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Mastocarcinoma therapy synergistically promoted by lysosome dependent apoptosis specifically evoked by 5-Fu@nanogel system with passive targeting and pH activatable dual function

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ABSTRACT

This manuscript describes a synergistic therapy for mastocarcinoma by pH and temperature dual-sensitive nanogel, and effects of microstructure, composition and properties of nanogel on the cellular response mechanism. The extracellular internalization of nanogels was obviously enhanced, due to the passive targeting function at $T > VPTT$. Interestingly, the increased cytotoxicity was further synergistically enhanced by an unexpected apoptosis as evoked by the 5-fluorouracil loaded nanogel (FLNG). The systemically evaluation of the effectors generated from different sub-cellular organelles including endosome, lysosome, autophagosome confirmed that it was a lysomal dependent apoptosis. Such specific apoptosis was mainly attributed to its activatable protonated PEI at low pH, which caused lysosomal membrane destruction and lysosomal enzyme cathepsin B (Cat B) leakage. This Cat B was then translocated to the mitochondria resulting in mitochondrial membrane permeability increase and mitochondrial membrane potential (MMP) decrease, followed by cytochrome c (Cyt C) release. Cyt C was the main molecule that evoked apoptosis as reflected by overexpression of caspase 9. Additionally, such lysosome dependent, apoptosis was further enhanced by the passive cellular targeting at $T > VPTT$. Thus, the tumor growth inhibition was synergistically enhanced by the extracellular temperature dependent passive targeting and intracellular pH activatable lysosomal dependent apoptosis.

1. Introduction

The chemotherapeutic effects of conventional small molecular drugs are limited due to the serious side effects against normal tissues. This side effect can be obviously overcome by nanomedicine which promises higher therapeutic efficacy and lower toxicity. Many nanomedicines are being tested in clinical and preclinical trials with five nano formulations approved by FDA in clinics [1]. Currently, most of the delivery systems focus on hydrophobic drugs. On the other hand, hydrophilic anticancer drugs account as high as 60% of chemotherapeutic agents. One of the broad spectrum hydrophilic drugs is 5-fluorouracil (5-Fu) that can suppress the synthesis of nucleic acid. Wide distribution of such hydrophilic drug in blood may cause even more side effects to normal tissues for its water solubility, which indicated that seeking nanocarreris for hydrophilic drugs is highly desirable [2–3]. Nanogel is a nanosized particle with three-dimensional crosslinked networks and high water adsorption capacity [4]. Compared with liposomes and micelles, the highly porous nanogel allows for incorporation of a plethora of drug molecules. On the other hand, the crosslinked structure ensures its superior serum stability [2,3]. Therefore, nanogel is an excellent candidate for delivery of hydrophilic anticancer drugs. Several hydrogel systems with different functions have been used against cancer cells [4–8].

On the cellular level, the drug utilization is still not so high partly because of the poor cellular internalization and low intracellular release. Thus, how to improve the cellular uptaking and intracellular

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release remains a challenge. As is known to all, in the tumor microenviroment, the temperature (~39 °C) is relatively higher than that in normal tissues owing to the higher metabolism of tumor cells [9,10]. Additionally, the extra-/intra-cellular pH(~5.0–6.8) tends to be mildly lower than that of blood and normal issues (pH 7.2–7.4) [11,12]. Such unique properties are extensively utilized to explore the stimuli-responsive nanocarriers for enhancing cellular internalization and intracellular drug release. Poly(*N*-isopropylacrylamide) (PNIPAM) shows high potential in controlled drug delivery due to its well-known lower critical solution temperature (LCST ~32 °C). The endocytosis of micelles/nanogels based on PNIPAM was obviously enhanced at the tumor microenvironment with a controlled drug release profile [13–15].

On the other hand, enormous effort has been devoted to developing intelligent nanosystems which could respond to specific intracellular stimuli, especially the endosomal/lysosomal pH value. For example, a pH sensitive nanogel was stable at physiological pH values but has the capacity to rapidly release the drugs to special intracellular compartments, such as endosomes (pH 5.5–6) and lysosomes (pH 4.5–5.0) [16–18]. Noted here, the drug releasing impetus originates from the balance of osmotic pressure. Polyethylenimine (PEI) with large amounts of pH protonated amino groups is a very popular polycation to create the pH sensitive carriers for controlling intracellular drug release [19–21]. Post endocytosis, the activatable amino groups along the chain backbone endow the tunable chain dimension and membrane disruption ability. The influx of protons due to the intra- and intercellular pH value difference eventually causes high osmotic pressure or rupture of the vesicles, resulting in release of the entrapped drug [22–24]. PEI-based nanogels effectively enhance cellular uptake and control the intracellular drug release [25–29]. But few studies focus on how the changes of the chain dimension affect intracellular organelles like lysosomes, as well as if there would be some new cellular response mechanism.

Here, a nanogel based on the thermosensitive monomer PNIPAM and pH activatable PEI was prepared to form a 5-Fu loaded nanogel (FLNG). The physical-chemical properties of such dual functional nanogel, the effect of the nanogel's properties on the cellular endocytosis, intracellular drug release and anti-tumor profile were extensively investigated. In addition, the effectors from intracellular organelles such as endosome, lysosome and autophagosome induced by nanogels were systematically investigated. The cell death mechanism like apoptosis and the corresponding pathway synergistically evoked by the carrier was further disclosed by confocal laser scanning microscopy (CLSM), flow cytometer (FCM), fluorescent microscopy (FM) and western blot.

2. Experiments

2.1. Materials

N,N-methylenebis-acrylamide (BIS), tert-butylhydroperoxide (TBHP) and *N*-isopropylacrylomide (NIPAM) were purchased from TCI Chemicals Pvt. Ltd. (Shanghai, China), and purified by recrystallization for three times. Polyethylenimine (PEI) with a molecule weight of 25 kDa and 5-fluorouracil (5-Fu) were purchased from Sigma-Aldrich Co. LLC. The PEI was used as received. Cell line 293T and a human breast cancer cell line MDA-MB-231 were obtained from the American Type Culture Collection (ATCC). Four-week-old female nude Balb/c mice were purchased from Shanghai Experimental Animal Center of Chinese Academic of Sciences (Shanghai, China).

2.2. Nanogel synthesis

0.84 mg of PEI (MW = 25 kDa) were dissolved in 20 mL MilliQ H_2O and the pH value of the solution was tuned to 7.4 with HCl. Then, 0.4746 g NIPAM and 4.746 mg BIS in crystal forms were weighted and added to the PEI solution, followed by adjusting the solution volume to 30 mL with MilliQ H_2O. After complete dissolution, the solution was transferred to a 100 mL ground-bottom flask at 50 °C with highly pure N_2. After 30 min, 250 μL TBHP with a concentration of 0.01 M was dropped into the solution under stirring, and the temperature was adjusted to 80 °C with N_2 for 2 h and then O_2 overnight. The nanogel solution was purified by centrifugation and dialysis, approximately 32.5 mL stock solution was obtained and stored at 4 °C for further experiments.

2.3. Size distribution and morphology by TEM

The hydrodynamic diameter (D) and size distribution (PD.I.) were determined by ZetaSizer (Nano-ZS, Malvern Instruments, Worcestershire, UK) equipped with a He—Ne laser (633 nm) at the scattering angle of 173°. The stock nanogel solution was diluted by MilliQ-H_2O to a concentration of 0.6 mg/mL and filtrated using 0.45 mm hydrophilic membrane filters (Millipore). In the typical TEM (Hitachi, H-7000 Electron Microscope) experiments, the nanogel solution at a concentration of 1 mg/mL was dropped onto 200-mesh Formvar-free carbon-coated copper grids (Ted PellaType-A; nominal carbon thickness 2–3 nm). After water evaporation by exposing the sample to air at room temperature, it was inversely covered on a small drop of hydrated phosphotungstate (PTA) solution with a mass fraction of 2%. The TEM images were obtained at 100 kV.

2.4. Chemical structure analysis by ^1H-NMR

The stock solution was lyophilized for 24 h to obtain a nanogel powder. Then about 1.5 mL D_2O was added into the nanogel powder with gentle shaking. The ^1H spectrum of nanogel was characterized by ^1H-NMR (400 MHz, Varian Inc.). The result is shown in Supplementary document 1. The NMR characterization here is mainly used to confirm that the NIPAM is successfully grafted to the PEI chain. The molecular weight of nanogel and PNIPAM was precisely calculated by light scattering as shown following.

2.5. Physical chemistry properties characterized by dynamic/static light scattering

Static laser light scattering (SLLS) was conducted using ALV CG-3 (ALV, Germany): the measurements of the nanogels were implemented by varying the scattering angles from 30° to 150°, with a stepwise increase of 5°. Four concentrations (ca. 0.225, 0.45, 0.9 and 1.8 mg/mL) were prepared and the Zimm equation was used in analysis. Additionally, in this experiment, the physical chemistry parameters like M, R_g, A_2, R_h, ρ ($\rho = \frac{Rg}{Rh}$, the ratio of radius of gyration to the hydrodynamic radius) and surface potential were systemically investigated by SLLS as listed in Table 1. The experiment details are shown in Supplementary document 2 [9,23].

2.6. Temperature sensitive properties

UV-VIS spectrometer (Cary-100, Varian) fitted with temperature

Table 1
Physical chemistry properties of the PNIPAM/PEInanogel.

R_h (nm)[a]	R_g (nm)[b]	M_W (g/mol)[b]	A_2[b]	ρ[c]	LC[d]	ξ(mV)[e]
151 ± 3	183 ± 5	8.91×10^8	1.48×10^{-9}	1.21	50%	10.2

[a] The R_h was determined by DLLS.

[b] The R_g, M_W, and A_2were obtained in SLLS, the detail experiments were described in Supplementary data 2.

[c] ρ was calculated by the ratio of R_g/R_h.

[d] The loading content indicated the $100*W_{drug}/W_{nanogel}$.

[e] The surface potential was tested by the Nano-ZS, Malvern Instruments.