffolds decompose in the presence of sodium ions, naturally occurring in the body, and can thus release entrapped therapeutics slowly over time [10]. While its use in gene delivery has been demonstrated in the forms of microspheres [8], poly-ionic complexes [11-13] or hydrogel [9], here we investigated for the first time the feasibility of entrapping PEGylated lipoplexes within a solid alginate scaffold system and examined its potential for sustained delivery of nucleic acids to the female reproductive tract. We investigated the entrapment efficiency, release kinetics of lipoplexes at different formulation conditions, as well as the efficiency of the released lipoplexes to be taken up by cells both *in vitro* and *in vivo*. To our knowledge, this is the first report which describes the use of biodegradable matrices for vaginal delivery of nucleic acids.

2. Materials and Methods

2.1. Materials

Dioleoyl trimethylammonium propane (DOTAP) and cholesterol were purchased from Sigma (St Louis, MO) and dioleoyl phosphatidylethanolamine (DOPE) was obtained from Northern Lipids (Vancouver, Canada). Polyethylene Glycol $(PEG)_{2000}$-C16Ceramide conjugate was from Avanti Polar Lipids (Alabaster, AL). Sodium alginate was purchased from Concept Biotech Inc (CBI, Taichung, Taiwan).

Oligonucleotides (5'-GTCAGAAATAGAAACTGGTCATC-3'; 5'-GATGACCAGTTTCTATTTCTGAC-3') with or without fluorescein isothiocyanate (FITC) labeling were obtained from Invitrogen (Carlbad, CA). Green fluorescent protein (GFP) targeted siRNA with sense sequence of 5'-GCACGACUUCUUCAAGUCCUU-3'was purchased from Sigma-Aldrich (St Louis, MO) in annealed form. Lamin A/C targeted siRNA with sense sequence of 5'CUGGACUUCCAGAAGAACAdTdT-3' and non-targeting siRNA with sense sequence of 5'-UUAUGCCGAUCGCGUCACAUU-3' were obtained from GenePharma (Shanghai, China).

HeLa cells were obtained from the American Type Culture Collection (Manassas, VA) and GFP-expressing HeLa cells were prepared according to protocols described in Gu *et al* [14]. All cells were maintained in 0.2% primocin (Invivogen, San Diego, CA) containing Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% heatinactivated fetal bovine serum (FBS; Bovogen, Keilor East, Australia) and 2mM L-glutamine (Invitrogen).

All other chemicals and solvents used were analytical grade.

2.2. Liposome Formulations

Non-PEGylated liposomes were prepared using a thin film hydration method as described previously [15]. Briefly, a dried lipid thin film consisting of DOTAP, cholesterol and DOPE (1:0.5:0.5 molar ratio) was hydrated with sterile 5% dextrose solution to give a final total lipid concentration of 5 mM. Following stabilization at room temperature for two hrs, liposomes were extruded through a series of Nucleopore^{TM} track-etched membranes using a Lipex^{TM} extruder (Northern lipids) under nitrogen pressure. The resulting small unilamellar liposomes (<100 nm) were subsequently complexed with oligonucleotides at an Nitrogen:Phosphate (N:P) ratio of 4.

PEGylated liposomes were prepared using the hydration of freeze-dried matrix (HFDM) method as described previously [16]. DOTAP, cholesterol, DOPE and PEG_{2000}C16Cermide with a molar ratio of 50:35:5:10 was used. The final freeze dried matrix was hydrated with sterile water and the final mixture contained 40 μg of oligonucleotides in 300 μL of isotonic sucrose solution.

2.3. Preparation of PEGylated Lipoplex-entrapped Alginate Scaffold (PLAS) Systems

2.3.1. Fabrication of Alginate Scaffolds, Characterisation, and Surface Morphology

Two different procedures were used to fabricate the alginate scaffolds: freeze-dried and air-dried methods. For the freeze dried method, 2% alginate (Concept Biotech Inc) solution was prepared using sterile isotonic dextrose solution. The mixture was then pipetted into plastic molds (2.2 cm x 2cm in size) and gradually frozen to around -80°C and subsequently dried in a lyophilizer (ALPHA 1–2 LDplus, Martin Christ, Germany) overnight. The dried matrix was subsequently cross-linked with calcium using 5% $CaCl_2$ solution and was then left at room temperature overnight. For the air-dried method, the frozen alginate block was immersed in 5% $CaCl_2$ solution without being lyophilised until the scaffold structure was formed completely. The scaffold system was then allowed air-dried overnight at room temperature. The dimensions of the scaffolds were measured before and after wetting with water. To examine the surface morphology, the scaffolds were attached to aluminium stubs using carbon tabs. The scaffolds were subsequently sputter coated with platinum using a Metaserv automatic mounting press and micrographs of the scaffolds were obtained using a Scanning electron microscope (SEM, JEOL JSM-7001F) at a voltage of 10 kV.

2.3.2. Entrapment Efficiency of PEGylated Lipoplexes within the Scaffold System

PEGylated lipoplexes were entrapped into the alginate scaffold system at alginate:lipid (A:L) ratios of 5:1 to 10:1 (*w*/*w*). Briefly, PEGylated lipoplexes, containing 20 to 80 μg oligonucleotides, were

gently mixed with the alginate solution by pipette and the resulting mixtures was added to 5 mL tubes and were slowly frozen. The scaffold system was then fabricated using the freeze-dried method described above. The entrapment efficiency of the PEGylated lipoplexes within the scaffold system was calculated by subtracting the amount of oligonucleotides lost in the $CaCl_2$ solution during the cross-linking step from the initial amount of oligonucleotides added in the formulation. The concentration of oligonucleotides in the $CaCl_2$ solution was determined using Quant-iT™ PicoGreen™ reagent (Invitrogen) following removal of the alginate scaffold. Briefly, the samples were diluted in 5% $CaCl_2$ solution to a concentration within the linear range of the standard curve (1-1,000 ng/mL, in $CaCl_2$ solution). Samples were then treated with 0.5% Triton-X 100 (Sigma), which destabilized the lipoplexes to release entrapped oligonucleotides. One hundred μL of diluted PicoGreen reagent (1:200 dilution) was then added to 100 μL samples in a 96-well plate according to the manufacturer's instruction. Fluorescence intensity was subsequently measured using a Fluostar™ plate reader at 485 nm excitation and 520 nm emission wavelengths. Sample concentrations were subsequently calculated using the standard curve. Three batches of samples were tested for each formulation condition (n=3) and the assay was performed in duplicates.

2.3.3. Particle characterisation

Particle size of the PEGylated lipoplexes was determined following lyophilisation in the presence of alginate. Formulations with alginate:lipid ratios of 6 and 8 were examined. Following hydration of the freeze-dried alginate scaffolds, the samples were centrifuged at 5000g for 10 mins to remove still-yet-to-be degraded alginate scaffold and the percentage of siRNA remaining in the supernatant was determined using Picogreen™ assay as described above. The size of the released lipoplexes was subsequently measured using Zetasizer Nano ZS™ (Malvern Instruments, Malvern, UK) as previously described [16]. Three or four batches of samples were examined for each formulation (n=3 or 4).

2.3.4. Release Kinetics

To determine the release rate of lipoplexes from the cross-linked alginate scaffold, formulations containing 10-15 μg of oligonucleotides were immersed in 5 mL of phosphate buffered saline (PBS) or citrate buffer (150 mM, pH 5.0). Citrate buffer was prepared at a sodium concentration of 6 g/L. The lipoplex scaffolds were prepared at alginate:lipid ratios ranging from 5:1 to 30:1. Samples were incubated at 37°C with gentle shaking (70 rpm) and the release of lipoplexes was monitored over time. At each time point, 250 μL of the samples was collected and the volume was replaced with fresh buffer for continued monitoring of the release. The concentration of the released oligonucleotides was determined using the Picogreen™ assay kit as described above. Standard curves (1 -1,000 ng/mL) were generated in citrate buffer and PBS. Three batches of alginate scaffold were prepared for each experiment and all assays were performed in triplicate.

2.3.5. In vitro Efficacy

To examine the influence of alginate on the uptake efficiency of PEGylated lipoplexes into cells or the gene silencing efficiency of siRNA, FITC-labeled oligonucleotides or GFP-targeted siRNA were incorporated into the PEGylated liposomes. The PEGylated lipoplex-entrapped alginate scaffold (PLAS) systems were prepared at alginate:lipid ratios ranging from 5:1 to 10:1. HeLa or GFP-expressing HeLa cells were seeded in 6-well plates 24 hrs prior to transfection at a density of 100,000 cells/well. The scaffold system containing 20 μg of FITC-oligonucleotides or GFP-targeted siRNA was hydrated with 1.5 mL PBS and the samples were then allowed to stabilize at room temperature for 2 hrs prior to addition to cells. Cells were incubated with 2 mL of media containing 2.6 μg of FITC-oligonucleotides/GFP siRNA overnight for cell uptake or 48 hrs for gene knockdown. PEGylated lipoplexes and alginate-only samples which contained the highest amount of alginate used in the experiment were both included in the experiment as a positive and a negative

control, respectively. Oligofectamine™ was also used according to the manufacturer's instructions. Final oligonucleotide/siRNA concentration for all formulations was 100nM. At the termination of the experiment, cells were trypsinised and resuspended in 2% paraformaldehyde (Sigma-Aldrich) in PBS. Samples were kept at 4°C in the dark before the fluorescence intensities of cells being quantified by BD-FACSCanto™ flow cytometry. All formulations were tested in triplicate (n=3).

2.4. Animals, Nucleic Acid Preparation/Administration, and Efficacy Assessment *in vivo*

K14E7 (10 week-old) and C57BL/6 (8 week-old) female mice were obtained from ARC (Perth, Australia). All experiments were approved by the University of Queensland Animal Ethics Committee. All uptake and siRNA delivery efficiency experiments were performed using C57BL/6 mice while paraffin sections from the vaginal tissues of K14E7 mice were used to assess the changes in the physical environment in the vaginal cavity during mice estrous cycle.

All formulations were administered intravaginally into Penthrox™ (Medical Developments International, Victoria, Australia) or ketamine (Parnell laboratories, New South Wales, Australia) and xylazil (Troy laboratories, New South Wales, Australia) anaesthetized mice with or without citric acid (5%) or nonoxynol-9 (N-9, Conceptrol™, Ortho Options) pre-treatment using a Gilson™ p20 pipette. Citric acid pre-treatment was performed similar to the procedure described previously [2]. Briefly, a cotton ball soaked with 5% citric acid aqueous solution was inserted into the vaginal tract for 2 hrs prior to nucleic acids treatment. Conceptrol™ gel which contains 4% N-9 was applied to the vaginal cavity 6 hrs prior to lipoplexes administration [17]. Vaseline™ was also applied at the vaginal introitus to prevent leakage for liquid-based formulations. For alginate scaffold system, freeze-dried scaffolds with or without PEGylated lipoplexes were administered intravaginally into mice using a pair of forceps. Eight µL of sterile saline was subsequently instilled into the vaginal cavity to help moisturising the scaffold. For all experiments, two to three mice were used per treatment group.

For the uptake study, FITC-labeled oligonucleotides (500-4,000 pmol) were incorporated into various lipid-based formulations including Lipofectamine™, cationic DOTAP liposomes, or PEGylated lipoplex-entrapped cross-linked alginate scaffold system. All formulations were prepared according to manufacturer instructions or as described earlier. For preparation of alginate scaffold system, alginate:lipid ratio of 8:1 was used and 2,000 pmol of FITC-Oligo was incorporated into the system. Lipofectamine™ (2 µL, Invitrogen) was used to complex with 500-4,000 pmol of FITC-Oligo, as previously described [18]. For DOTAP liposomal formulations (contained 500-4,000 pmol of FITC-Oligo), complexes were formed in a concentration not to exceed 80 µg/mL before being concentrated to 12 µL using a 30K Ultra-free centrifugation filter (Millipore, New South Wales, Australia) immediately prior to administration into mice. At 24 hrs post-administration, cervical and vaginal tissues were dissected and were immersed in Tissue-Tek O.C.T. Compound (ProSciTech, Queensland, Australia). The tissues were then snap-frozen in an ethanol/dry ice bath. Frozen sections (8 µm) were cut using a Leica CM 1850 cryostat (Leica Microsystems, Wetzlar, Germany) and were mounted on slides. Sections were subsequently stained with propidium iodide (PI, 3.3 µg/mL) in Vectashield™ for co-localisation of FITC-oligonucleotides before viewing using a Zeiss Axioskop 2 Plus fluorescence microscope. Percentages of co-localisation (% Area) were calculated in 5 randomly selected areas for various treatment groups using ImageJ™.

For target-gene knockdown experiment, Lamin A/C targeted siRNA was used. Each mouse received approximately 600pmole (8µg) of siRNA formulated in the PLAS system (alginate:lipid ratio of 8:1) per dose. Two doses were administered intravaginally for each mouse on two consecutive days and the mice were sacrificed 24 hrs following the last treatment. Three mice were

used in each treatment group (n=3). Proteins were subsequently extracted from vaginal tissues and Lamin A/C levels were assessed for each specimen via western blotting using LaminA/C antibodies (1:1000, Cell Signaling Technology, Danvers, MA, USA). β-tubulin (1:1000, Sigma) was used as a loading control. Lamin A/C levels were quantified using ImageJ.

2.5. Paraffin-embedded tissue sections

Haematoxylin and eosin staining of the paraffin-embedded tissue sections from K14E7 mice was performed by the University of Queensland histological service.

2.6. Statistical Analysis

Data are presented as mean or mean ± standard error of the mean (SEM). An unpaired Student's t-test (two-tailed, $p<0.05$) was used to determine statistically significant differences.

3. Results

3.1. Conventional liposomes fail to deliver nucleic acids into vaginal tissues

We first sought to evaluate several commonly used transfection agents, Lipofectamine™, Oligofectamine™, or DOTAP-containing liposomes, for their effectiveness in delivering oligonucleotides into the vaginal tissues using previously described techniques [3, 18]. To determine uptake into vaginal tissues, we used 23 bp DNA oligonucleotides, fluorescently tagged with FITC, as a model molecule for siRNA. As physical changes in the vaginal cavity throughout the estrous cycle can significantly affect the delivery of bioactives into vaginal tissues [2, 19], we first investigated its impact on lipoplexes. Mice were monitored for their estrous cycle via daily examination of the outer vaginal tissues, with red and swollen tissues indicating estrous or proestrous. Consistent with previous findings [2, 19], changes in mouse vaginal anatomy were observed at different phases of the cycle, with the outer layer of the epithelial cells becoming cornified and hyperkeratotic when mice were in estrous (Fig. 1). To examine the effect of the cornified layer on the delivery of lipoplexes, DOTAP liposome-complexed FITC-labelled oligonucleotides (500-4,000 pmol) were administered intravaginally when mice were in estrous. Cervicovaginal tissues were dissected 24 hrs post-treatment and frozen sections were stained using propidium iodine to identify vaginal epithelium. Lipoplexes administered at this stage of the estrous cycle resulted in strong uptake by cell debris present in the vaginal tract, however, no delivery was detected in the cervicovaginal epithelium (Fig. 2A). Similarly, Lipofectamine™ or Oligofecatamine™ were unable to deliver oligonucleotides into the vaginal epithelium of mice in estrous, even at higher dosages (up to 4,000 pmol, data not shown). We next sought to determine the oligonucleotide delivery efficiency in non-estrous mice using the same vectors. Disappointingly, despite extended sedation of mice (1 hr) to prevent leakage of the administered complexes, no uptake of the complexes into vaginal tissues at 24 hrs post-treatment was observed (Fig. 2B). This was observed irrespective of the dose of lipoplexes administered (500-4,000 pmol FITC-oligonucleotides) or the delivery vectors used, Lipofectamine™ (Fig. 2B) or DOTAP liposomes (data not shown).

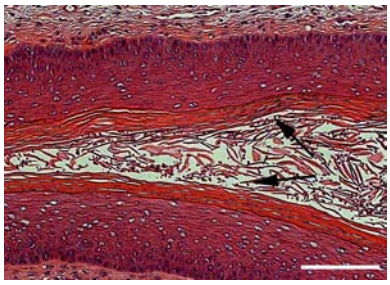

Figure 1 Hematoxylin and eosin staining of mouse cervicovaginal section in estrous. Outer layer of the epithelial cells become cornified (arrows) when mice enter into estrous which creates a physical barrier for the effective delivery of bioactives into the epithelium. The scale bar represents 200 μm. [Colour in print].

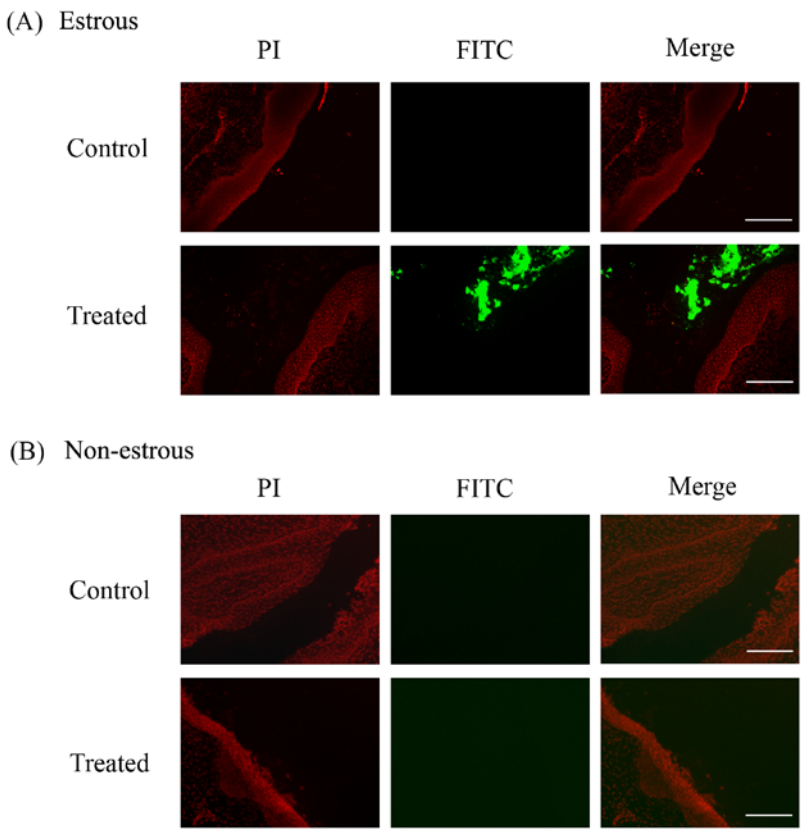

Figure 2 Intravaginal delivery of lipoplexes. (A) No delivery of FITC-Oligonucleotides to the vaginal tissues was observed when DOTAP liposome-oligonucleotides complexes (500 pmol) were administered in estrous/proestrous state of the mouse estrous cycle. In contrast, these complexes were taken up efficiently by cell debris present in the vaginal tract. (B) No delivery of Lipofectamine™-complexed FITC-oligonucleotides (500 pmol) into vaginal tissues was observed even when the administration was performed when mice were not in estrous. All images were obtained at twenty-four hrs post-treatment and frozen tissue sections were stained with propidium iodide (PI) in Vectashield™. The scale bars represent 200 µm. [Colour in print].

3.2. Citric acid pre-treatment of the vaginal cavity improves the delivery of liposome-complexed oligonucleotides

To enhance the delivery efficiency, we sought to pre-treat the vaginal cavity to improve its receptiveness to the delivery vectors. Pre-treatment of the vaginal cavity with nonoxynol-9 (N-9) for 6 hrs has been shown to increase susceptibility to infection of human papilloma virus (HPV) due to disruption of the stratified genital epithelium [17]. However, we found that pre-treatment with N-9 did not result in an improvement in the delivery efficiency of lipoplexes to vaginal tissues (data not shown). In contrast to N-9, pre-treatment with 5% citric acid for 2 hrs resulted in the delivery of lipoplexes into vaginal tissues, as shown by the presence of FITC-oligonucleotides within the vaginal tissue (counterstained with PI, Fig. 3). The improved delivery may be a result of the citric acid inducing a transient and reversible opening of the tight junction in the vaginal epithelium [19]. However, in contrast to the study reported by Kanazawa and colleagues, where a significant uptake of naked plasmid DNA was achieved following electroporation in citric acid pre-treated mice [2], we demonstrated that the delivery of oligonucleotides using conventional DOTAP-containing liposomes was still minimal under this treatment condition suggesting that these liposomes may not be able to mediate a clinically significant siRNA-derived therapeutic response intravaginally.

3.3. Development of PEGylated lipoplex-entrapped alginate scaffold (PLAS) system

The inefficient delivery of lipoplexes in the vaginal tissues was likely due to (1) the lack of retention of the lipoplexes in the vaginal cavity following administration and/or (2) inefficient transport of these lipoplexes through the mucus layer lining the cervicovaginal epithelium even following citric acid treatment. Mucus is primarily composed of mucins, which can interact extensively with cationic liposomes [20] through its heavily glycosylated regions [21]. PEGylation has recently been reported to enhance particle stability in the presence of mucin [22] and

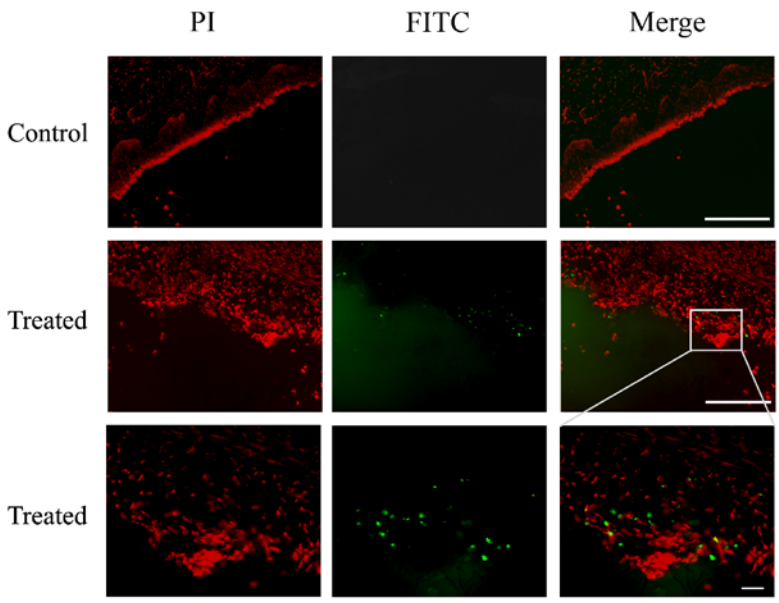

Figure 3 Citric acid pre-treatment improved the vaginal delivery efficiency of lipoplexes. Some delivery of FITC-Oligonucleotides was observed in the vaginal tissues following lipoplexes administration when the vaginal cavity was pre-treated with 5% citric acid for 2 hrs. The bottom panel shows magnification of the area outlined in the middle right image. The oligonucleotides (2,000 pmol) were delivered using DOTAP liposomes and no fluorescein autofluorescence was detected in the control animal (treated with 5% citric acid only). All images were obtained at twenty-four hrs post-treatment and frozen tissue sections were stained with propidium iodide (PI) in Vectashield™. The scale bars represent 200 µm (top two panels) and 20 µm (bottom panel). [Colour in print].

significantly increase the transport rate of biodegradable nanoparticles in human cervical mucus [23]. Based on this, and to address these two issues, we formulated PEGylated liposomes to facilitate the transport of oligonucleotides across cervicovaginal mucosal layer and thus enhance epithelial cell uptake. The physical characteristics of these particles were presented in Wu *et al* [16]. Additionally, we fabricated alginate scaffolds to entrap the PEGylated lipoplexes in order to facilitate greater retention of these particles in the vaginal cavity following intravaginal administration. We subsequently investigated the effect of various formulation methods and parameters on physical scaffold characteristics, entrapment efficiency, release kinetics, and efficacy.

3.3.1. Scaffold characteristics

Alginate scaffolds were fabricated using either a freeze-dried or an air-dried method (Fig. 4). For the freeze-dried method, the water crystal formed during the freezing step was removed by sublimation giving rise to porous structure (Fig. 5A). This was in direct contrast to the scaffold prepared using the air-dried method where the space which the water crystal occupied collapsed during the drying step. The resultant scaffold was therefore less porous (Fig. 5B), which may not allow efficient entry of the vaginal fluid (which can be as low as 2mL/day in humans [24]). Additionally, the alginate scaffold prepared using the freeze-dried method expanded less upon exposure to water (22%) as compared to the air-dried method (50%) (Fig. 5C, $p<0.005$). Since the greater expansion of the air-dried scaffold system could potentially result in vaginal discomfort following application, the freeze-dried fabrication method was thus employed for all our subsequent experiments.

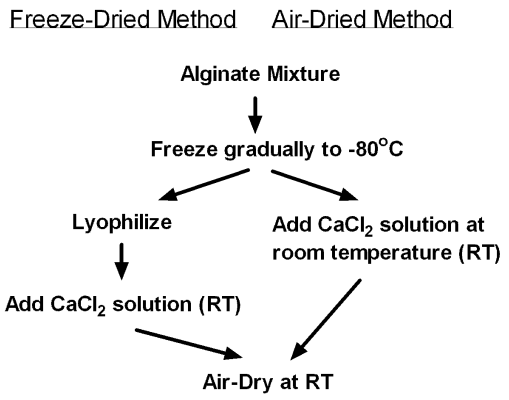

Figure 4 Flow chart of the fabrication of alginate scaffolds via freeze-dried and air-dried methods.

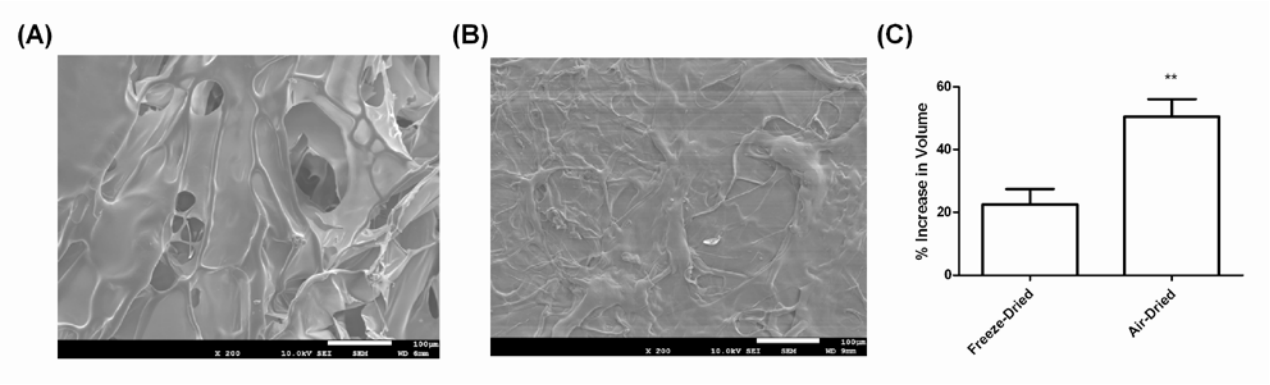

Figure 5 Characteristics of the alginate scaffold prepared using either the freeze-dried or the air-dried method. SEM image of the scaffold fabricated using the freeze-dried method (A) showed more porous structure compared to ones prepared using the air-dried method (B). (C) Comparison of the percentage of expansion in volume when the scaffolds prepared using these two different methods were moisturised with water. Bars represent the mean value (n=6-8) and error bars represent the corresponding SEM. **p<0.005, compared to freeze-dried method.

3.3.2. Entrapment efficiency, particle characterisation, and release kinetics

We next investigated the efficiency of incorporating PEGylated liposomes into alginate scaffold using various alginate:lipid (*w*/*w*) ratios. PEGylated liposomes were formulated using the Hydration of the Freeze-Dried Matrix (HFDM) method we previously developed, where entrapment efficiency of oligonucleotides was found to be over 90% [16]. The lipoplexes were then mixed with the alginate solution prior to the freeze-drying step (Fig. 4). Importantly, the efficiency of the HFDM-formulated lipoplexes to deliver siRNA *in vitro* was not diminished after being subject to freeze drying process twice (data not shown). The entrapment efficiency of these lipoplexes within the scaffold was determined by subtracting the amount of oligonucleotides removed by the $CaCl_2$ solution used to cross-link the scaffold device from the initial amount. We found that the entrapment efficiency increased with increasing amount of alginate used, with 50% entrapment efficiency achieved at alginate:lipid ratios of 8:1 or 10:1 compared to 40% when formulated at alginate:lipid ratios of 5:1 or 6:1 (Fig. 6A, $p<0.005$ and $p<0.05$ for 8:1 and 10:1 formulations, respectively). We next examined the impact of alginate on the particle size of PEGylated lipoplexes following lyophilisation. PLAS systems formulated with alginate:lipid ratios of 6 and 8 were examined. Following hydration and centrifugation at 5000g for 10 mins to remove still-yet-to-be degraded alginate scaffold, more than 80% of the siRNA still remained in the supernatant, which allowed us to objectively assess the particle size of the released lipoplexes. The average particle size of the lipoplexes was found to be 300.68 (SD=58) nm and 368.93 (SD=53) nm for formulations prepared at alginate:lipid ratios of 6 and 8, respectively. The polydispersity indexes (PI) were around 0.4 for both formulations. It must be noted that the particle size observed for these alginate scaffold-embedded lipoplexes is considerably larger than "free" lipoplexes (<200nm) and could be contributed by the association of alginate on the surface of these liposome particles.

While the pH of the vaginal tract in mice is around 6.5 [25], the release kinetics were assessed using with a slightly more acidic citrate buffer (pH5) to mimic the pH of human vaginal tract (pH ~4) [26, 27]. As shown in Fig. 6B, the liposome-alginate scaffold formulated at an alginate:lipid ratio of 10:1 exhibited a slightly slower release rate with 25% of the lipoplexes being released at 1 hr following incubation as opposed to 40% for formulations prepared at lower alginate:lipid ratios (5:1 – 8:1). This amount of lipoplex release (25%, 1 hr, *w*/*w* 10:1) was also found to increase significantly to 50% when the sodium content in citrate buffer was increased by 40% at the same pH (data not shown), suggesting that the release rate is dependent on the rate of calcium-sodium exchange in the alginate system. Importantly, given that the sodium content in the vaginal fluid is

>4 times less than the amount of sodium contained in citrate buffer used here [24] (Fig. 6B), one would expect that the release rate of lipoplexes to be much slower *in vivo*. Indeed, when PBS (contained 40% less sodium than citrate buffer) was used, only 10% of the lipoplexes were released following 24 hrs of incubation as opposed to 60% in citrate buffer for formulation prepared at alginate:lipid ratio of 30:1 (data not shown).

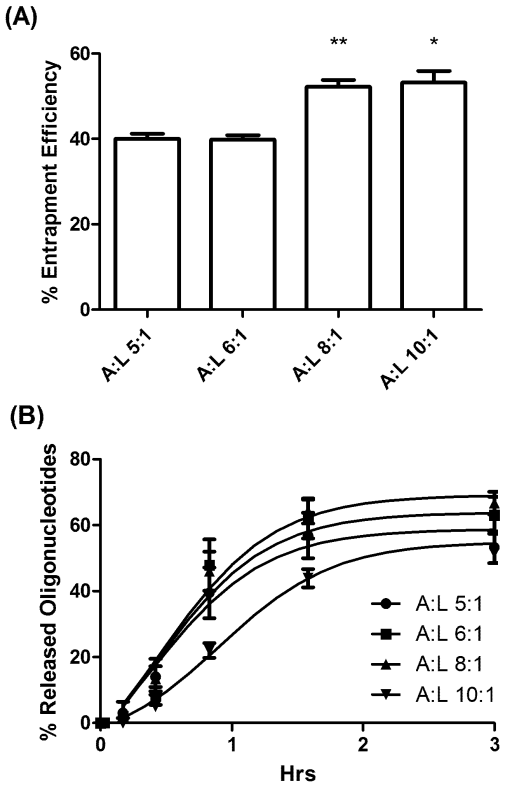

Figure 6 Entrapment efficiency and release kinetics of the PEGylated lipoplex-entrapped alginate scaffold (PLAS) system. (A) The entrapment efficiencies of PEGylated lipoplexes within the alginate scaffold for samples prepared at alginate:lipid (A:L) ratios ranging from 5:1 to 10:1. Bars represent the mean value (n=3) and error bars represent the corresponding SEM. *p<0.05, **p<0.005, compared to the formulation prepared at an A:L ratio of 5:1. (B) The release of PEGylated lipoplexes from the scaffold systems over time in citrate buffer (pH 5) for formulations prepared at A:L ratios ranging from 5:1 to 10:1.

3.3.3. In vitro and in vivo efficacy

To investigate how the presence of alginate affects the delivery efficiency of PEGylated lipoplexes, we evaluated cell uptake using FITC-labelled oligonucleotides. The alginate system was formulated as described earlier but without cross-linking with calcium to eliminate effects of release kinetics on the uptake efficiency between different formulations. We found that the cell uptake efficiency decreased with increasing alginate concentration in the formulation, with 50% reduction in cell uptake efficiency observed when alginate:lipid ratio increased from 5:1 to 10:1 (Fig. 7A). The reduced uptake of the lipoplexes was likely due to the negative charge of alginate on the surface of the particles. It must be noted, however, that even at alginate:lipid ratio of 10:1, significant uptake of lipoplexes into cells was still observed (approximately 6.6 fold increase in average fluorescence intensity compared to untreated cells). This indicated the association of liposomes with siRNA following their release from the alginate scaffold as no such uptake could be observed when naked FITC-labelled oligonucleotides were incorporated within the scaffold system (data not shown). We next assessed the ability of these alginate scaffold systems to deliver siRNA by assessing the level of green fluorescence protein (GFP) knockdown in GFP-expressing HeLa cells using GFP-targeted siRNA. We used an alginate:lipid ratio of 8:1 for this experiment given acceptable entrapment and cell uptake efficiencies achieved at this ratio (Fig. 6A and 7A). Since the association of alginate with PEGylated particles could affect both their cellular uptake and endosomal release, it was not surprising that the gene silencing efficiency of PEGylated lipoplexes was lowered by about 50% when incorporated with alginate scaffold at a ratio of 8:1 (alginate:lipid) compared to unincorporated PEGylated lipoplexes (Fig. 7B). It must also be noted that the level of GFP knockdown by siGFP is significantly lower when delivered using PEGylated lipoplexes (35

%) compared with Oligofectamine^{TM} (58 %). This is likely to be contributed to by PEG's interference with cellular uptake and the release of siRNA from the endosomal compartment [28], though its presence is critical for the muco-penetrative property of these particles [23].

While the delivery efficiency of PEGylated lipoplexes *in vitro* diminished in the presence of alginate, we hypothesised that the longer retention of the scaffold in the vaginal cavity along with the potential of PEG to facilitate the transport of lipoplexes across the mucosal barrier would result in superior delivery efficiency *in vivo* compared to cationic lipoplexes. Using the same administration technique, dose, and time point, it was found that the amount of FITC-labelled oligonucleotides delivered into vaginal tissues using the PLAS system (A:L 8:1) (Fig. 8) was significantly more than that observed with cationic lipoplexes (Fig. 3). Indeed, following quantification of 5 randomly selected images from each treatment group, we showed a 6-fold increase in the percentage of uptake of FITC-Oligonucleotides into vaginal tissues for PLAS system compared with cationic lipoplexes (16.84% vs. 2.68%, Fig. 9). We next assessed the level of target gene knockdown by siRNA using Lamin A/C siRNA-containing PLAS system (A:L 8:1) in mice. Despite the observed variability between mice within each treatment group, the knockdown of Lamin A/C in vaginal tissues for mice which have received the Lamin A/C siRNA treatments was evident compared to mice which have received the control siRNA treatments (85% knockdown, Fig. 10). These results therefore demonstrated the promise of the PLAS system as an efficient vaginal siRNA delivery system.

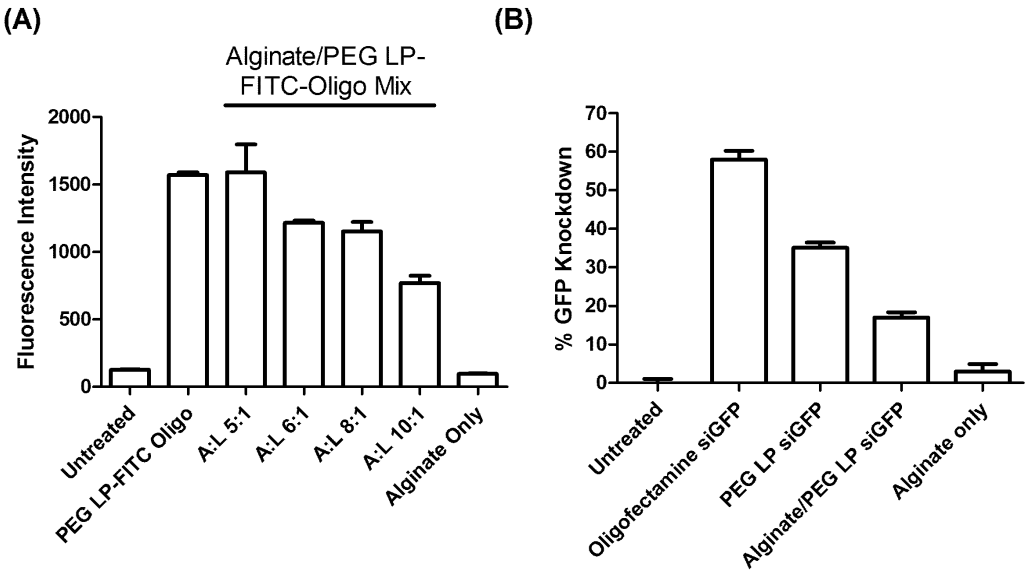

Figure 7 *In vitro* efficacy of the PEGylated lipoplex-entrapped alginate scaffold (PLAS) system. (A) Cell uptake efficiency of the FITC-Oligonucleotides-entrapped PEGylated lipoplexes (LP) in the presence of alginate for formulations prepared at alginate:lipid (A:L) ratios ranging from 5:1 to 10:1. Experiment was performed using HeLa cells at oligonucleotides concentration of 100nM and cells were examined for fluorescence intensity by FACS following an overnight incubation. (B) The ability of the PEGylated lipoplexes to deliver siRNA at an A:L ratio of 8:1 was examined using GFP-targeted siRNA in GFP-expressing HeLa cells. Cells were incubated with the formulation for 48 hrs prior to analysis by FACS. Bars represent the mean value (n=3) and error bars represent the corresponding SEM.

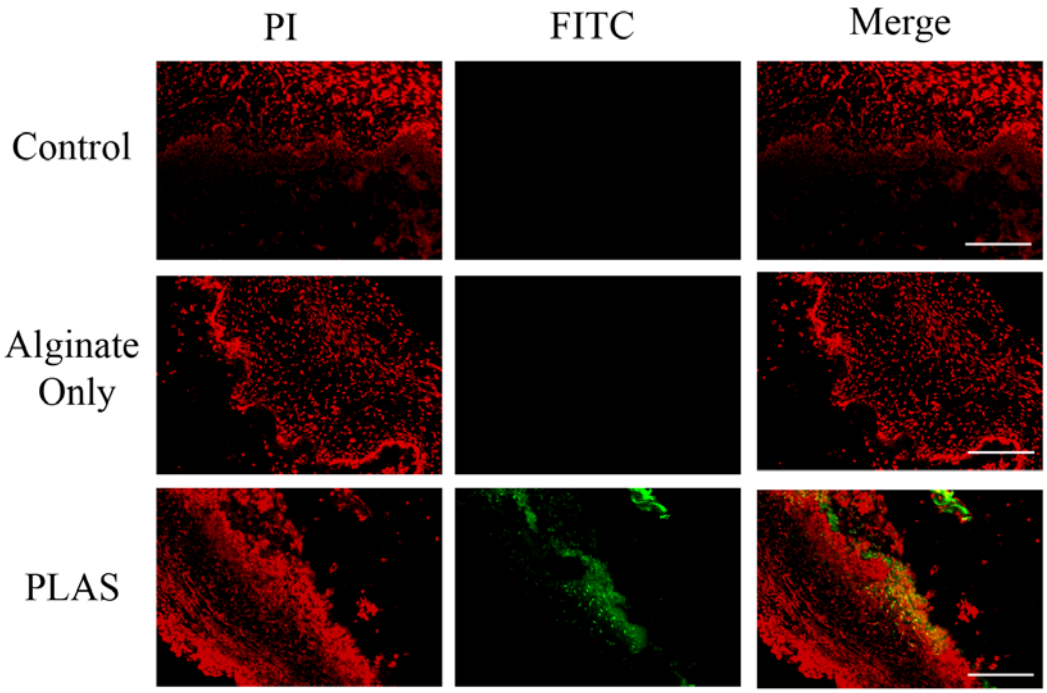

Figure 8 Vaginal delivery efficiency of the PEGylated lipoplex-entrapped alginate scaffold (PLAS) system. Clear uptake of FITC-Oligonucleotides into vaginal tissues was observed following intravaginal implantation of the FITC-Oligonucleotides-containing PLAS system (2,000 pmol, bottom panel). The scaffolds were prepared at an alginate:lipid (A:L) ratio of 8:1 and three mice were used for the treatment group. All mice were treated with 5% citric acid for 2 hrs prior to administration. No fluorescein autofluorescence was detected in either the control mice (treated with 5% citric acid only) or mice treated with alginate scaffolds which do not contain lipoplexes. All images were obtained at 24 hrs post-treatment and frozen tissue sections were stained with propidium iodide (PI) in Vectashield™. The images shown for the lipoplex/alginate hybrid system were representative of three mice. The scale bars represent 200 µm. [Colour in print].

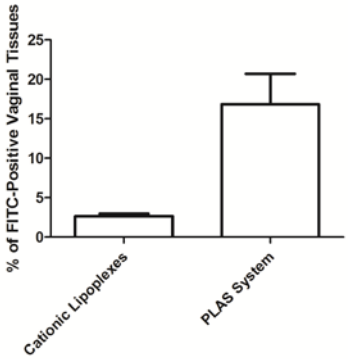

Figure 9 Percentage of FITC-positive vaginal tissues following vaginal administration of FITC-Oligonucleotides delivered using cationic lipoplexes or PEGylated lipoplex-entrapped alginate scaffold (PLAS) system.

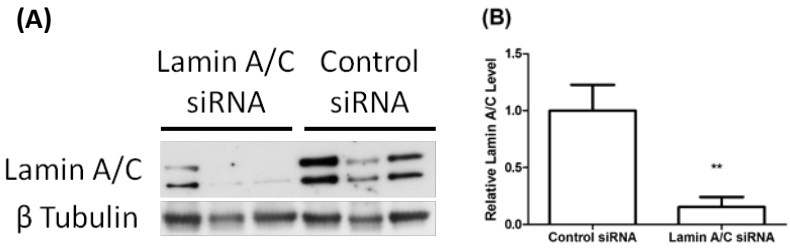

Figure 10 Knockdown of Lamin A/C in vaginal tissues using siLamin A/C-containing PLAS (A:L 8:1) system. (A) Western analysis of the lamin A/C level. Each lane represents the Lamin A/C level in the vaginal tissue in each individual mouse. Mice received two doses of Lamin A/C or control siRNA treatment (600pmole/dose) intravaginally and three mice were used per treatment group. All mice were pre-treated with 5% citric acid 2 hrs prior to siRNA administration. (B) Quantitative analysis of the combined LaminA and C levels between treatment and control groups. **p<0.01, significantly different from control treatment group.

4. Discussion

To date, several groups have reported varying success in delivering siRNA to the vaginal tract, from simple application of lipoplexes into the vaginal cavity with [3] or without [18] progesterone treatment, to thorough cleaning of the vaginal tract prior to siRNA administration [7]. The discrepancy in the administrative methods between these studies has prompted us to investigate the challenges of delivering siRNA to vaginal tissues under normal physiological conditions without progesterone treatment, which may more accurately represent the human vaginal tract [29]. This is because progesterone treatment cannot only arrest mice in the diestrus phase of the estrous cycle but is also typically associated with a thinned vaginal epithelia that may facilitate epithelial penetration, a morphology unlike any human estrous phase [29]. Its use has also been associated with increased rates of vaginal infections and decreased immune response in the vagina [30].

In contrast to previous reports [3, 18], we found that conventional lipoplexes are not retained in the vaginal cavity following administration and are unable to reach the cervicovaginal epithelium under normal physiological conditions (not hormone treated, Fig. 2). This was unsurprising as particles as small as 59 nm have been shown to unable to diffuse through human cervical mucus due to their extensive interaction with mucins fibers, despite its average pore size of 340 nm [31, 32]. Mucus thus presents as one of the most significant barriers to effective vaginal delivery [21]. To address this, groups have shown that dense coatings of low MW PEG can shield nanoparticles from adhesive interactions with mucus enabling these particles to diffuse more freely in the presence of mucin fibers [23, 33, 34]. This is critical as we found that even when the vaginal cavity was pre-treated with citric acid, which could induce transient and reversible openings of the tight junction in the vaginal epithelium [19], minimal uptake of conventional lipoplexes into vaginal tissues was achieved suggesting that a significant amount of lipoplexes were still trapped in the mucus layer and were rapidly cleared (Fig. 3). The importance of developing muco-penetrative particles was further demonstrated when N-9, a non-ionic detergent widely used in vaginal gels, lubricants, and condoms [35], failed to enhance the delivery of lipoplexes to the underlying epithelium despite its ability to disrupt vaginal epithelium [17, 36] (data not shown). While it is possible that the liposome structure could have been destroyed in the presence of the detergent (N-9) leading to ineffective delivery, the ability of N-9 to dissociate hydrophobically-bundled mucin fibers could have also contributed to this given its ability to decrease the average mucus pore size from 200-500 nm to 130 nm [37, 38].

It was thus hypothesised that PEGylated lipoplexes would serve as a more appropriate delivery vector for vaginal siRNA delivery. However, the ability of these lipoplexes to be retained in the vaginal cavity following administration was a concern due to its liquid nature, a feature that is similar to currently available formulations [3, 6, 7, 18]. To address this, we formulated a solid PLAS system where PEGylated lipoplexes could be released from solid alginate scaffolds gradually overtime. Importantly, unlike the poly(ethylene-co-vinylacetate) (EVAc) disc which has previously been used to deliver plasmid DNA in a sustained manner in the vaginal cavity [39], alginate is biodegradable thus presents an attracting alternative. While the particle size of these lipoplexes increased significantly from 200nm to around 350nm in the presence of alginate, given that the pore size of cervicovaginal mucus ranges from 50-1800nm with a size of 340 +/- 70nm [38], these PEGylated muco-inert particles could still penetrate the mucus layer lining cervicovaginal tissues readily.

The mechanism by which the PEGylated lipoplexes is released from the scaffold was likely through a combination of surface desorption, diffusion, and alginate degradation. The reduced interaction between lipoplexes and alginate scaffold due to the presence of PEG likely has resulted in the initial burst release of our particle from the scaffold system (Fig. 6B) [40]. However, the sustained release nature of the PLAS system following this initial burst release indicated that that PEGylated

lipoplexes were entrapped within the scaffold system at least to some extent rather than merely being immobilized on its surface. Given that the increased alginate concentration in the formulation could significantly prevent the loss of lipoplexes during the cross-linking procedure (Fig. 6A), it was not surprising to see that the release rate of lipoplexes decreased with increasing alginate concentration (Fig. 6B). Importantly, we found that the remains of the alginate scaffolds (*w*/*w* 8) could still be observed in the vaginal tract at 24 hrs post intravaginal administration. This was in direct contrast to the *in vitro* study where the scaffolds were almost completely disappeared within 3 hrs of incubation in citrate buffer (Fig. 6B). This discrepancy was likely due to the difference in sodium level in the buffer compared to the vaginal fluid [24] as well as the vast difference in the fluid volume present between *in vitro* (5 mL) and *in vivo* (50 μL) conditions. Indeed, when OptiMem^{TM} or PBS was used, we observed minimal degradation of these scaffold systems at 24 hrs post incubation (data not shown), which is consistent with many other scaffold device made of fibrin [41] or collagen [40].

Importantly, the released PEGylated lipoplexes from the alginate system were shown to be able to enter cells efficiently and resulted in target gene knockdown, though the efficiency was diminished with the increased level of alginate in the formulation (Fig. 7). It must be noted that while a few reports have shown superior delivery of plasmid DNA or siRNA using alginate [11] or collagen [42] based scaffold systems compared to the one reported in this manuscript, the transfection agent used in those studies were non-PEGylated and the cells were also embedded directly within the scaffold system for tissue regeneration purposes. A direct comparison of the delivery efficacy between these studies is therefore difficult. Despite this, the observed reduction of the gene silencing efficiency of siRNA in the presence of alginate (Fig. 7B) was similar to that reported for PEG-polyethyleneimine(PEI)-alginate nanoparticles where their gene silencing efficiency was found to be inferior than that of parent PEG-PEI nanoparticles, though the exact degree of reduction was not reported [13]. The mechanism by which alginate decreases the delivery efficiency of PEGylated particles, but not non-PEGylated ones, is not well understood at present [13]. Nevertheless, we showed that in the *in vivo* setting, the PLAS system delivered oligonucleotides into vaginal tissues at a much higher efficiency than cationic lipoplexes (6-fold increase, Fig. 3, 8, and 9). The enhanced delivery was likely due to a combination of the improved penetration of PEGylated lipoplexes across the mucosal layer compared to non-PEGylated counterparts as well as the ability of the scaffold to remain in the vaginal cavity following administration. The promise of this PLAS system to deliver nucleic acids intravaginally was further demonstrated when an 85% knockdown of Lamin A/C was observed following two consecutive treatments of Lamin A/C-targeted siRNA in mice (Fig. 10). While this is encouraging, future effort should focus on optimising formulation method and parameters, such as the degree of cross-linking, particle size [31], amount of PEGylation [33], type of alginate [10, 43], or formulation procedures [44], in order to improve the mucus-penetration, release kinetics and vaginal retention time of the PLAS system. These optimisations along with the establishment of a feasible treatment regimen will be critical in the future in order to achieve clinically significant therapeutic response following intravaginal administration and will therefore be the next set of challenges. In particular, enhancement of the muco-penetrative ability of the PLAS system will be crucial for its future clinical applicability as the need of pre-treatment of vaginal cavity with citric acid could potential lead to increased susceptibility to infections. Despite this, the simple formulation procedure described here for the PLAS system provided us with an attractive reservoir platform for vaginal delivery of nucleic acids. It avoids the use of organic solvents which are typically required for fabrication of other types of scaffold systems [42, 45]. Ultimately, the successful development of such system carries great potential in the vaginal administration of siRNA for localized diseases.

5. Conclusion

With the recent identification of siRNA targets for cervical cancer (E6, E7 and Grb10) [14, 46], HIV infections (CCR5) [47], or HSV-2 infections (UL-29 and UL-27) [3], vaginal application of siRNA has great appeal and importance. To our knowledge, this is the first report on the combined use of biodegradable scaffold and liposomes for mucosal delivery of siRNA. We demonstrated for the first time the feasibility of entrapping PEGylated lipoplexes in a solid, negatively charged alginate scaffold system and we found that the delivery of nucleic acids into vaginal tissues was significantly enhanced compared to conventional lipoplexes following intravaginal administration. Using the PLAS system, a significant siRNA-mediated knockdown of the target gene was observed in vaginal tissues following intravaginal administration. Overall, this novel hybrid system presents an innovative approach for the vaginal delivery of nucleic acids and its ease of administration and preparation offers vital features for rapid clinical development and patient compliance.

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