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Chitosan-*grafted*-poly(methacrylic acid)/graphene oxide nanocomposite as a pH-responsive *de novo* cancer chemotherapy nanosystem

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ABSTRACT

The aim of this study was the design and development of a novel *de novo* drug delivery system for cancer chemotherapy. For this purpose, chitosan (CS) functionalized using phthalic anhydride followed by 4-cyano, 4-[(phenylcarbothioyl) sulfanyl] pentanoic acid as a chain transfer agent (CTA) to afford CS-CTA macroinitiator. The synthesized CS-CTA macroinitiator was then copolymerized with methacrylic acid (MAA) monomer using reversible addition–fragmentation chain transfer (RAFT) polymerization technique to produce chitosan-*graft*-poly(methacrylic acid) (CS-*g*-PMAA) graft copolymer. Afterward, graphene oxide (GO) nanosheets were incorporated into the synthesized copolymer through the physical interactions. The CS-*g*-PMAA/GO nanocomposite was loaded with doxorubicin hydrochloride (DOX) as a universal anticancer drug. The biocompatibility, DOX-loading capacity, and pH dependent drug release behavior of the developed nanocomposite were also investigated. As the experimental results, as well as superior biological and physicochemical features of CS and GO, we envision that the developed CS-*g*-PMAA/GO nanocomposite may be applied as *de novo* drug delivery nanosystem for cancer chemotherapy.

1. Introduction

The increasing demand for *de novo* drug delivery systems (DDSs) encouraged polymer, materials, and biological scientists to more and more research efforts toward innovative design and development of efficient DDSs. In this context, the advent of nanoscience and nanotechnology create endless opportunities to achieve more efficient nanoscale DDSs, defined as nanomedicines [1–3]. This field has progressed significantly, since the first use of nanoparticles (NPs) in the late 1960s by Speiser and colleagues [4,5]. Nanomedicines have some superior features including targetability and multifunctionality as well as possibility to entrap poorly soluble drugs and to overcome multi-drug resistance [2]. In this context, graphene and its derivatives such as graphene oxide (GO) are the most promising candidates mainly due to their unique physicochemical as well as biological features [1,6,7].

Graphene, is a two-dimensional (2D) single layer of carbon atoms in the nanometer size scale that attracted a great deal of interest in both academic and industrial communities due to its superior properties including extremely high specific surface are, zero effective mass, high thermal conductivity, impermeable to gases, and displays high mobility of charge carriers, while it is optical transparency [8–10]. According to above mentioned features, graphene and its derivatives have received tremendous attention in numerous fields ranging from industrial to biomedical. The most important biomedical applications of graphene and its derivatives are biosensor platform [11], bio-imaging [12], tissue engineering [13], as well as drug delivery [1,13]. Among these, drug delivery purpose for cancer therapy is particular of interest due to extremely high specific surface area of graphene and its derivatives that led to efficient loading of various anticancer drugs *via* π–π stacking and hydrophobic interactions as well as the synergic effect of photothermal therapy (PTT; due to their high near-infrared absorbance) approach [1,6,13].

However, the most important problem regarding the practical usage of graphene is the low processability and strong interaction between individual graphene nanosheets that restrict its application. This problem can be solved through the oxidation of graphite to graphene oxide (GO), subsequently modification of GO through both chemical and physical approaches [8,14,15]. In this context, the physical modification of GO using macromolecules is an efficient and safe approach due to following

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reasons. This modification technique is simple, cheap, and safe, because it requires no chemicals or biological agents [8,16]. Among the macromolecules for modification of GO, natural polymers has attracted more attention due to their inherent physicochemical as well as biological features [17,18].

Natural polymers are generally produced by plants, animals, and microorganisms. These macromolecules are categorized into three main classes including polypeptides, polysaccharides, and polyesters [19,20]. The most important characteristics of natural polymers that qualified them for biomedical applications are their extraordinarily elevated stability, variable/controllable solubility, superior structural design, three-dimensional geometry, low immunogenicity, biodegradability, excellent biocompatibility and cytocompatibility, antigenicity, and often specific tissue/cell targeting [21,22]. Among natural polymers, polysaccharides are considerable of interest due to their abundances, low cost (in most cases) as well as general features of natural polymers that mentioned above [23,24]. In this respect, chitosan (CS) has attracted a substantial amount of research effort for design and development of chitosan-based biomaterials for biomedical applications. CS is a linear polycationic polysaccharide composed of *N*-acetyl-D-glucosamine and D-glucosamine units. This polysaccharide can be produced through the deacetylation of the chitin (the second most abundant polysaccharide after cellulose in nature) using both alkaline deacetylation and enzymatic degradation approaches [24,25].

However, the main drawback of CS is lack of solubility at pH higher than its pKa (pH~5.5–6.5) due to interchain hydrogen bonding. This issue can be solved through the chemical modification of CS through its amine and/or hydroxyl functional groups [25,26]. Some chemical reactions such as quaternization, alkylation, chelation of metals, acetylation, reaction with aldehydes and ketones (to give Schiff's base), and polymer grafting have been suggested for chemical modification of CS [26,27]. Among these, the polymer grafting approach with tailored surface properties is particular of interest especially in the case of biomedical applications. In general, three grafting approaches including "grafting from", "grafting to", and "grafting through" can be applied for this purpose [28,29]. It is worth noting that the "grafting from" approach is the most commonly used method toward the synthesis of CS-based copolymers. In this context, the grafting of pH-responsive polymers (*e.g.,* poly(methacrylic acid)) onto CS using reversible deactivation radical polymerization (RDRP) technique can be led to advanced biomaterials for biomedical applications.

The RDRP approach is divided into three main categories including atom transfer radical polymerization (ATRP) [30], nitroxide-mediated radical polymerization (NMRP) [31], and reversible addition of fragmentation chain transfer (RAFT) polymerization [32]. Among these, the RAFT technique attracted a great deal of interest mainly due to its applicability to wide variety of vinyl monomers, easy experimental set-up, and its potential for synthesis of homo-polymers, block copolymers, and post-modified polymers with controlled molecular weight, narrow dispersity, and complex macromolecular structures. Furthermore, another advantage of this technique against ATRP approach is no metal contaminants in the synthesized copolymers [32,33].

According to superior features of GO and chemically modified CS, the association of these biomaterials can be resulted to advanced drug delivery nanosystems. Based on this fact, we encouraged to design and development of a chitosan-*grafted*-poly(methacrylic acid)/graphene oxide [(CS-g-PMAA)/GO] nanocomposite as a pH-responsive *de novo* cancer chemotherapy nanosystem. Firstly, CS was modified with phthalic anhydride followed by 4-cyano, 4-[(phenylcarbothioyl) sulfanyl] pentanoic acid as a chain transfer agent (CTA) to afford CS-CTA macroinitiator. The macroinitiator obtained was copolymerized with MAA monomer using RAFT polymerization technique to produce CS-*g*-PMAA graft copolymer. The GO nanosheets were incorporated into the synthesized CS-*g*-PMAA copolymer, and the obtained CS-*g*-PMAA/GO nanocomposite was loaded with doxorubicin hydrochloride (DOX) as a universal anticancer drug. The anticancer drug delivery performance of the DOX-loaded CS-*g*-PMAA/GO nanocomposite was studied in terms of biocompatibility, DOX-loading capacity, and pH dependent drug release behavior.

2. Experimental

2.1. Materials

Chitosan (medium molecular weight, extent of deacetylation 75–85%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). 4-Cyano, 4-[(phenylcarbothioyl) sulfanyl] pentanoic acid was synthesized in our laboratory [32]. Dicyclohexyl carbodiimide (DCC), dimethylaminopyridine (DMAP), graphite, sodium nitrate ($NaNO_3$), potassium permanganate ($KMnO_4$), hydrochloric acid (HCl), and sulfuric acid (H_2SO_4) were purchased from Sigma-Aldrich and were used as received. Methacrylic acid monomer was purchased from Merck (Darmstadt, Germany), and distilled twice under reduced pressure before use. All other chemical reagents were purchased from Merck or Sigma-Aldrich and purified according to the standard methods. Doxorubicin hydrochloride (DOX) was purchased from Zhejiang Hisun Pharmaceutical Co., Ltd., Taizhou, China. Phosphate buffered saline (PBS), fetal bovine serum (FBS), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide), and other biological reagents were purchased from Invitrogen (Carlsbad, CA, USA) and were used as received.

2.2. Synthesis of N phthaloylchitosan

A 50-mL three-neck round-bottomed flask equipped with a condenser, gas inlet/outlet, and a magnetic stirrer, was charged with CS (2.00 g), phthalic anhydride (0.80 g, 5.6 mmol), and dried *N*, *N* dimethylformamide (DMF; 30 mL). The reaction mixture was deaerated by bubbling highly pure argon for some minutes, and then stirred for about 12 h under argon protection at 125 °C. At the end of this time, the flask was cooled using an ice/water bath, and the reaction was terminated by purring the content of the flask in excess cold methanol, filtered, and washed several times with methanol. The product obtained was dried in vacuum at room temperature.

2.3. Synthesis of CS-CTA macroinitiator

The CS-CTA macroinitiator were synthesized through the Steglich esterification of *N*-phthaloylchitosan using 4-cyano, 4-[(phenylcarbothioyl) sulfanyl] pentanoic acid as follows. A 50-mL three-necked round-bottom flask equipped with condenser, gas inlet/outlet, and a magnetic stirrer, was charged with 4-cyano, 4-[(phenylcarbothioyl) sulfanyl] pentanoic acid (1.00 g, 3.7 mmol), DCC (1.65 g, 8 mmol), and dried DMF (30 mL). The content of the flask was deaerated using argon gas for some minutes followed by stirring for about 4 h at room temperature under argon protection. At the end of this time, the content of the reactor was filtered using filter paper (Whatman) in order to remove dicyclohexyl urea salts. The solution was transferred into a dried 50-mL three-necked round-bottom flask equipped with condenser, gas inlet/outlet, and a magnetic stirrer, then *N* phthaloylchitosan (2.00 g), and DMAP (0.25 g, 2 mmol) were added to the reactor, and the content of the flask was stirred for 48 h under argon protection at 50 °C. The reaction was terminated through the pouring the content of the flask into a large amount of methanol. The product filtered, washed several times with methanol, and dried in reduced pressure at room temperature.

2.4. Graft copolymerization of MAA onto CS using RAFT polymerization technique

A dry polymerization ampoule was charged with macro-RAFT agent [(CS-CTA) 2.00 g], MAA monomer (1.5 mL, 25 mmol), AIBN (2.0 mg, 12

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