Fabrication of Biocompatible and Tumor-Targeting Hyaluronan Nanospheres by a Modified Desolvation Method

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ABSTRACT: The aim of this work was to maximize the tumor targetability of biocompatible hyaluronan (HA) by construction of a novel nanocarrier, using HA as the single material. HA was prefunctionalized with active amino groups, desolvated by acetone, and cross-linked by glutaraldehyde. The process was further optimized with regard to yield, stability, and particle size. The cytotoxicity of HA nanospheres (HA-NPs) was evaluated by thiazolyl blue tetrazolium bromide and reactive oxygen species assays. A549 cells and H22-bearing Kunming mice were employed to characterize the tumor targeting of fluorescein isothiocyanate-conjugated HA-NPs. Nanospheres prepared according to the optimal formulation were characterized by a maximum yield (90%), high stability over 7 days, regular spheres (97.42 nm in diameter), and negative charge (−32.7 mV in zeta potential). *In vitro* results revealed that HA-NPs had little cytotoxicity and efficiently accumulated into A549 cells in a HA-dependent manner. Following systemic administration in mice, HA-NPs selectively accumulated in the tumor as demonstrated by the frozen section examination and flow cytometry analysis. In conclusion, this work successfully prepared HA-NPs and explored their potential applications for tumor targeting in terms of safety and efficacy. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:1529–1537, 2014

Keywords: hyaluronan; CD44; cancer; desolvation; drug targeting; nanospheres; polymeric drug delivery systems

INTRODUCTION

Hyaluronan (HA) is a linear, negatively charged polysaccharide, which is composed of repetitive disaccharide units. It is involved in the regulation of several key biological processes by binding to its endogenous receptors including clusters of differentiation 44 (CD44), HA-mediated motility receptor, lymphatic vessel endothelial HA receptor-1, and HA receptor for endocytosis.[1] Among these receptors, CD44 is reported to be expressed highly on the surface of tumor cells, especially the highly invasive tumor cells and stem cells, a discovery that is widely utilized in specific targeting therapy.[2,3]

In the field of pharmaceutics, delivery systems based on HA–CD44 interactions have been developed to facilitate drug/gene delivery. More specifically, HA-based polymeric prodrugs account for a large proportion of the existing delivery systems. Successful examples include doxorubicin, epirubicin, butyrate, paclitaxel, fluorouracil, and camptothecin.[4–9] In spite of the enhanced therapeutic efficacy, this strategy exhibits some drawbacks, a main one of which is the rapid clearance rate of HA in the blood circulation.[10]

Various nanoparticulate systems for CD44 targeting are available at present. Thanks to the abundant carboxyl and hydroxyl groups, HA could be readily modified on lipid-based nanoparticle, FeO particles, or liposome.[11–13] Alternative systems include micelles constructed by introducing hydrophobic segments (i.e., fatty amine, histidine, deoxycholic acid, cholanic acid, and ceramide)[14–18] to the HA backbone, and nanocomplexs formed between positively charged chitosan, polyetherimide, or platinum and negatively charged HA.[19–21]

It is clear that HA, the targeting molecule, only accounts for a small part within these nanoparticulate systems. Qhattal and Liu[13] concluded that the uptake efficiency of HA-grafted liposomes in CD44-overexpressing tumor cells rises with increasing grafting density. It is therefore reasonable to assume that nanospheres consisting entirely of HA would have the strongest targetability. In addition, because of the continuous distribution of HA from the interior to the exterior of the nanospheres, HA-NPs would remain its targetability during the course of degradation, providing an advantage over conventional carriers. Another advantage of HA-NPs is associated with their inherent hydrophilicity, which minimizes serum protein binding and subsequent reticuloendothelial uptake.

To the best of our knowledge, there were few literatures reporting successful preparation of HA-NPs without using composite materials. In this study, we aimed to fill this gap by developing HA-NPs via a modified desolvation method. HA-NPs were further optimized in terms of yield, stability, and particle size, and evaluated for their cytotoxicity as well as targetability.

MATERIALS AND METHODS

Materials

Hyaluronan with molecular weights (MW) of 101 and 8 kDa were purchased from Dali (Liuzhou, Guangxi, China), whereas HA with MW of 1.44 mDa was from Freda (Jinan, Shandong, China). Thiazolyl blue tetrazolium bromide (MTT), trypan blue (TB), and sodium 3-nitrobenzenesulfonate (TNBS) were obtained from Sigma–Aldrich (St. Louis, Missouri, USA). Fluorescein isothiocyanate (FITC) and adipic dihydrazide (ADH) were purchased from Aladdin (Shanghai, China). Reactive oxygen species (ROS) assay kit was purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China).

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Table 1. Formulations Used in the Optimization Study of HA-NPs

Formulation	Molecular Weight of HA (kDa)	HA–ADH (mg)	Acetone (mL)	Glutaraldehyde (μmol)
A	101	5	/	75
B	101	5	3.5	/
C	101	5	/	75
D	101	/	3.5	75
E	/	5	3.5	75

Formulation A and B were used to evaluate the effect of acetone on nondesolvated HA–ADH and the effect of glutaraldehyde on free amino groups. Formulations C, D, and E were used to assess the effect of acetone, HA–ADH concentration, and MW of HA on particle size, respectively. / means variable.

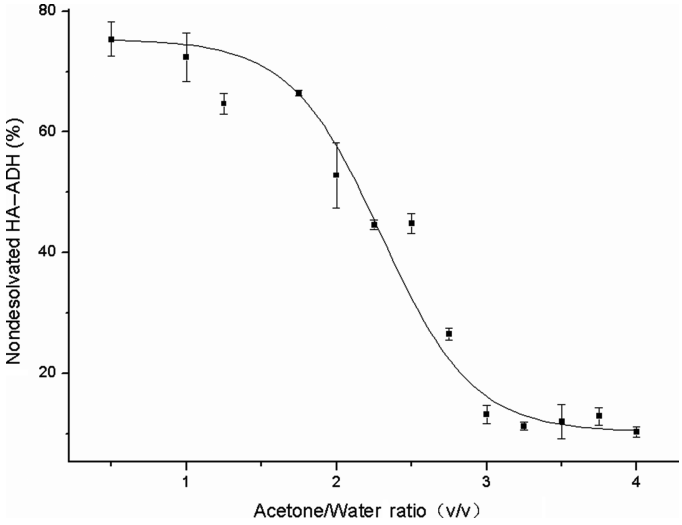

Figure 1. Percentage of nondesolvated HA–ADH in correlation with acetone volume added during the desolvation procedure.

4′,6-Diamidino-2-phenylindole) (DAPI), collagenase type IV, and DNase I were purchased from Biosharp (Hefei, Anhui, China). Sephadex G50 was purchased from Pharmacia (Uppsala, Sweden). Roswell Park Memorial Institute (RPMI-1640) medium and fetal bovine serum (FBS) were purchased from Hyclone (Logan, Utah, USA). Adenocarcinomic human alveolar basal epithelial cells (A549) and murine hepatocellular carcinoma (H22) cells were provided by the Key Laboratory of Drug Targeting and Drug Delivery Systems.

Derivatization of HA

Adipic dihydrazide (391.5 mg, 2.02 mmol) was added to a solution of HA (180 mg) in water (45 mL). After adjusting the pH of the reaction mixture to 4.75 with HCl solution, the reaction was initiated by adding 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide (86.4 mg, 0.44 mmol) under stirring at room temperature. The reaction was stopped 2 h later by adjusting the pH to 7.0. The mixture was exhaustively dialyzed against 0.1 M NaCl solution, 25% ethanol, and pure water, and then lyophilized to give HA–ADH.[22] ^{1}H-NMR (Unity Inova 400, Varian (Palo Alto, California, USA), 400 MHz, D_2O, δ, ppm): 2.235–2.207 (s, 4H, $-COCH_2$), 2.005–1.967 (t, 3H, $-NCOCH_3$), 1.638 (s, 4H, $-COCH_2CH_2$). The substitution degree of different conjugates was estimated to vary from 12.6% to 22.4% according to the ^{1}H-NMR spectrum.

Preparation and Characterization of HA-NP

Hyaluronan–ADH was dissolved in 1 mL water and desolvated by adding acetone dropwise under stirring. After addition of glutaraldehyde, the mixture was subjected to reaction for 12 h. HA-NPs were obtained after removal of acetone. Further purification was performed on Sephadex G50 for cell experiment and in *vivo* study. The optimization was conducted according to different formulations as listed in Table 1. All experiments were performed in triplicate.

The average particle size, polydispersity index (PDI), zeta potential, and count rate were measured using a dynamic light scattering analyzer (Zetasizer Nano ZS90, Malvern, UK). Before scanning electron microscope (SEM, JSM-6510LV, Jeol, Tokyo, Japan) and transmission electron microscopy (TEM, H-6001V, Hitachi, Tokyo, Japan) observation, nanospheres were coated with gold and stained with phosphotungstic acid.

In order to determine the cellular uptake by flow cytometry (FC500, Beckman Coulter, Brea, California, USA),

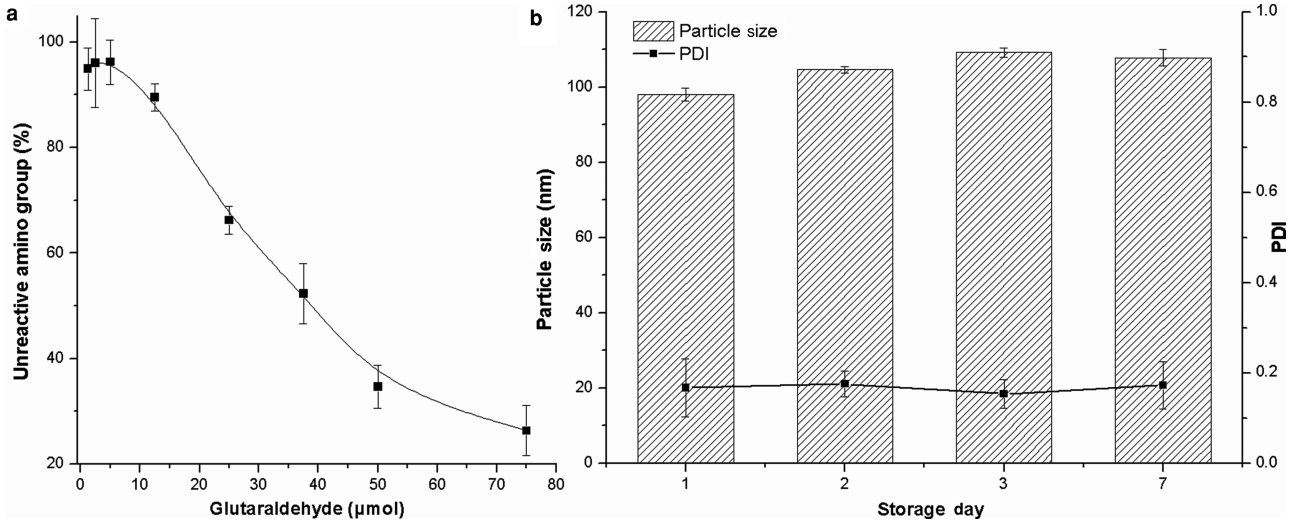

Figure 2. (a) Influence of the amount of glutaraldehyde on free amino groups of HA-NPs. (b) Diameter and size distribution variation of HA-NPs cross-linked with 75 μmol glutaraldehyde over 7 days after incubation with PBS at 4°C.

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