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Hybrids of carbon dots with subunit B of ricin toxin for enhanced immunomodulatory activity

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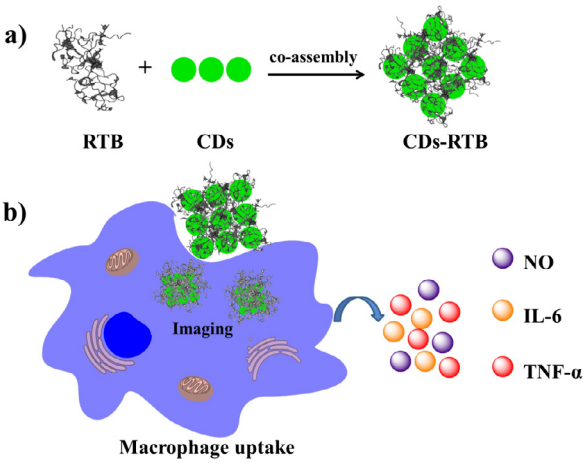
GRAPHICAL ABSTRACT



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ABSTRACT

Although Ricin toxin binding subunit B (RTB) can promote the activation of macrophages and modulate the cell-mediated immunity, its applications are severely limited due to the intrinsic properties of proteins, like poor stability and low efficacy of cellular uptake. In this work, the stable nanoparticles were prepared by supramolecular assembling of carbon dots (CDs) and RTB. The formed CDs-RTB possesses robust stability and can protect RTB against enzymatic hydrolysis. More importantly, CDs-RTB can promote macrophages proliferation, improve the generation of NO, IL-6 and TNF-α in RAW264.7 cells and increase the expression of mRNA, indicating the enhanced immunomodulatory activity of CDs-RTB. This work highlights the potential of using CDs as a simple and stable platform to assemble RTB and effectively promotes the application of RTB as the immunostimulant.

1. Introduction

Ricin toxin, one of the most lethal toxins, is a heterodimeric protein toxin produced by the seeds of *Ricinus communis* [1,2]. This

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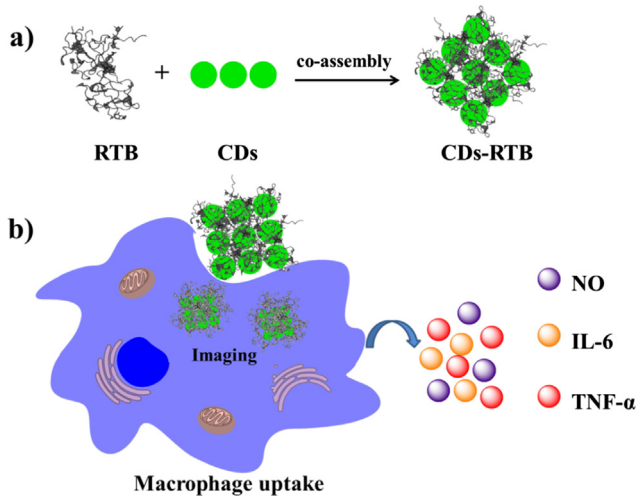
toxin consists of A and B chains that are linked by a disulfide bond. The B-chain of the ricin toxin (RTB) with the molecular weight of 34 kDa is a galactose-binding lectin protein that binds to the membrane of eukaryotic cell by interacting with cell-surface molecules, such as galactose and glycolipids [3,4]. Several reports have demonstrated that RTB can function as a carrier fused to other molecules [5–7], or act as a target for antibody-based intervention therapeutics against infectious disease [8]. Furthermore, our previous works have proved that RTB can promote the activation of macrophages and modulate the cell-mediated immunity [9,10]. However, the applications of RTB are limited due to the intrinsic properties of proteins, including poor stability in biological environments, low bioavailability, short half-life period and enzymatic hydrolysis [11–14]. To address these issues, two strategies are commonly used. One is modification of RTB, mainly including PEGylation [15,16] and recombinant fusion protein [17,18]. The other is to load RTB into the different matrixes, such as hydrogels [19–21], liposomes [22,23], inorganic nanoparticles [24,25] and carbon materials [26,27]. Although these methods could improve the efficacy of protein therapeutics, some new problems, such as complicated synthesis, cytotoxicity of carriers and reducing protein activity spring up [15,21,25]. Therefore, it is urgent to develop an effective, safe and simple delivery vector to meet these challenges.

Carbon dots (CDs), as a class of fluorescent materials, have attracted a great deal of interest due to their intriguing properties, for instance ultrasmall size, water solubility, photostability, cell membrane permeability and biocompatibility [28–31]. They can be easily obtained from graphite and organic molecules such as citric acid or glucose [32–34], and emit blue, green, and red fluorescence [35,36]. To date, CDs have been widely used in energy conversion [37,38], ion detection [39–41], bioimaging fields [42,43] and drug delivery [44,45]. For example, very recently, we have assembled CDs with EFGP for intracellular protein delivery [46]. Inspired by this work, we anticipate that CDs also could load RTB through supramolecular interactions and deliver them into cells.

In this work, RTB was assembled with CDs to form the hybrid nanoparticles as shown in Scheme 1. The formed CDs and RTB (CDs-RTB) nanoparticles possess small size and robust stability. Moreover, CDs-RTB also could activate macrophages effectively and enhance immunomodulatory activity.



Scheme 1. Schematic illustration of the preparation (a) and cellular uptake (b) of CDs-RTB.

2. Materials and methods

2.1. Materials

All chemicals and reagents were purchased from commercial sources and used without further treatment, unless indicated otherwise. D-Glucose was purchased from Beijing Chemical Works, and the purity was 99.5%. Tetraethylenepentamine was purchased from Sinopharm Chemical Reagent Co., Ltd., and the purity was 90%. RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Carlsbad, CA, USA). LPS, trypsin and Hoechst 33258 staining were purchased from Sigma-Aldrich (St. Louis, MO, USA). BCA kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). NO detection kit was purchased from R&D Systems (Abingdon, UK). IL-6 and TNF-α ELISA kits were purchased from eBioscience (Vienna, Austria). Trizol reagent kit and M-MuLV reverse transcriptase were purchased from Invitrogen (Carlsbad, CA, USA). Ethidium bromide (EB) was purchased from KeyGen Biotech Co. Milli-Q water was collected from a Milli-Q system (Millipore, USA).

2.2. The instruments for characterizations

Size distributions of the nanoparticles were determined by DLS with a Zeta-sizer Nano-ZS (Malvern Instruments Ltd.). The measurement was carried out at 25 °C and the scattering angle was fixed at 90°. Zeta potential measurement was performed using a Zeta-sizer Nano-ZS (Malvern Instruments Ltd.). The measurement was carried out at 25 °C, and the model was smoluchowski, the F (Ka) value was 1.50, the viscosity was 0.8872, RI was 1.330, dielectric constant was 78.5. Transmission electron microscopy (TEM) images were taken by a JEOL JEM-1011 (Japan) at the accelerating voltage of 100 kV. UV–vis absorption spectra were recorded via a Shimadzu UV-2450 UV–vis scanning spectrophotometer. Fluorescence emission spectra were conducted on a LS-55 fluorophotometer.

2.3. Preparation of CDs, RTB and CDs-RTB

CDs were prepared by means of a modified thermal pyrolysis route starting from tetraethylenepentamine and glucose according to our previous work [28,36]. Briefly, D-Glucose and tetraethylenepentamine were loaded into a beaker at an equivalent stoichiometric ratio. The transparent solution was heated to 125 °C and kept for 30 min, then heated to 180 °C and maintained for 30 min. The final reaction products were completely solubilized, and then subjected to dialysis against water to remove small molecules for 1 d. The resulting product was freeze-dried to obtain brown solid.

The RTB was acquired following the protocol that has been reported [9,10]. The pET28a-RTB recombinant plasmid was transformed into competent BL21 (DE3) strain. A single colony was picked from a selective plate, inoculated into LB medium containing 100 μg mL^{-1} ampicillin and cultivated overnight at 37 °C with shaking (160 rpm). Expression of recombination RTB protein was induced with isopropyl-β-d-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM for 12 h and the cells were pelleted by centrifugation at 5000*g* for 20 min at 4 °C. The bacterial cell pellets were lysed by ultrasonicate. Inclusion bodies were harvested, washed, and solubilized in 6 M urea containing a mixture of denaturant and salt. The suspension was stored at 4 °C overnight and then centrifuged at 10,000*g* for 20 min. The supernatant, after filtering through a 0.45 mm filter (Millipore, Bedford, USA), was purified by Ni-NTA agarose (Qiagen, Hilden, Germany) affinity chromatography. The purified protein was then refolded by dialysis in phosphate-buffered saline in a diminishing concentration of