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In situ doxorubicin–CaP shell formation on amphiphilic gelatin–iron oxide core as a multifunctional drug delivery system with improved cytocompatibility, pH-responsive drug release and MR imaging

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ABSTRACT

An amphiphilic gelatin–iron oxide core/calcium phosphate shell (AGIO@CaP-DOX) nanoparticle was successfully synthesized as an efficient anti-cancer drug delivery system, where doxorubicin (DOX) as a model molecule was encapsulated by electrolytic co-deposition during CaP shell formation. The shell of CaP precipitate played a pivotal role, not only in acting as a drug depot, but also in rendering the drug release rate in a highly pH-dependent controlled manner. Together with MR imaging, highly biocompatible drug-carrying CaP shell and efficient cellular internalization, the AGIO@CaP-DOX nanoparticles developed in this study area promising multifunctional nanodevice for nanotherapeutic approaches.

1. Introduction

Advanced delivery systems for anticancer drugs often use environmental stimuli to trigger a desired dose for therapeutic medication, while enhancing medical efficacy with fewer side effects. For example, the extracellular pH in tumor tissue is lower than that in normal tissue, and the pH-responsive carriers would accelerate drug release in tumor tissue [1]. In addition, the acidic cellular environment, such as in endosome and lysosome, also increases the efficacy of anticancer drugs via pH-initiated release of drugs [2]. The common delivery nanocarriers, such as liposomes [3], nanohydrogels, mesoporous silica nanoparticles and micelles [4], can easily solubilize and encapsulate poorly water-soluble drugs into their core structure. However, these candidates are also physico-chemically unstable and, consequently, subject to unexpected drug leakage. Therefore, it is essential to prevent or minimize natural leakage upon practical medication and, in the meantime, to improve efficiency in the delivery of drug carriers via surface modification, such as forming a thin shell on the surface of drug-carrying nanoparticles [5,6]. Numerous studies in the literature over the past decades have employed organic substances to modify the surface of carriers with improved biofunctionalities [7], including biocompatibility [8], stimuli-triggered release [9] and molecular recognition (targeting capability). However, reports addressing the use of inorganic substances such as silica [10,11], titania [12] and calcium phosphates [13,14] in the development of nanoparticulate drug delivery systems are comparatively less extensive.

Calcium phosphate (CaP) precipitates have long been well recognized as highly biocompatible and bioactive substances, based on their homology with natural inorganic materials such as teeth and bones. CaP has received much attention in the biomedical community, with several advantages over existing organic and inorganic counterparts [15]. First, CaP is able to bind and encapsulate drug or nucleic acids [16,17], and its extraordinary bioactivity and biocompatibility enables protection of the encapsulated molecules from enzymatic degradation, while efficiently delivering into cells [18]. Second, the delivery activity is probably related to the fact that CaP rapidly dissolves in the acidic environment and remains stable in a natural environment. Third, CaP is expected to be dissociated in endosomes/lysosome to induce endosomal/lysosomal membrane disruption with high osmotic pressure, and release its cargo into the cytoplasm [19]. Recently, the use of CaP as a surface modifier to form a shell structure on a drug-containing core phase for drug delivery with improved cytocompatibility has received considerable attention [20,21]. However, advanced drug delivery systems with multifunctionality have been an emerging trend in the development of modern biomedical technology, especially in nanomedicinal applications. The combining merits of improved cytocompatibility, strong MR imaging contrast, and highly environmental response from a controlled assembly of individual components ensure a promising drug delivery vehicle for

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advanced therapeutic purposes. In this work, a core–shell nanoparticle using a self-assembled amphiphilic gelatin was developed in the present authors' laboratory to direct the aggregation of iron oxide nanocrystallites [22] and form amphiphilic gelatin–iron oxide (AGIO) nanoparticles. Following rapid precipitation of CaP mineral with a controlled Ca/P ratio, a thin layer along the surface of AGIO can be constructed. To further facilitate drug loading during CaP shell formation, an anticancer drug doxorubicin (DOX) was employed as a model molecule to explore the feasibility of simultaneous co-deposition of the DOX–CaP layer on the AGIO surface. With the design, it was found that the shell of CaP precipitate played a pivotal role not only in acting as a drug depot, but also in rendering the drug release rate with a highly controlled, pH-dependent manner. While the core–shell nanoparticles are well characterized in terms of nanostructural evolution, chemical structure, imaging modality and drug release behavior, these drug-loaded nano-objects also demonstrated high biocompatibility and efficient cellular internalization toward the HeLa cells, suggesting a promising multifunctional nanodevice for nanotherapeutic approaches.

2. Experimental

2.1. Materials

Absolute ethanol (99.5%), benzyl ether (99%), 1,2-hexadecanediol (97%), oleic acid (90%), oleylamine (>70%) and iron(III) acetylacetonate were purchased from Aldrich Chemical Co. Gelatin type A (Bloom 300), hexanol anhydride, calcium chloride, ammonium dihydrogen phosphate and sodium hydroxide were supplied by Sigma. Fluorescein isothiocyanate (FITC, Sigma) was used to label the nanoparticles for visualization under a fluorescence microscope. Doxorubicin hydrochloride was used as the model drug obtained from Sigma.

2.2. Synthesis of monodisperse iron oxide nanoparticles

Monodisperse iron oxide nanoparticles were synthesized by a method developed by Sun et al. [23]. Briefly, iron oxide nanoparticles were synthesized by mixing 2 mmol $Fe(acac)_3$ (iron III acetylacetonate), 10 mmol 1,2-dodecanediol, 6 mmol oleic acid, 6 mmol oleylamine and 20 ml benzyl ether under a constant flow of nitrogen. The mixture was stirred and preheated to reflux (200 °C) for 30 min, and then brought to 300 °C for another 1 h under nitrogen. The black–brown mixture was allowed to cool to room temperature, and then 50 ml ethanol was added for precipitation process. The products were collected by centrifugation at 6000 rpm for 10 min and then washed four times with an excess of pure ethanol. The product, iron oxide nanoparticles, was centrifuged to remove solvent, and redispersed in hexane.

2.3. Synthesis of AGIO nanoparticles

Amphiphilic gelatin was developed in the present authors' laboratory [22]. Briefly, 1.25 g of gelatin was taken up in 20 ml water, and the suspension was gently mixed with 2 ml of 0.1 M NaOH solution, and then the solution was stirred for 0.5 h at 70 °C. Subsequently, 4 ml of hexanol anhydride were added to 20 ml of gelatin hydrolyzate with stirring at 70 °C. After a reaction time of 5 h, the mixture was cooled to room temperature and adjusted with dilute sodium hydroxide to a pH value of 7.4. The resulting solutions were collected by dialysis tubing cellulose membrane after dialysis with ethanol solution (25% v/v) for 24 h. To obtain the powder form, the gel was then dried in the oven at 60 °C.

To prepare the AGIO nanoparticles, 5 mg of iron oxide nanoparticles were centrifuged at 6000 rpm for 10 min, and then redispersed in 0.5 ml chloroform to form a uniform organic phase. 100 mg of amphiphilic gelatin as a polymer binder was dissolved in 2 ml deionized (DI) water. After amphiphilic gelatin had been completely dissolved in the solution, the organic phase was added into the reaction solution. The mixture was emulsified for 1 min with an ultrasonicator at 50 W. During ultrasonication, the mixture was heated to evaporate the organic solvent. Afterwards, the mixture was stirred and heated again to 50 °C on a hot plate to ensure complete removal of the organic phase. The final products were washed three times with DI water, and then centrifuged at 12,000 rpm for the collection of the precipitates.

2.4. Synthesis of AGIO@CaP nanoparticles

Five milliliters of DI water was mixed with 60 μl of 0.1 M (NH_4)-H_2PO_4 and 100 μl of 0.1 M NaOH (pH 10). Then, 100 μl of 0.1 M $CaCl_2$ and 2 mg of AGIO nanoparticles prepared as described were added consecutively, while the suspension was stirred at 400 rpm under room temperature (typically 25 °C). The suspension was stirred for an additional 30 min before analysis and storage. The final product was collected via centrifugation at 12,000 rpm and decantation of the supernatant.

2.5. Characterization of nanoparticles

The morphology of AGIO nanoparticles and AGIO@CaP core–shell nanoparticles was examined using transmission electron microscopy (TEM; JEM-2100, Japan). Fourier transform infrared (FT-IR) spectra were obtained with attenuated total reflectance FT-IR (ATR-FTIR) spectroscopy, and the results were recorded on a spectrometer (Bomem DA8.3, Canada) which can be used to identify the crystallographic phase of CaP coated on AGIO nanoparticles. Meanwhile, energy dispersive X-ray (EDX) spectroscopy was used to calculate the Ca/P molar ratio of the AGIO@CaP nanoparticles. The magnetization of the AGIO, AGIO@CaP and AGIO@CaP-DOX nanoparticles was measured using a superconducting quantum interference device (SQUID; MPMS-XL7) at 298 K and ±10000 G applied magnetic field. Before carrying out the SQUID analysis, the AGIO, AGIO@CaP and AGIO@CaP-DOX nanoparticles were vacuum dried for 2 days at 60 °C.

MR in vitro assays were performed using a 7 Tesla Rodent MRI Scanner (BioSpec 70/30, Bruker Topspin, Ettlingen, Germany).T_1 relaxivity (r_1) and T_2 relaxivity (r_2) were determined by diluting AGIO, AGIO@CaP and AGIO@CaP-DOX nanoparticles with 0.5% agarose gel at Fe concentrations of 0.00, 0.06, 0.10, 0.25 and 0.47 mM. A quadrature-volume coil with an inner diameter of 112 mm was used for RF transmission and reception. Both T_1 and T_2 weighted images were obtained by the multi-slice multi-echo (RARE; TE = 10.5 ms, TR = 1300 ms, SLTH = 1 mm, acquisition matrix 384×192 field of view (FOV) = 5.0×2.5 cm^2, NEX = 10) and fast spin echo (Turbo RARE; TE = 33 ms, TR = 2500 ms, SLTH = 1 mm, acquisition matrix 384×192 FOV = 5.0×2.5 cm^2, NEX = 6) sequences, respectively.

2.6. Synthesis of AGIO@CaP-DOX nanoparticles

Five milliliters of DI water was mixed with 60 μl of 0.1 M (NH_4)-H_2PO_4 and 100 μl of 0.1 M NaOH (pH 10). To this solution were added 100 μl of 0.1 M $CaCl_2$, 2 mg of AGIO nanoparticles and 2 ml of the solution with 25 μg ml^{-1} of DOX, while the suspension was stirred at 400 rpm under room temperature (typically 25 °C). After reaction for 1 h, the suspension was washed three times with DI water and then centrifuged at 12,000 rpm to collect the products. Free DOX was removed by centrifugation at 12,000 rpm and