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The role of glycyrrhetinic acid modification on preparation and evaluation of quercetin-loaded chitosan-based self-aggregates

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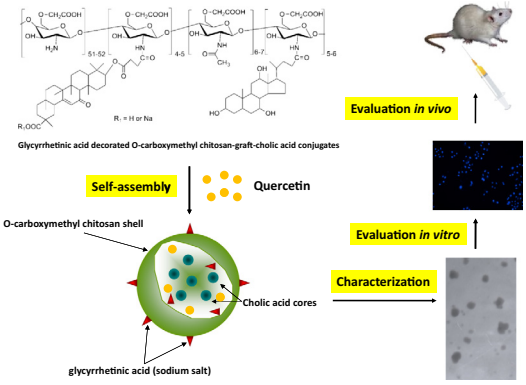
HIGHLIGHTS

- Glycyrrhetinic acid decorated polymer (GA-CMCA) was used to deliver quercetin (QC).
- QC-GA-CMCA had smaller size and narrower size distribution than unmodified ones.
- GA modification on conjugates could alter the *in vitro* release pattern of QC.
- QC-GA-CMCA showed enhanced cytotoxicity and cell apoptosis rate.
- QC-GA-CMCA could prolong drug circulation time in rats.

GRAPHICAL ABSTRACT



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ABSTRACT

Quercetin (QC), a type of plant-based chemical, has been reported to own anticancer activity *in vivo*. However, the poor water solubility limits its pharmaceutical application. In this study, two kinds of QC-loaded self-aggregates based on O-carboxymethyl chitosan-cholic acid conjugates (CMCA) were developed to improve the drug bioavailability in which glycyrrhetinic acid (GA) modification was utilized in the nanocarrier fabrication (QC-GA-CMCA) or not (QC-CMCA). These self-aggregates were prepared by a modified ultrasound-dialysis method and the role of GA modification on the evaluation of QC-loaded self-aggregates was investigated. Transmission Electron Microscopy (TEM) images revealed the formation of spherical particles of both self-aggregates. Dynamic Light Scattering (DLS) analysis and UV–VIS spectroscopy showed that the QC-GA-CMCA had smaller size, narrower size distribution, higher drug loading and entrapment efficiency than corresponding QC-CMCA aggregates. QC-GA-CMCA showed more obvious sensitivity to acidic pH condition based on the zeta potential measurements at various pHs, and fastest drug release was observed at pH 5.7 for QC-CMCA while at pH 6.5 for QC-GA-CMCA. In addition, QC-GA-CMCA demonstrated enhanced cell cytotoxicity and higher cell apoptosis rate *in vitro*, and also higher AUC value and a prolonged residence time of drug *in vivo*.

1. Introduction

Self-aggregates developed from amphiphilic polymer have acquired considerable interest in pharmaceutical field due to their potential use for delivery of various therapeutic agents [1,2]. Such self-assembly system with a core–shell structure can provide a

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protective inner core for encapsulated agents and a hydrophilic shell which can help these nano-sized particles avoid the uptake of the reticuloendothelial system. It can also enhance the drug accumulation in target sites via the enhanced permeability and retention effect [3]. O-carboxymethylated chitosan (OCMC), a water-soluble derivative of chitosan, is synthetized by substitution reaction of chitosan and monochloroacetic acid. The improved properties of OCMC including compatibility, stability, bioactivity and aqueous solution property confer it a good option for the construction of self-aggregates. Hydrophobic molecules such as deoxycholic acid, stearic acid and oleic acid have been successfully used to modify OCMC for the development of amphiphilic polymer and subsequently the formation of self-aggregates in aqueous media [4].

Quercetin (QC), a naturally occurring polyphenolic flavonoid, is distributed universally in various edible plants [5]. As an efficient antioxidant of plant origin, QC possesses varied range of therapeutic benefits including antiviral [6], anti-inflammation [7], antidiabetic [8] and anti-anaphylaxis effects [9]. Recently, it has been extensively investigated for its anticancer activity due to its nature of free radical scavenger. Multiple cellular mechanisms at molecular levels are attributed to its antitumor and antiproliferative effects, mainly including down-regulation of mutant P_{53} protein [10], arrest of G1 phase [11], inhibition of relevant enzymes such as tyrosine kinase [12], protein kinase C [13], COX-2 and matrix metalloproteinases [14]. Moreover, QC could also reduce the action of some toxic agents such as cisplatin [15]. Another study revealed the good hepatoprotective activity of QC in the amelioration of paracetamol-induced hepatotoxicity [16]. However, an obstacle toward the application of QC is its poorly aqueous solubility and stability in physiological medium. Many attempts have been carried out by researchers to facilitate its administration using drug delivery systems. For years, enhanced QC bioavailability and bioactivity have been achieved by encapsulating QC into various drug carriers such as lipid nanocapsules [17], self-nanoemulsifying system [18], poly(lactic-co-glycolic-acid)-based nanocapsules [19] and copolymers-based micelles [20]. To the best of our knowledge, few literatures is available about the utilization of chitosan-based self-aggregates for QC delivery and this paper will focus on this filed.

Glycyrrhetinic acid (GA), the hydrolysis product of glycyrrhizin, is extensively used as a ligand for liver targeting due to the abundant receptors for GA on hepatocyte membranes. According to the published work, two typical approaches to introduce GA into nanocarriers are summarized as follows: Firstly, GA is used as a hydrophobic group to modify the hydrophilic polymers for the construction of self-assembled nanoformulations, such as GA-graft-hyaluronic acid conjugates [21] and GA-modified sulfated chitosan [22]. Alternatively, using poly(ethylene glycol) (PEG) as a hydrophilic linker to conjugate GA molecule onto the surfaces of nanocarriers aims to provide GA with more opportunities to interact with the GA receptors on hepatocyte membrane, such as GA-PEG–b-poly(γ-benzyl L-glutamate) conjugates [23] and GA modified chitosan/PEG nanoparticles [24]. Our previous study demonstrated a new design strategy that O-carboxymethylated chitosan (OCMC) was firstly hydrophobically modified with cholic acid to prepare novel self-aggregates named CMCA and then GA, as a liver targeting ligand, was covalently conjugated to the chitosan backbone to develop GA-CMCA for targeted delivery [25]. The results concluded a portion of GA could be presented on the surfaces of self-aggregates as its hydrophilic sodium salt form at pH 7.4 after alkalization of GA-CMCA, which may provide nanocarriers with enhanced targeting ability. However, the effect of the GA modification strategy on the development of drug-loaded chitosan-based self-aggregates is still a vacancy and thus it is necessary to explore the role of this GA modification strategy on the preparation and evaluation of drug-loaded self-aggregates. This is an important concern for the deep understanding of these self-aggregates.

In this study, two kinds of QC-loaded self-aggregates (QC-CMCA and QC-GA-CMCA) based on CMCA were prepared, in which the CMCA polymer was modified with GA or not. The main purpose of this work was to evaluate the effect of the particular GA modification on the *in vitro* and *in vivo* characteristics of QC-loaded chitosan-based self-aggregates. Thus, comparable studies between the QC-CMCA and QC-GA-CMCA, especially their physicochemical properties, *in vitro* drug release, *in vitro* antitumor efficiency, cell apoptosis effect and *in vivo* pharmacokinetics were carried out.

2. Materials and methods

2.1. Materials

OCMC (Mw = 17 kDa, Mw/Mn = 1.475) was degraded from commercial OCMC (Mw = 156 kDa, Mw/Mn = 1.376, degree of deacetylation = 90%, degree of carboxymethylation = 82%) which was provided by Aoxing Biotechnology Co., Ltd. (Zhejiang, China). GA (purity >98% by HPLC) was purchased from Zelang Pharmaceutical Co. (Nanjing, China). Cholic acid (CA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), 4-dimethylaminopyridine (DMAP), N-Hydroxy succinimide (NHS), quercetin, sulforhodamine B (SRB), trichloroacetic acid, acetic acid and Tris base were obtained from Sigma–Aldrich (USA). The methanol (Tianjin Siyou Co., Ltd., China) was of high performance liquid chromatography grade. Other chemical reagents and solvents were of analytical grades and were used without further purification. Deionized water was used in all experiments.

Wistar rats (200 ± 10 g) used for pharmacokinetics study were obtained from the Experimental Animal Center of Shandong University (Jinan, China). The rats were housed in cages for 1–2 weeks before experimentation and fed with food and water *ad libitum*. Before the experiments, all rats were kept under fasting overnight but with free access to water. All care and handing of animals were performed with the approval of Institutional Authority for Laboratory Animal Care of Shandong University.

2.2. Synthesis and characterization of CMCA and GA-CMCA conjugates

CMCA and GA-CMCA conjugates were synthesized according to the published literature [25]. First, CA (0.3 mol/mol sugar residues of OCMC) was dissolved in DMF and equal amounts (2 equiv./CA) of EDC and NHS were added and stirred at room temperature for 30 min. Then the mixture was added dropwise into OCMC solution and allowed to proceed at room temperature for 36 h. The reactant was dialyzed against water/methanol mixture (1:4, v/v) and subsequently distilled water. CMCA conjugates were isolated by lyophilization. GA was conjugated to CMCA via a succinate linker. The hydroxyl group on GA was firstly modified with succinic anhydride in the presence of DMAP and purified in a silica gel column to donate suc-GA. After activation with EDC and NHS, the activated suc-GA was dropped into CMCA solution and further stirred for 36 h. The reaction mixture was thoroughly dialyzed against $NaCO_3$ solution (1 mM) and finally lyophilized to obtain alkalized GA-CMCA.

The molar ratios of CA to amino groups in CMCA and the amount of GA covalently bounded to GA-CMCA were determined by a UV/vis spectrophotometer.

2.3. Preparation of QC-loaded self-aggregates

QC-loaded self-aggregates were prepared using a modified ultrasound-dialysis method. Firstly, 100 mg conjugates were