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Layer-by-layer nanoparticle platform for cancer active targeting

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A B S T R A C T

Nanoparticles as drug delivery carriers have been investigated over the last few decades, particularly for cancer treatment. The rationale in developing such nanoparticles is to maximize drug efficacy while minimizing toxic side effects. This can be most effectively achieved through target specific drug delivery. A novel biocompatible nanoparticle platform prepared using the core-shell self-assembly technique is reported. The core consists of calcium phosphate which is biocompatible and pH-sensitive, and the shell is composed of biocompatible polymers (hyaluronic acid, CD44 targeting moiety; and chitosan, physical cross-linker). Cisplatin was selected as a model drug and incorporated between the core and the shell. The nanoparticle composition was optimized for high serum stability and low protein binding. These nanoparticles demonstrated target specific delivery in human lung cancer cells (which overexpress CD44 receptors). The targeting ability of the nanoparticles was confirmed with an 8-fold increase of drug efficacy (IC_{50}) compared to cisplatin. Furthermore, the pH-sensitive core of the nanoparticle platform led to controlled drug release through destabilization in acidic conditions. This platform technology provides a simple approach for the design of targeted biocompatible nanoparticles for cancer therapy.

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1. Introduction

The rapid growth that has occurred in the field of nanotechnology has enabled easier access to the use of nanoparticles as drug delivery systems for combating diseases such as cancer (Goldberg et al., 2007). Nanoparticles have great potential to improve drug efficacy through increased uptake and controlled drug release (Kuo et al., 2013; Law et al., 2008). According to recent studies, engineered nanoparticles equipped with target specific ligands significantly enhance particle internalization into the target cells (Gabizon et al., 2004; Choi et al., 2010), thereby improving drug efficacy (Yang et al., 2010; Shen et al., 2012; Eck et al., 2008) Additionally, nanoparticles have been engineered with stimuli-responsive properties such as temperature (Ganta et al., 2008; Ling et al., 2014) and pH-sensitive moieties (Petros et al., 2008; Kirpotin et al., 1996) in order to enhance intracellular delivery and drug release (Saito et al., 2003). For instance, pH-responsive moieties have been shown to facilitate controlled drug release through their ability to switch drug release on-and-off *via* a pH-trigger (Mura et al., 2013; Elbayoumi and Torchilin, 2009)

Despite improvements achieved in drug efficacy with the use of nanoparticles, there are associated safety concerns. Biocompatibility is a crucial factor in nanoparticle safety (Naahidi et al., 2013; Kohane and Langer, 2010). Biodegradation of nanoparticles through natural pathways is ideal for clinical applications. On the other hand, non-biodegradable nanoparticles may cause immunogenicity and severe inflammation as well as long-term side effects (Mokhtarzadeh et al., 2016). Non-biocompatible materials may induce undesirable interactions between nanoparticles and biological systems (Dobrovolskaia and McNeil, 2007; Dobrovolskaia et al., 2008). These interactions depend on the physicochemical properties of nanoparticles and the biological environment (Aggarwal et al., 2009)For instance, nanoparticles that are 100–200 nm in size accumulate passively in tumor tissues due to the enhanced permeation and retention (EPR) effect (Hobbs et al., 1998; Maeda, 2010). In contrast, particles greater than the submicron size easily undergo clearance *via* the mononuclear phagocyte system (MPS) or are taken up by the liver and the spleen (Petros and DeSimone, 2010). Furthermore, immunoglobulins or complement proteins have a high affinity for hydrophobic or positively charged moieties (Wang et al., 2012) These interactions may result in loss of drug efficacy as well as increase in toxicity (Domanski et al., 2004; Agashe et al., 2006).

In order to overcome the above limitations, two major strategies have been investigated: active targeting (Davis, 2009)

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and controlled drug release (Oh et al., 2007) As noted above, active targeting has been utilized to increase therapeutic efficacy of anticancer drugs. In addition, pH-sensitive characteristics have been used to control drug release rates. For example, human epidermal receptor 2 (HER 2) specific antibody-labeled and doxorubicin-loaded liposomes resulted in improved cellular uptake into HER2-overexpressing SK-BR3 cells, and therefore higher drug efficacy (Gao et al., 2009) Another example is the use of the pH-sensitive polymeric nanoparticles which effectively released drug at the target tumor site *via* a pH-dependent structural transformation of the polymer backbone, triggered by the acidic tumor environment.

In the current study, a novel nanoparticle platform equipped with active targeting and pH-sensitive functionalities is reported. The key strategies here were to use biocompatible materials with simple preparation methods, which may facilitate the development of nanoparticle drug products for cancer therapy. The nanoparticles were prepared by core-shell layer-by-layer assembly with the drug particles sandwiched between the core and the shell. Calcium phosphate was used as a pH-sensitive core and hyaluronic acid (HA) (targeting moiety) was self-assembled with chitosan to form the shell. The physicochemical properties of the nanoparticles, such as particle size and zeta-potential, as well as how these properties change throughout the preparation process were investigated. *In vitro* release was tested under different pH conditions to investigate pH-triggered drug release from the nanoparticles. Nanoparticle uptake, therapeutic efficacy, and cytotoxicity were all investigated using a CD44-overexpressed lung cancer cell line.

2. Materials

Calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$), potassium phosphate mono basic (K_2HPO_4), polyethylene glycol (PEG, M_w 8 kDa), carboxymethyl cellulose (CMC, M_w 90 and 250 kDa), chitosan (high purity, M_v 110–150 kDa), chitosan oligosaccharide lactate (M_n 5 kDa), sodium alginate (medium viscosity), cis-diammineplatinum (II) dichloride (cisplatin), fluorescein isothiocyanate (FITC), dimethyl sulfoxide anhydrous (DMSO), dimethyl formamide anhydrous (DMF), and diisopropylethylamine (DIPEA) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium hyaluronate (HA, 10 kDa) was purchased from Lifecore Biomedical (Chaska, MN). Dulbecco's modified eagle medium (DMEM) was purchased from Life Technologies. All other chemicals and reagents used were of cell culture or reagent grade, and all aqueous solutions were prepared with distilled deionized water (MiliQ® water).

3. Methods

3.1. Preparation of the platform nanoparticles

3.1.1. Calcium phosphate nanocore

Different polymers were investigated in order to obtain a stable calcium phosphate nanocore (nanoCaP). Table 1 describes the polymeric stabilizers investigated. The nanoCaP particles were prepared based on a previously reported method (Cheng and Kuhn, 2007). Polymers were dissolved in MilliQ® water (0.2%, v/v), and the polymer solutions were filtered through a 0.22 μm syringe filter. Potassium phosphate (30 mM) and polymer solutions were mixed under stirring conditions (magnetic stirrer at 1500 rpm) for 30 min. Calcium nitrate (30 mM) was then added to the mixture resulting in a final volume ratio of 1.5:1:1 (calcium:phosphate: polymer), and this mixture was stirred for 1 h using a magnetic stirring plate. The resultant nanoparticles were collected *via* centrifugation at 14,500 rpm for 40 min, and washed with MilliQ® water three times. Finally, the nanoCaP particles were dispersed in MilliQ® water using sonication for 10 s. All preparation steps were performed at room temperature. The nanoCaP suspensions were stored at 4 °C for one week, and the particle size changes were monitored using a dynamic light scattering (Malvern Zetasizer ZS90).

Table 1
List of polymeric stabilizers. Two different types of stabilizers (cationic or anionic) were used for stabilization of calcium phosphate.

Stabilizer		MW (Da)	Solubility
Cationic	Chitosan oligosaccharide	<5000	water-soluble
	Chitosan glutamate	100,000 ~ 200,000	water-soluble
Anionic	Sodium alginate	90,000 ~ 250,000	water-soluble
	Carboxymethyl cellulose	100,000 ~ 350,000	water-soluble

3.1.2. Optimization of nanoCaP

The nanoCaP suspensions were prepared using the procedures described above. Different ratios and concentration of calcium, phosphate, and carboxymethyl cellulose (CMC) were investigated to generate the desired particle size range (100–150 nm) and stability. Furthermore, different molecular weights of CMCs were investigated to optimize the particle size.

3.1.3. Drug loading

Aquated cisplatin (aqCDDP) which does not contain chloride ions was used as a model anticancer drug. The preparation method for aqCDDP was previously reported (Woodman et al., 2015). Briefly, 90 mM $AgNO_3$ was added to cisplatin solution (1 mg/ml) at a molar ratio of 2:1, and the mixture was stirred under dark conditions at room temperature. After 24 h, the precipitates (silver chloride) were removed *via* centrifugation three times at 13,000 rpm for 30 min. The resultant solution was filtered through a 0.2 μm syringe filter. The final concentration of aqCDDP was determined using an atomic absorption spectrophotometer with a Pt filter (AAS model 5100, Perkin Elmer, Shelton, CT). One milligram of aqCDDP was mixed with nanoCaP (1 mg), and the mixture was stirred for 48 h at 700 rpm under dark conditions. In order to remove unreacted aqCDDP, the drug-loaded nanoparticles (nano-CaP^{CDDP}) were collected *via* centrifugation at 14,500 rpm for 40 min and washed with MilliQ® water three times. Finally, nanoCaP^{CDDP} particles were dispersed in PBS *via* sonication for 10 s. To determine drug loading efficiency, dried nanoparticles were solubilized in 1 N HCl and incubated for 30 min at room temperature, and then the Pt content was analyzed using an atomic absorption spectrometer. The entrapped aqCDDP content in the nanoparticles was calculated using the following equation:

$$Drug\ loading\ efficiency = \frac{mass\ of\ aqCDDP\ in\ nanoparticles}{mass\ of\ aqCDDP\ loaded\ nanoparticles}$$

3.1.4. Targeted nanoparticles

Chitosan (CS) was dissolved in 0.1 M acetic acid (1 mg/ml), and the chitosan solution was adjusted to pH 6.5. The nanoCaP^{CDDP} suspensions were added to the chitosan solution at a 1:1 volume ratio, and the mixtures were incubated for 10 min at room temperature. The resultant nanoparticles were collected *via* centrifugation at 14,500 rpm for 40 min and washed twice with MilliQ® water to remove excess chitosan. The nanoparticle pellets were then resuspended in MilliQ® water to obtain chitosan-coated nanoparticles (CS-nanoCaP^{CDDP}). HA in DI water (10 mg/ml) was reacted with CS-nanoCaP^{CDDP} for 10 min at room temperature. Finally, targeted nanoparticles (HA-CS-nanoCaP^{CDDP}) were collected and washed using the procedure described above. HA-CS-

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