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Predicting the *in vivo* accumulation of nanoparticles in tumor based on *in vitro* macrophage uptake and circulation in zebrafish

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ABSTRACT

Nanoparticles have resulted in great progress in biomedical imaging and targeted drug delivery in cancer theranostics. To develop nanoparticles as an effective carrier system for therapeutics, chemical structures and physicochemical properties of nanoparticle may provide a reliable means to predict the *in vitro* characteristics of nanoparticles. However, *in vivo* fates of nanoparticles, such as pharmacokinetics and tumor targeting efficiency of nanoparticles, have been difficult to predict beforehand. To predict the *in vivo* fates of nanoparticles in tumor-bearing mice, differences in physicochemical properties and *in vitro* cancer cell/macrophage uptake of 5 different nanoparticles with mean diameter of 200–250 nm were comparatively analyzed, along with their circulation in adult zebrafish. The nanoparticles which showed favorable cellular uptake by macrophages indicated high unintended liver accumulation *in vivo*, which is attributed to the clearance by the reticuloendothelial system (RES). In addition, blood circulation of nanoparticles was closely correlated in adult zebrafish and in mice that the zebrafish experiment may elucidate the *in vivo* behavior of nanoparticles in advance of the *in vivo* experiment using mammal animal models. This comparative study on various nanoparticles was conducted to provide the basic information on predicting the *in vivo* fates of nanoparticles prior to the *in vivo* experiments.

1. Introduction

In recent decades, nanotechnology-based medical techniques have made great progress in biomedical imaging and targeted drug delivery [1–3]. In particular, as a drug carrier system, nanoparticles have been extensively studied for tumor-targeted drug delivery. It has been shown that nanoparticles specifically accumulate in tumor sites, due to the unique property of nanoparticle structure. It is because the abnormal structures of angiogenic blood vessels in tumors allow nano-sized particles to penetrate into the tumor tissue, and the defective lymphatic systems often cause local retention of the particles. This effect is called the enhanced vascular permeability and retention (EPR) effect, and is regarded as the basic targeting strategy of nanoparticles [4]. In addition to the EPR effect, nanoparticles present prolonged blood circulation characteristics which lead to their tumor-targeted delivery in a controlled manner, compared to the free form of drug.

Based on the high tumor targeting efficiency of nanoparticles, nanoparticle development as a drug carrier system generally follows the order of 1) rational design, 2) synthesis and optimization for desired physicochemical properties, 3) *in vitro* test, 4) *in vivo* test, and 5) preclinical or clinical trials, *etc.* In the process of development, the physicochemical properties of nanoparticles may provide useful information in predicting stability, solubility, drug-loading efficacy, toxicity, and tumor-targeting efficiency. However, unfortunately, many of the developed nanoparticles which showed favorable *in vitro* results failed to advance past the *in vivo* stage due to the unintended non-specific accumulation of nanoparticles *in vivo*. In particular, many of the nanoparticles are delivered to the liver or spleen by non-specific targeting, but not to the tumor sites, resulting in low tumor-targeted accumulation and unfavorable pharmacokinetics.

Indeed, the reticuloendothelial system (RES) is regarded as one of the primary causes of low levels of nanoparticle accumulation in tumor sites. As a part of the immune system, RES has a role in removing the systemically administered nanoparticles before they reach the target tumor tissues. Notably, one of the major RES organs is the liver,

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which contains Kupffer cells, which account for the majority (80–90%) of total fixed tissue-macrophages in the body [5,6]. Intravenously or intraperitoneally injected nanoparticles are often trapped in the liver and fail to be delivered to the tumor [7,8]. In practice, many of the nanoparticles with diameters ranging from 150 to 300 nm were found in the liver and spleen as a result of rapid hepatic clearance [8,9]. Since Kupffer cells represent approximately 35% of the non-parenchymal liver cell population in normal adult mice [10], nanoparticles in the *in vivo* experiment using the mouse model usually show especially high accumulation in the liver. It is critical to understand the mutual relationship between the *in vivo* tumor-targeting of nanoparticles and their properties revealed in pre-*in vivo* studies. A reliable prediction for *in vivo* fates of nanoparticles may reduce costs and facilitate the development of drug-carrying nanoparticles. However, the correlation between pre-*in vivo* characteristics and non-specific liver targeting of nanoparticles has not been fully understood.

Herein, we carefully compared five different nanoparticles to identify the correlation between the characteristics of individual nanoparticles and relevant *in vivo* behaviors. In order to rule out the size-dependent effects of nanoparticles in the *in vivo* distribution, we prepared 5 different nanoparticles with hydrodynamic diameter of 200–288 nm, including polystyrene nanoparticle (PSNP), titanium dioxide-encapsulated carboxymethyl dextran nanoparticles (TDNP), dextran sulfate nanoparticle (DSNP), hyaluronic acid nanoparticle (HANP), and glycol chitosan nanoparticle (GCNP). In particular, *in vitro* cancer cell/macrophage uptake, availability of blood circulation in zebrafish, and biodistribution in tumor-bearing mice were extensively analyzed to identify the potential correlation. A comprehensive study on the correlation of the *in vitro* and the *in vivo* behaviors of the nanoparticles was conducted to contribute to a more reliable prediction of the *in vivo* distribution and the pharmacokinetics of nanoparticles, which will lead to more efficient development of nanoparticles as drug carriers.

2. Materials and methods

2.1. Materials

A polystyrene-based nanoparticle, FluoSpheres® Carboxylate-Modified Microspheres (0.20 μm), was purchased from Life technologies™ (Warrington, PA). Carboxymethyl dextran (Sigma-Aldrich, St. Louis, MO), titanium dioxide (TiO_2; ≥99%, Sigma-Aldrich), sodium hyaluronate (M_w = 230 KDa; Lifecore Biomedical, Chaska, MN), dextran sulfate sodium (M_w = 36–50 kDa; MP Biochemicals, Santa Ana, CA), and glycol chitosan (M_w = 250 KDa, degree of acetylation = 82.7%; Sigma-Aldrich) were purchased as base-materials for formulating nanoparticles. Additional purification was performed before use by dialyzing against distilled water, followed by lyophilization. Dopamine, *N*-hydroxysuccinimide (NHS), 5β-cholanic acid, 1-ethyl-3-(3-dimethyl laminopropyl)-carbodiimide hydrochloride (EDC), 1-hydroxybenzotriazole (HOBt), adipic acid dihydrazide (ADH), and ethylenediamine were all purchased from Sigma-Aldrich. A near infrared fluorescence (NIRF) dye, FNR-675, was provided from BioActs (Incheon, Korea). Methanol and anhydrous dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). The listed chemicals were of analytical grade and used without further purification.

2.2. Preparation of nanoparticles

PSNP was diluted in phosphate buffered saline (PBS) to be used without further modification. TDNP, HANP, and GCNP of similar particle size were prepared as previously reported [11–13], and DSNP was prepared by the identical method that was used to prepare HANP and GCNP. Briefly, TDNP was prepared by chemically conjugating carboxymethyl dextran to TiO_2 nanoparticles through dopamine functionalization. The dopamine functionalization was performed by adding aqueous dopamine solution (192 μM in distilled water, 100 μL) to TiO_2 (5 mg/2.5 mL formamide) and mixing by stirring for 2 h. The resulting mixture was centrifuged at 13,000 rpm and washed with formamide. The dopamine-functionalized TiO2 nanoparticles were then suspended in 5 mL formamide. Separately, carboxymethyl dextran (100 mg/10 mL formamide) was stirred into EDC and NHS mixture overnight to activate the carboxylic acid groups. Then, dopamine-modified TiO_2 nanoparticles (5 mg) were added to the carboxymethyl dextran solution drop by drop. After 10 h, NaOH solution (0.1 M, 200 μL) was added and the mixture was dialyzed (molecular cut off = 50 KDa) against sodium borate buffer (pH 8.6) at 4 °C. After the dialysis, the mixture was sonicated (sonic vibracell VCX 750, 30% amplitude) and filtered through a 0.8 μm acetate syringe filter to remove any large aggregates. The resulting solution was then freeze-dried. The product was dissolved in PBS and filtered through a 0.45 μm acetate syringe filter before use. The self-assembled nanoparticles – DSNP, HANP, and GCNP – were prepared by chemically conjugating 5β-cholanic acid to each polymer. Hydrophobic 5β-cholanic acid was chemically conjugated to the hydrophilic dextran sulfate, hyaluronic acid, or glycol chitosan by forming amide bonds in the presence of EDC and NHS. The degree of substitutions (DS, defined as the number of 5β-cholanic acid molecules per 100 sugar residues) were controlled to be 9, 10 and 4.7, respectively. The resulting amphiphilic polymer conjugates were dispersed in PBS by using probe-type sonicfire (Ultrasonic Processor, GEX-600; 90 W, 2 min) to be self-assembled into nanoparticles. Finally, the nanoparticles were chemically labeled with NIRF dye, FNR-675 (0.1 wt.%), for *in vitro* and *in vivo* imaging analyses.

2.3. Physicochemical characterization of nanoparticles

The hydrodynamic sizes of the nanoparticles were measured by dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern Instruments, Westborough, MA) with a 633 nm He-Ne laser at 25 °C. The zeta potentials of the nanoparticles were determined in distilled water and PBS (pH 7.4) by electrophoretic light scattering. All measurements were performed in triplicates and described as mean value ± standard deviation. The morphologies of the nanoparticles were observed using transmission electron microscopy (TEM, CM30 electron Microscope, Philips, CA). TEM samples (final concentration of 0.1 mg/mL, diluted in distilled water) were prepared in grid. HANP, DSNP, and GCNP were negatively stained with 2 wt.% uranyl acetate, and PSNP and TDNP were observed without staining.

2.4. In vitro cellular uptake of nanoparticles

Murine macrophage cells (Raw 264.7) and squamous cell carcinoma cells (SCC7) were purchased from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 medium, respectively. Both types of media were supplemented with 10% fetal bovine serum (FBS, Invitrogen, Burlington, Canada), penicillin (100 units/mL), and streptomycin (100 μg/mL). The cells were grown in a humidified incubator at 37 °C containing 5% CO_2 (Thermo Fisher Scientific Inc., USA), and maintained by splitting when 80% confluency was reached.

To visualize *in vitro* cellular uptake of nanoparticles, Raw 264.7 and SCC7 cells (5×10^4 cells/35 mm^2 cover glass-bottomed dish) were exposed to the fluorescence-labeled nanoparticles (25 μg/mL each) for 0.5, 1, 3, and 6 h. After the incubation, the cells were washed twice with PBS (pH 7.4) and fixed with 4% paraformaldehyde solution for 10 min. The nuclei of the cells were stained with diamidino-2-phenylindole (DAPI), and the cells were observed under a confocal laser scanning microscopy (Olympus, Tokyo, Japan) equipped with 405 diode (405 nm) and He-Ne-Red (633 nm) lasers. The NIRF signals in the cytoplasm of RAW 264.7 and SCC7 cells were quantified with