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Urothelium-adherent, ion-triggered liposome-in-gel system as a platform for intravesical drug delivery

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ABSTRACT

Instillations of therapeutic agents into the urinary bladder have limited efficacy due to drug washout and inadequate attachment to and penetration into the bladder wall. Instilled nanoparticles alone have low stability and high susceptibility to washout, while gel-based systems are difficult to administer and retain. To overcome disadvantages of current technologies, a biodegradable, in situ-gelling liposome-in-gel (LP-Gel) system was developed for instillation into the bladder, composed of nano-sized, fluidizing liposomes incorporated into a "smart" biopolymeric, urine-triggered hydrogel. The liposomes are optimized for their fluidizing composition in order to enhance cellular penetration through the urothelial barrier, while the hydrogel co-delivers the suspended nanocarriers and enhances adhesion on the mucin layer of the urothelium. The composite system thus mimics both the lipid membranes and mucosal layer that comprise the urothelial barrier. LP-Gel showed appreciable cytotoxicity in rat and human bladder cancer cells, and instillation into rat bladder showed enhanced adhesion on the urothelium and increased penetration into the bladder wall. Instillation of paclitaxel-loaded LP-Gel showed drug retention for at least 7 days, substantially higher than free drug (few hours), and with negligible systemic levels. The LP-Gel platform system thus facilitates prolonged drug localization in the bladder, showing potential use in intravesical applications.

1. Introduction

The ability of the urinary bladder to function as a storage organ is crucially dependent on the bladder permeability barrier presented by the urothelium [1,2], which is built from densely-packed hexagonal umbrella cells joined by tight junctions and covered with a highly hydrophilic mucin layer [3]. The glycosaminoglycans (GAGs) present in the mucin layer [4] prevent foreign substances from adhering to the urothelium and reaching the underlying cellular components, thereby hindering diffusion of instilled drug solutions into the bladder wall.

Current treatment modalities for urinary bladder cancer involve tumor resection followed by chemotherapy [5], and intravesical instillation of drug solutions directly into the bladder [6,7]. However, drug efficacy is limited by the permeability barrier of the bladder urothelium [8], which prevents drug diffusion into the bladder wall, by drug dilution during bladder filling and subsequent washing out during voiding, necessitating repeated drug infusions. Primary chemotherapeutic agents used in bladder cancer are gemcitabine [9], epirubicin [10], cisplatin [11], paclitaxel [12], mitomycin C [13], etc., which need multiple instillations. Paclitaxel has been used in combination therapy for urinary bladder cancer, but its low solubility requires the use of Cremophor® excipient for stabilization in an aqueous solution, resulting in severe systemic toxicity [14]. Although nanocarriers may be used to enhance the efficacy of drug delivery [15], their attendant limitations restrict duration of drug retention in the bladder. Recent studies with intravesical drug-loaded inorganic nanoparticles, such as calcium carbonate [16], magnetic nanoparticles [17,18,19], nanogold particles [20], and biodegradable nanoparticles, such as liposomes [21], gelatin nanoparticles [22,23], Abraxane [24], hyperbranched polyglycerol [25], and other polymers [26] have shown appreciable anti-tumor effects, but remain limited by their low urothelial adhesion (up to 2 h) and are heavily dependent on surface functionalization.

A number of thermo-sensitive hydrogels, such as PLGA-PEG [27], poloxamer [28], pluronic [29] and chitosan [30,31], have been used along with nanoparticles, but are limited by the difficulty in administering thermosensitive materials. Further, in the case of chitosan gels co-encapsulation of magnetic nanoparticles and an external magnetic field were required for retention in the bladder [31]. The thermosensitive hydrogels used so far need specific handling temperatures, precise drug/polymer ratios and specific pH to ensure its sol-gel transition is at body temperature [32], thereby limiting the therapeutic moieties that may be delivered, and also are mostly Newtonian fluids [33] that require high pressure and extended delivery time for injection through small bore catheters. Although these nanoparticle-hydrogel

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combination systems have widened the scope of moieties that may be used for intravesical delivery, attaining the core goals of enhanced urothelial attachment, bladder wall permeability and sustained drug retention and release in the bladder still remains a challenge.

Ion-triggered hydrogels have not been utilized so far in intravesical applications, but may prove useful due to simple preparation protocols and triggered gelation that is enabled by the high concentration of ions in urine. Gellan is a biopolymeric hydrogel that is biodegradable and FDA-approved as a direct food-additive [34], is mucoadhesive in nature [35], and undergoes ion-triggered in situ gelation [36]. Gellan has been widely used as a substrate for tissue engineering, ophthalmic and wound healing applications due to its bioactivity [37]. Gellan can be used to increase adhesion on the hydrophilic mucin layer of the urothelium, and can be utilized to form an in situ carrier matrix for drug-loaded nanoparticles. Further, there is a need for amphiphilic, degradable, non-toxic nanoparticles which can fluidize the urothelial barrier for enhanced penetration and can co-encapsulate hydrophilic and hydrophobic drugs. Soya phosphatidylcholine liposomes were chosen for this purpose since they are composed of predominantly unsaturated phospholipids that form fluidizing lipid vesicles.

The present study thus designed and evaluated a novel "smart" in situ-gelling liposome-in-gel composite system (**LP-Gel**), using paclitaxel (PTX)-loaded fluidizing liposomes incorporated into gellan hydrogel, for direct instillation in the urinary bladder. LP-Gel utilizes urine to undergo ion-triggered gelation to form a cross-linked gellan matrix, and also enables enhanced attachment to the mucin layer on top of the urothelial surface due to the mucoadhesive property of gellan. The system thus mimics both the lipid membranes and mucin layer present in the urothelium, thereby allowing better interaction with and adhesion to the bladder wall. This can serve to localize drug release at the target site and minimize systemic toxicity

2. Materials and methods

2.1. Materials

Milli-Q water was used (Millipore, Bedford, MA, USA). Methanol and chloroform (HPLC grade, 99.9% purity) were obtained from Spectrochem (Mumbai, India). Sodium chloride (GR grade, 99.5% purity) was obtained from SRL (Mumbai, India). Soya lecithin (soya phosphatidylcholine, SPC) was purchased from Himedia (Mumbai, India). Gellan gum (high acyl, food grade Kelcogel®) was obtained from CP Kelco (Atlanta, GA, USA). Rhodamine-6G dye was purchased from Sigma Aldrich (St Louis, MO, USA). Paclitaxel was purchased from Dabur Pharma (Mumbai, India).

2.2. Preparation of liposome-in-gel system (LP-Gel)

Liposomes were prepared by thin-film hydration method, where SPC was dissolved in 2:1 chloroform:methanol mixture, evaporated by vacuum, and the lipid film was hydrated with 0.9% w/v saline. Size of the liposomes was reduced by sonication. For dye-loaded liposomes, the rhodamine-6G dye was added to hydration medium, and for PTX-loaded liposomes, PTX was dissolved along with lipids during thin film formation (1:2 (w/w) drug: lipid). Excess dye was removed by centrifuging the suspension (25,000 rpm, 4 °C, 30 min), removing the supernatant and resuspending the liposomes in saline. The suspension was sonicated using a probe sonicator to reduce the size of the liposomes.

0.1% (w/v) gellan hydrogel was prepared in 0.9% (w/v) saline. LP-Gel was prepared by mixing the gellan solution with the liposome suspension (with or without loaded dye or PTX) with constant stirring, to get a final lipid concentration of 2 mg/mL and gellan concentration of 0.1% (w/v).

2.3. Characterization

The mean diameter and surface charge of liposomes was determined by dynamic light scattering (DLS) (BI200SM Brookhaven, NY, USA) and zeta potential measurements (Phase Analysis Light Scattering with ZetaPALS, Brookhaven, NY, USA), respectively. Liposomal suspensions (2 mg/mL) were prepared for DLS and ZETA measurements, and mean diameter and size distribution of the particles was obtained with NNLS analysis (Brookhaven, NY, USA), while average surface charge was calculated from ten ZETA cycles.

For calculation of encapsulation of PTX, after formation of liposome-PTX, it was centrifuged (25,000 rpm, 4 °C, 30 min), and the supernatant discarded. The pellet was then resuspended in saline, and the suspension was sonicated using a probe sonicator to reduce size of the liposomes. The suspension was then centrifuged again (25,000 rpm, 4 °C, 10 min), and any unencapsulated PTX and debris was discarded as a pellet. The supernatant containing the PTX-liposomes was used for encapsulation measurements.

The PTX trapped in the liposomes was quantified by disruption of liposomes using organic solvents and estimating the PTX using UV/vis spectroscopy (Perkin Elmer Lambda25, Waltham, MA, USA) at the characteristic peak of 228 nm, and encapsulation efficiency was calculated. The UV–Vis method and HPLC method were found to be equivalent for estimation of paclitaxel levels.

Paclitaxel drug release (from 3 mL of LP-Gel-PTX) was carried out by placing the formulation in a dialysis membrane (Dialysis Membrane-50, Himedia, Mumbai, India) and observing release in a USP dissolution apparatus (Type II Electrolab TDT-08L, Mumbai, India) with saline:methanol (3:1 v/v) as sink medium (150 mL) at 37 °C (paddle speed 200 rpm). The amount of sink medium used (150 mL) is in proportion to that is needed to completely solubilize total PTX present in the formulation (i.e. 3 mg in 3 mL formulation), such that the detectable amount of PTX is within the limits of the calibration curve (where maximum PTX concentration used is 20 μg/mL). Amount of paclitaxel loaded and released was estimated using UV/vis spectroscopy (Perkin Elmer Lambda25) at the characteristic peak of 228 nm.

Cryo-FEG-Transmission electron microscopy (cryo-FEG-TEM; JEM-2100F, JEOL, Peabody, MA, USA) and scanning electron microscopy (FEI QUANTA, USA and Hitachi S-3400N, Tokyo, Japan) were used for imaging. Injectability test was done using UTM (universal testing machine, Tinius Olsen Benchtop Materials Testing Machines H5KS, Horsham, PA, USA) with increasing concentrations of gellan (0.1–0.2% w/v) in phosphate buffered saline (PBS) filled in syringes and extruded through a 22 gauge needle (1 mL/min) using standard ASTM uniaxial compression test (QMat 5.14 S-series). Viscosity was measured with a rheometer (Physica MCR 301, Anton Paar, Graz, Austria), using the cone and plate set-up, and using shear rates ranging from 0.1 to 100 s^{-1} at 37 °C.

2.4. Cellular internalization and cytotoxicity

NBT-II rat urinary bladder cancer (National Centre for Cell Science (NCCS), Pune, India) and T24 human urinary bladder cancer (ATCC, Manassas, VA, USA) cell lines were used for cytotoxicity studies. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Himedia, Mumbai, India) for NBT-II, and McCoy's 5A medium (Himedia, Mumbai, India) for T24 cells. All media was supplemented with 10% fetal bovine serum (FBS, Himedia, Mumbai, India), 0.2% Antibiotic and antimycotic solution (Himedia, India) and cultured at 37 °C in 5% CO_2 to obtain an adherent monolayer (~80–90% confluency) over 72 h in T25 flasks. Cells were detached using Trypsin-EDTA solution (Himedia, India). Cellular uptake was evaluated by incubating cells with rhodamine-6G dye-loaded LP-Gel for 3 h. Fluorescence in the cells was observed with confocal laser scanning microscopy CLSM (Olympus Fluoview FV500, Tokyo, Japan), with excitation wavelength at 570 nm and emission wavelength at 590 nm for Rhodamine-6G. The mean