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Vaginal delivery of siRNA using a novel PEGylated lipoplex-entrapped alginate scaffold system

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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 the 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 the 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.

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 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,

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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 (Oligo, 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 2 mM 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 2 h, liposomes were extruded through a series of Nucleopore™ track-etched membranes using a Lipex™ 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 were 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 × 2 cm 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 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 (1–1000 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 microliter 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 duplicate.

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 5000 *g* for 10 min 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).