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Layer-by-layer assembly of liposomal nanoparticles with PEGylated polyelectrolytes enhances systemic delivery of multiple anticancer drugs

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ABSTRACT

Layer-by-layer (LbL)-engineered nanoparticles (NPs) are a promising group of therapeutic carriers used in an increasing number of biomedical applications. The present study uses a controlled LbL process to create a multidrug-loaded nanoplatform capable of promoting blood circulation time, biodistribution profile and controlling drug release in the dynamic systemic environment. LbL assembly is achieved by sequential deposition of poly-L-lysine (PLL) and poly(ethylene glycol)-*block*-poly(L-aspartic acid) (PEG-*b*-PLD) on liposomal nanoparticles (LbL-LNPs). This generates spherical and stable multilayered NPs ~240 nm in size, enabling effective systemic administration. The numerous functional groups and compartments in the polyelectrolyte shell and core facilitate loading with doxorubicin and mitoxantrone. The nanoarchitecture effectively controls burst release, providing different release kinetics for each drug. LbL-LNPs are pH-sensitive, indicating that intracellular drug release can be increased by the acidic milieu of cancer cells. We further demonstrate that the LbL nanoarchitecture significantly reduces the elimination rates of both drugs tested and markedly extends their systemic circulation times, paving the way for efficacious tumor drug delivery. Because this delivery system accommodates multiple drugs, improves drug half-life and diminishes burst release, it provides an exciting platform with remarkable potential for combination therapeutics in cancer therapy.

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1. Introduction

In recent years, many polymeric and lipid carriers have been developed and shown to be successful in clinically relevant cancer models [1]. Nevertheless, there is tremendous scope for improvement of the therapeutic effects of drugs administered to patients with cancer [2]. In particular, successful therapy is highly dependent on the efficient accumulation of nanoparticles (NPs) at the tumor site [3]. This relies on effective delivery via the prolonged blood circulation, programmed/controlled drug release, high payload capacity and multifunctionality of the nano-delivery system [4].

Polyelectrolyte layer-by-layer (LbL) NPs of the appropriate size and surface functionalization have drawn significant scientific interest because of their technological importance in cancer drug delivery [5]. LbL-NPs fabricated by the alternate deposition of charged polyelectrolytes onto various colloids are becoming prominent delivery systems because of their high loading capacity for therapeutic modalities, excellent biocompatibility, multifunctionality, tunable drug release and responsiveness to stimuli [5]. This unique approach has been transferred from two-dimensional (2-D) flat surfaces to three-dimensional (3-D) colloidal surfaces, and allows the fabrication of polycationic and polyanionic materials at the nanometer-scale level [6]. Although there are a small number of studies describing the application of the LbL approach to the development of drug delivery systems, the main emphasis of most of these studies has been the barrier-controlled release of the core content, and no attempts have been made to build a systemic delivery system targeting tumors [7]. However, there is mounting evidence that NPs with long half-lives in the circulation preferentially accumulate in tumors, and if this applies to LbL-NPs, they have the potential to greatly advance clinical translation [8]. Moreover, responsiveness to stimuli such as pH also confers tumor specificity superior to that of pH-insensitive, non-erodible,

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electrostatically assembled NPs [9]. A pH-based strategy takes unique advantage of the tendency of the reduced tumor pH to disassemble the deposited layers, resulting in drug release in close proximity to the cancer cells, but not under normal physiological conditions [10].

Although only one of a plethora of potential carriers, liposomes represent the most common translational nanocarriers and are already approved by the Food and Drug Administration (USA) for various clinical chemotherapeutic approaches [11]. Liposomes have attracted significant attention due to their ability to carry and protect a range of hydrophilic and hydrophobic molecules. In addition, liposomes have a better biocompatibility profile than some micellar systems, making them an ideal choice for drug delivery [12]. However, application of the LbL architectural approach to liposome production presents some daunting challenges relating to: (a) the colloidal stability of LbL-functionalized NPs under physiological conditions; (b) the amount of drug encapsulation in the core and shell; (c) the effective particle size of core–shell NPs and most importantly; (d) surface deposition of polyethylene glycol to prolong their in vivo blood circulation time [13,14]. Considering all of these challenges, we chose a combination of poly-L-lysine (PLL) and poly(ethylene glycol)-*block*-poly (L-aspartic acid) (PEG-*b*-PLD) as the respective polycationic and polyanionic polymers. Positively charged PLL is known to enhance the cellular uptake of NP systems, whereas PEG-*b*-PLD provides colloidal stability and pH sensitivity. Owing to the amphoteric nature of PEG-*b*-PLD, it undergoes protonation–deprotonation cycles and is protonated at the acidic pH found in endosomes, which destabilizes the LbL-NPs and results in the release of the therapeutic load at the tumor site [15]. Moreover, the modular design enables the development of a multidrug delivery system by loading different therapeutic molecules in the core and in the shell layers [15]. Recently, a few reports described the fabrication and systemic application of LbL assembled nanocarriers. However, these studies either focused on the size and stability of the system or employed single drug-loading [6,14]. For the first time, we have incorporated dual therapeutic modalities in the core and shell functional architectures and evaluated the systemic performance of this delivery system, with a view to its clinical translation for cancer therapy.

In this study, we sought to improve the in vivo stability and pharmacokinetic profile of NPs intended for systemic drug delivery by combining the unique advantages of the LbL technique with those of liposomes. To achieve this, LbL liposomal nanoparticles (LbL-LNPs) were prepared with up to 11 alternating layers of PEG-*b*-PLD and PLL. Using a modular design, doxorubicin (DOX) was incorporated into the core and mitoxantrone (MTX) was loaded onto the surrounding shell layers, creating a novel dual-drug delivery system. The present study describes the detailed mechanistic basis for assembly of LbL-LNPs, and their physicochemical characterization. In vivo studies showed that three-layered and 11-layered LbL-LNPs extended drug plasma half-life. In addition, cytotoxicity analyses of blank and drug-loaded LbL-LNPs were performed in MCF-7 cells. Overall, this work highlighted the value of the LbL architecture as a robust multi-therapeutic platform with potential for in vivo cancer drug targeting.

2. Materials and methods

2.1. Materials

DOX hydrochloride was donated by the Dong-A Pharmaceutical Company (Yongin, South Korea). MTX hydrochloride was procured from Shaanxi Top Pharm Chemical Co. Ltd (Xi'an, China). Methoxy-PEG-*b*-PLD sodium salt (MW: 12,000) was purchased from Alamanda Polymers (Huntsville, AL, USA). The block lengths were 113 and 50 repeating units for PEG and PLD, respectively. PLL (MW: 15,000-30,000), cholesterol and dimethyldioctadecylammonium bromide (DDAB) were purchased from Sigma-Aldrich (St Louis, MO, USA). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was obtained from NOF America Corporation (White Plains, NY, USA). All other chemicals were of reagent grade and used without further purification.

2.2. Fabrication of LbL-LNPs

Liposomal nanoparticles (LNPs) were prepared using a thin-film hydration technique. Firstly, the lipid phase was prepared by dissolving DPPC, cholesterol and DDAB (8:4:1 M ratio) in an organic solvent mixture of chloroform/methanol (3:1). The organic solvent was removed from the lipid phase by evaporation at 60 °C in a rotary evaporator (EYELA, Tokyo, Japan) under a vacuum. The resulting thin lipid film was hydrated using ultra-pure water. The large unilamellar vesicles were extruded using a mini-extruder (Avanti Polar Lipids, Inc., Alabaster, AL, USA) with two 50 nm-pore 19 mm polycarbonate filters for 21 cycles, to obtain monodispersed nanosized LNPs. DOX-loaded LNPs were prepared by adding 10% w/w of DOX to the lipid mixture prior to this thin-film hydration process.

LbL-LNPs were generated as reported previously, with slight modifications [13]. Briefly, polymer (PLL and PEG-*b*-PLD) and liposome solutions were prepared at a concentration of 1 mg ml^{-1}. Typically, small aliquots of each polymer solution (20-30 μl) were added to the liposome dispersions and vortexed for 5 min. The mixture was immediately sonicated (Sonics, USA) for another 5 min and kept aside for the next polymer addition. It is worth noting that no intermittent washing or centrifugation steps were included in this procedure. Rather, the exact amount of polymer required for surface charge reversal was determined by careful titration.

2.3. Characterization of LbL-LNPs

The physicochemical characterization of LbL-LNPs including the size, shape and solid state characteristics are presented in the supplementary section.

2.4. Quartz crystal microbalance-dissipation (QCM-D) analysis: monitoring LbL build-up in real time

QCM-D analysis was carried out using the Q-Sense® E1 system, equipped with a QFM 401 Q-Sense® flow module (Stockholm, Sweden) [17]. Liposomes (1 mg ml^{-1}) were passed into the measurement cell for 15 min at a uniform flow rate of 100 μl min^{-1}, using a peristaltic pump. Between each polymer deposition, a 5 min rinsing step with water was performed. Q-Sense® Analysis software was used to model the results. Detailed methodology is presented in the supplementary section.

2.5. Drug loading

To calculate the amount of DOX entrapped in the LNPs and LbL-LNPs, the formulations were filtered through an Amicon centrifugal ultrafiltration device (MWCO 10,000 Da, Millipore, Billerica, MA, USA) for 10 min at 5000 rpm. The free drug present in the resulting supernatant was measured using a UV/vis spectrophotometer (PerkinElmer U-2800, Hitachi, Tokyo, Japan) at a wavelength of 482 nm.

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