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Pluronic F127 nanomicelles engineered with nuclear localized functionality for targeted drug delivery

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ABSTRACT

PKKKRKV (Pro-Lys-Lys-Lys-Arg-Lys-Val, PV7), a seven amino acid peptide, has emerged as one of the primary nuclear localization signals that can be targeted into cell nucleus via the nuclear import machinery. Taking advantage of chemical diversity and biological activities of this short peptide sequence, in this study, Pluronic F127 nanomicelles engineered with nuclear localized functionality were successfully developed for intracellular drug delivery. These nanomicelles with the size ~100 nm were self-assembled from F127 polymer that was flanked with two PV7 sequences at its both terminal ends. Hydrophobic anticancer drug doxorubicin (DOX) with inherent fluorescence was chosen as the model drug, which was found to be efficiently encapsulated into nanomicelles with the encapsulation efficiency at 72.68%. In comparison with the non-functionalized namomicelles, the microscopic observation reveals that PV7 functionalized nanomicelles display a higher cellular uptake, especially into the nucleus of HepG2 cells, due to the nuclear localization signal effects. Both cytotoxicity and apoptosis studies show that the DOX-loaded nanomicelles were more potent than drug nanomicelles without nuclear targeting functionality. It was thus concluded that PV7 functionalized nanomicelles could be a potentially alternative vehicle for nuclear targeting drug delivery.

1. Introduction

Pluronic copolymers consist of hydrophilic poly (ethylene oxide) (PEO) and hydrophobic poly (propylene oxide) (PPO) arranged in ABA triblock structure: PEO–PPO–PEO. In aqueous solutions beyond their critical micelle concentration (CMC), these copolymers can self-assemble into nanomicelles with size varying from 10 nm to 100 nm. The formed nanomicelles generally feature a core-shell structure in which the inner hydrophobic core can be used to incorporate poorly soluble drugs and provide an improved pharmacokinetics as well as possess the protection effect from in-advance leakage. These unique characteristics of Pluronic micelles have been extensively explored for biomedical applications including the delivery of drugs into the central nervous system across the blood brain barrier [1], oral delivery of drugs [2] and tumor-specific delivery of antineoplastic agents [3]. Moreover, the availability of terminal hydroxyl groups of Pluronic polymer makes it easy to chemically conjugate so as to achieve multiple functionalities like active tumor targeting and improvement of the physical stability [1–4]. Hence, Pluronic micelles are considered to be one of the ideal platforms for nanomedicine [1].

F127, P123 and L121 are the most widely focused Pluronic copolymers. In particular, F127 (PEO_{100}–PPO_{65}–PEO_{100}) has been used in a variety of pharmaceutical formulations including gene delivery [5–7], tissue engineering and diagnosis [8–11], drug carriers [1,12–18]. Fan et al. [7] fabricated hollow nanospheres from β-cyclodextrin modified F127 for gene delivery. These hollow nanospheres were demonstrated to enable comparable or even higher gene transfection efficiency in comparison with polyethylenimine (PEI, 10 kDa), while showing a 100 times lower cytotoxicity than PEI 10 kDa. Lee et al. [11] recently generated a polydioxanone/Pluronic F127 (PDO/F127) scaffold for tissue-engineered bone formation. Their experimental results suggest that PDO/F127 scaffold could provide a suitable environment for the osteoblastic differentiation of the periosteal-derived cells. Zhang et al. [12] synthesized a new type of Pluronic F127/chitosan-based nanocapsules with a core-shell structure, which shows interesting thermal sensitivity and wall-permeability as well as the capability of encapsulating small therapeutic agents. Further experimental studies indicate that these nanocapsules are very promising for treating diseases, particularly when the treatment is combined with cryotherapy.

Despite of the above achievements, the lack of active targeting capability impedes the maximum therapeutic effects for Pluronic copolymers as drug carriers. Therefore, there have been recent intensive efforts to address this issue aiming at improving the anti-cancer effect, by developing novel targeting drug delivery systems [19–21]. One of the molecules most widely used as targeting ligand to develop ligand-drug conjugate is folic acid [22], which displays high affinity for the folate receptors overexpressed in most of malignant tumor

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cells. Lin et al. [20] developed a type of multifunctional magnetic nanoparticles (MNPs) based on folate-modified F127 (FA-PF127) and polyacrylic acid-modified iron oxides (PAAIO). These MNPs could be simultaneously applied as a diagnostic and therapeutic agent that specifically targets cancer cells. Zhang et al. [21] recently reported on the obtaining of folic acid functionalized Pluronic P123/F127 mixed micelles encapsulating paclitaxel (FPF-PTX). The aim of this study was to combine the potential of folic acid to selectively target the cancer cells and the capacity of Pluronic block copolymers to overcome multidrug resistance (MDR) in MDR tumors resulting in their sensitivity to various anticancer agents [3]. They found out that the cellular uptake of FPF-PTX was higher than that of the micelles without the folate ligand (PF-PTX). Furthermore, in vitro studies of cytotoxicity, cell apoptosis and cell cycle arrest have also revealed that FPF-PTX exhibited more potent effects than control samples represented by PF-PTX and free PTX. These results were well correlated with in vivo studies showing an enhanced antitumor efficacy of FPF-PTX micelles.

In this study, a seven amino acid peptide PKKKRKV (Pro-Lys-Lys-Lys-Arg-Lys-Val, PV7), was chosen to harness the Pluronic micelles in order to achieve a nuclear targeting drug delivery system, since this amino acid sequence is capable of recognition by nucleus transport proteins facilitating the nucleus internalization [23–25]. Anticancer drug, doxorubicin (DOX), was chosen as the model drug to assess the therapeutic effect of the PV7 functionalized F127 nanomicelles for intracellular drug delivery. Multiple mechanisms have been proposed to explain the cytostatic and cytotoxic effects of DOX but the main mechanism is considered to be associated with its incorporation in between two nitric bases of DNA double helix and subsequent tumor cell apoptosis. Thus, nuclear-targeted delivery of DOX would be expected to enhance its anticancer efficacy. Both in vitro cytotoxicity and apoptosis studies were performed to evaluate the biological activity of DOX in aiding with the targeting delivery system.

2. Materials and methods

2.1. Materials

F127, N-Hydroxysuccinimide (NHS), N, N, N-triethylamine (TEA), Nile red (NR), maleic anhydride, N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC · HCl,), deuterated dimethyl sulfoxide (DMSO-*d6*) were purchased from Aladdin and used as received. Doxorubicin hydrochloride (DOX · HCl, Zhejiang Hisun Pharmaceutical Co., Ltd, China) was desalinated before use. Chloroform ($CHCl_3$), was dried by refluxing over CaH_2, and distilled before use. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, EDTA-trypsin or EDTA free trypsin, Dulbecco's phosphate buffered saline (DPBS) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Gibco Invitrogen Corp. 4% paraformaldehyde was purchased from DingGuo Chang Sheng Biotech. Co., Ltd. The dialysis membranes (Spectra/Por 7, MWCO 8000–12,000 Da) were purchased from Spectrum Laboratories Inc. Annexin V-FITC Apoptosis Detection Kit and propidium iodide (PI) were obtained from KeyGEN Biotech. Co. Ltd.

2.2. Synthesis of F127–PV7

2.2.1. Synthesis of F127–COOH

F127 (2.0 g, 0.16 mmol) and maleic anhydride (0.34 g, 3.5 mmol) were dissolved in distilled $CHCl_3$ (10 mL) and the resulting solution was allowed to react for 24 h under stirring at 70 °C. Following completion of the reaction, the solution was concentrated and poured twice into an excess amount of ice cold diethyl ether to precipitate the reaction product. Then, F127–COOH was dried under a vacuum dehydration and collected as white power.

2.2.2. PV7 conjugation onto F127–COOH

F127–COOH (0.5 g, 0.04 mmol) was dissolved in distilled water by ultrasound treatment. To above solution 1.2 equiv EDC · HCl (9.2 mg, 0.048 mmol) and NHS (5.5 mg, 0.048 mmol) were added. The mixture was stirred at room temperature for 5 h to activate the carboxyl groups of F127–COOH, then PV7 (0.1 g, 0.12 mmol) was added into the mixture and kept stirred overnight. Thus the obtained F127–PV7 was purified by exhaust dialysis and then collected for freeze-drying until further use.

2.3. Assessment of physical properties

The structural characterization of F127–PV7 was confirmed by Fourier transform infrared spectroscopy (FT-IR) and proton nuclear magnetic resonance spectroscopy (^{1}H NMR, recorded on a Bruker DMX-500 NMR spectrometer with DMSO-*d6* as solvents). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was performed using the Applied Biosystems 4700 Proteomics (TOF/TOF) Analyzer (Framingham, MA, USA). The UV Nd:YAG laser was operated at a 200 Hz repetition rate wavelength of $\lambda = 355$ nm. Accelerated voltage was operated at 20 kV under batch mode acquisition control. The size of the micelles was measured by dynamic light scattering (DLS) (Malvern Instruments Ltd., Worcestershire, UK). The micellar morphology was determined by transmission electron microscopy (TEM, Tecnai-12 Bio-Twin, FEI, Netherlands) at an acceleration voltage of 100 kV. The samples for TEM analysis were prepared by dropping micelle solution on the copper grid followed by staining with phosphotungstic acid and dried at room temperature for 24 h. The fluorescence spectra were recorded on a Hitachi F2500 luminescence spectrometer.

2.4. Micelle formation

Micelles of F127 and F127–PV7 in aqueous media were prepared by dialysis method at room temperature. The block polymer (4.0 mg) was dissolved in 2.0 mL DMF at initial concentrations of 2.0 mg mL^{-1}. The mixtures were transported into dialysis membrane tubes (MWCO = 8000–12,000 Da) and dialyzed against deionized water (1000 mL) for 24 h by refreshing the water several times.

2.5. Critical micelle concentration (CMC) measurement

The CMC values of F127 and F127–PV7 were determined by using pyrene as the hydrophobic florescent probe [26]. The typical procedure is described as follows: a solution of pyrene (7×10^{-6} mol L^{-1}) in 100 μL acetone was added to a cuvette. After the evaporation of the acetone, 2 mL aqueous F127 or F127–PV7 solutions at different concentrations were added to the relevant cuvettes containing pyrene residue. The solutions are then sonicated for 5 s and kept in the dark for 24 h to reach the equilibrated solubilization of pyrene in aqueous phase. The fluorescent spectra were recorded on a Hitachi F-2500 luminescence spectrometer with the excitation wavelength of 310 nm. The emission fluorescence at 350 nm, 370 nm and 397 nm was monitored. The CMC was estimated by extrapolating the intensity of I_{397} at low and high concentration regions.

2.6. DOX loading and in vitro drug release measurement

DOX-loaded F127 or F127–PV7 micelles (F127/DOX or F127–PV7/DOX) were prepared by dialysis protocol. Briefly, 10 mg F127–PV7 or 40 mg F127 was dissolved in 8 mL DMF, to which 2 mg DOX · HCl desalinated by adding TEA (0.16 μL) was added. The mixture was transported in the dialysis tube to remove the unloaded DOX and the solvent DMF by dialysis in 500 mL of PBS buffer solutions (pH = 7.4, 10 mM) for 24 h at room temperature. The PBS buffer solutions were refreshed several times. For determination of drug loading