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Full Length Article

The effects of ligand valency and density on the targeting ability of multivalent nanoparticles based on negatively charged chitosan nanoparticles

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ABSTRACT

It has been shown that multivalent ligands could significantly enhance the binding avidity compared with the monovalent ones; therefore, once incorporated into nanoparticles, they promote superior targeting ability without increasing the ligand density. Although ligand valency and density play a key role on the targeting ability of corresponding nanoparticles, these facotrs remain largely unexplored and detailed studies are lacking. Herein, a series of multivalent ligands with certain valencies (FA_n, n indicates the valency of ligand: $n = 3, 5, 7$) has been conveniently synthesized by conjugating different copies of folate ligands with poly(acrylic acid) (PAA). Negatively charged chitosan nanoparticles (CTS-SA NPs) have been utilized as proper multivalent platforms because they can strongly suppress non-specific protein adsorption and cellular uptake without interfering with the targeting ability of multivalent ligands. Subsequently, the structure of CTS-SA NPs has been modified using different amounts of FA_n to form multivalent nanoparticles (FA_n-CTS-SA NPs) with various valencies and densities. A series of specific investigations of them suggested that the cellular uptake of multivalent nanoparticles has largely varied with the ligand valency variation even at similar ligand densities; and also largely varied with ligand density variation even at the same ligand valencies. The intermediate valency and density values determined in the current study (*ie.*, 5 and 2.4 wt%, respectively) have provided the best cellular uptake, facilitating superior targeting ability at relatively low ligand valency and density. Unexpectedly, no conspicuous difference has been observed during endocytotic inhibition assays with single inhibitors, which may be attributed to the synergetic endocytotic mechanism with multiple pathways of multivalent nanoparticles. The optimal multivalent nanoparticles have also exhibited excellent biocompatibility, long-term stability *in vitro* and enhanced circulation time *in vivo*, thus demonstrating their potential for targeted drug delivery.

1. Introduction

Targeted drug delivery has emerged as a propitious alternative to other cancer therapies due to its unique accumulation behavior at the tumor site and minimum side effects on normal tissues [1,2]. Over the past years, researchers have attempted to improve this strategy by increasing the ligand density of targeted nanoparticles [3,4]. However, most of the targeting ligands are exogenous antibodies [5] or hydrophobic molecules [6–9]. It is worth mentioning that high ligand density may induce *in vivo* immunogenicity [10,11], reduce the aqueous solubility, or even cause aggregation of nanoparticles [7,12,13], eventually triggering their recognition and clearance by the reticuloendothelial system (RES). Therefore, the way to maximize the targeting ability without increasing the ligand density remains an essential and challenging issue in the field of targeted nanoparticles.

A multivalent ligand comprises of multiple copies of ligands conjugated onto a central scaffold (such as branched molecules [14,15], calixarene [16], β-cyclodextrin [17], and polymers [18–20]) to generate a ligand cluster. Multivalent interactions occur when multiple ligands of one entity bind simultaneously to multiple receptors of another one (*eg.*, cell surface [20,21]) or to multiple binding sites of one receptor [22–24], strongly enhancing the binding avidity over

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the monovalent ligand [25–27]. For example, Mark R. Hardy and coworkers have previously indicated that synthetic tetra- and trivalent derivatives of oligosaccharides have exhibited superior avidity toward hepatic Gal/GalNAc lectin in contrast with the monovalent analogue, even under the same concentration [14]. The excellent potential of multivalent ligands has been also emphasized by studying a series of monomeric, dimeric and multimeric aptamer-siRNA conjugates, with the multivalent comb-type aptamer-siRNA (Comb-Apt-siR) showing the strongest intracellular uptake of targeted MCF-7 cancer cells [19].

Additionally, several studies have focused on multivalent ligands modified solid surfaces, showing that these could improve the adhesion of targeted molecules or cells to surfaces. For instance, Chen's group has reported that β-cyclodextrin-based heptavalent lysine [CD$(Lys)_7$] surfaces possess higher plasminogen binding ability compared with the monovalent ones [28], and the heptavalent mannose conjugates (CD-M) modified surfaces have exhibited superior capacity toward capturing the mannose-specific type 1 fimbriated bacteria [29]. We have previously reported that the multivalent REDV ligands modified-alginate surface (cREDV-ALG) have better promoted the adhesion and proliferation of human umbilical vein endothelial cells (HUVECs) compared with the monovalent surface [30].

Starting from 2010 and accounting on their promising potential as discussed, further attempts on multivalent ligands have been developed toward improving the targeting ability of drug delivery nanoparticles [31–35]. Paula T. Hammond has reported that multivalent folate-containing nanoparticles based on functionalized linear-dendritic polymers (LDPs) have significantly enhanced the cellular uptake of the dendritic nanoparticles [31]. Subsequently, Davide Prosperi et al. have synthesized a tetravalent mannose ligand based on per-substituted calixarene, showing that its functionalization with gold nanoparticles has remarkably improved the targeting efficiency to mannose receptor-overexpressing cancer cell HeLa over the monovalent analogue [34]. A recent study reported by Jian Yin has revealed that amphiphilic β-cyclodextrins (β-CDs) have self-assembled into heptavalent mannose-functionalized nanoparticles, facilitating the targeted delivery of anticancer drugs toward MDA-MB-231 breast cancer cells, while inhibiting the tumor growth *in vivo* [35].

Despite the progress that multivalent drug delivery systems have been achieved so far, various issues still exist. For instance, it has been proposed that the incorporation of PEG corona into multivalent nanoparticles would hinder the ligands' binding to cellular receptors and this shielding effect inhibits their recognition and endocytosis by tumor cells [33,36,37]. On top of these limitations, the synthesis of dendrimers is laborious, and several unique features of dendrimers, namely their well-defined structure and abundant terminal groups, are minimized or even lost after chemical functionalization and encapsulation of theranostic agents [38,39]. The modulation of the ligand valency toward targeting ability optimization is not possible in the case of calixarene and β-cyclodextrin based multivalent ligands, because only per-substituted ligands can be obtained [28,29,34,35]. The ligand valency and density impact on the targeting ability of multivalent nanoparticles remains largely unexplored in the literature, and detailed studies are lacking. Hence, the production of multivalent nanoparticles is complicated and challenging. Further work is required for a comprehensive understanding not only of the optimization process of multivalent ligands, but also for the construction of appropriate multivalent nano-platforms.

Chitosan is a biocompatible, natural polysaccharide commonly used in drug delivery [40,41]. However, its cationic nature leads to chitosan nanoparticles to strongly interact with the negatively charged serum components, and triggers severe aggregation and rapid clearance from circulation [42,43], thus limiting its *in vivo* applications. In order to mitigate the aforementioned limitation, we have previously reported that negatively charged chitosan nanoparticles could possess improved stability under biological conditions [43]. Herein, we report the modification of chitosan nanoparticles with succinic anhydride (SA) which is a small molecule that provides a negative surface, thus achieving excellent stability in plasma solution, while avoiding the adverse shielding effect [42]. Furthermore, a series of multivalent folate ligands having different valencies has been synthesized by conjugating the folate with poly(acrylic acid) (PAA) scaffold. PAA plays a key role due to its hydrophilic nature that enhances the water solubility of hydrophobic ligands. Also, its flexible configuration allows for the multivalent ligands responding to the conformational requirements of receptors and exploring the optimal spatial arrangement to bind the targeted cells [44,45]. Accounting on these facts, miscellaneous amounts of multivalent ligands with different valencies have been incorporated onto biocompatible chitosan nanoparticles. This has allowed us to design and construct a series of specific multivalent nanoparticles with different ligand valencies and densities, and their effects on the targeting ability and uptake mechanism of multivalent nanoparticles have been studied. Multivalent nanoparticles are expected to provide superior targeting ability at low ligand density by optimizing the ligand valency and density.

2. Materials and methods

2.1. Materials

Chitosan (CTS, 5–20 mPa s, 0.5% in 0.5% acetic acid at 20 °C) with deacetylation degrees of 75.0–85.0% was purchased from TCI Co. Ltd. (Japan). FITC-labeled chitosan (FITC-CTS) was synthesized according to our previous study [9]. Poly(acrylic acid) (PAA, number-average molecular weight of 3600, M_{PAA} = 3600), folate (folic acid), sodium tripolyphosphate (TPP), succinic anhydride (SA), 1-dimethylaminopropyl-ethylcarbodiimide hydrochloride (EDC), *N,N′*-dicyclohexylcarbodimide (DCC), and *N*-Hydroxysuccinimide (NHS) were purchased from Aladdin (Shanghai, China). Folate-free RPMI 1640 cell culture medium was purchased from Gibco (Carlsbad, CA). Cell Counting Kit-8 (CCK-8) was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Dulbecco's modification of Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin (pen-strep) were purchased from DingGuo Biotech. Co. (Shanghai, China).

KB cells (a human epidermoid carcinoma cell line) were grown in folate-free RPMI 1640 containing 10% FBS and HepG2 (a human hepatic carcinoma cell line) were grown in DMEM containing 10% FBS under standard cell culture conditions (37 °C, humidified, 5% CO_2).

Female SD rats (220 ± 20 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). All the animal experiment procedures were carried out in strict compliance with the "Guide for the Care and Use of Laboratory Animals".

2.2. Measurements

^{1}H NMR spectra were collected on a Bruker 400 MHz spectrometer. FT-IR and MALDI-TOF MS was performed on Bio-Rad FTS 6000 and Bruker AutoflexIII LRF200-CID respectively. Dynamic light scattering (DLS) experiments were performed with a Zetasizer Nano-ZS90 instrument (Malvern Instruments Ltd., UK) at room temperature. The size of nanoparticles was examined on a Tecnai G^2F20 transmission electron microscope (FEI, USA). UV–vis spectra were obtained on SHIMADZU UV-2550 (Japan).