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Communication

Graphene oxide complex as pH-sensitive antitumor drugs†

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pH-sensitive nanostructured antitumor drugs from GO-CONH-Schiff base (GCS) were prepared from the chitosan-xanthone schiff base (CS) modified graphene oxide (GO) complex. The successful synthesis of GCS was confirmed using various spectroscopic techniques including FT-IR, XPS, UV-vis and TGA. The resulting GCS showed superb antitumor activity with pH-sensitive release of antitumor parts CS while less cytotoxicity of GCS to the normal human cells was obtained. The release of CS were stable and thorough in the solution with pH 1 (the pH value for gastric juice), suggesting that as-synthesized pH-sensitive drugs could provide new insights into design of advanced nanostructured oral drugs.

1. Introduction

Chitosan is an unbranched cationic biopolymer in acidic media since it carries a positive charge at pH below 6.5. Thus, chitosan shows attractive interactions with numerous negatively charged materials, such as most living tissues (e.g. skin, bone, hair), polysaccharides (e.g. alginate), polyanions, bacteria and fungi, enzymes and microbial cells. It has been shown that the chitosan-based materials have many valuable bioactivities, including hemostasis, bacteriostasis, fungistasis, anticancer and anticholesteremic activity.[1-3] Due to its well-known polymeric characters, chitosan has been used intensively in various drug delivery systems. For example, N-Trimethyl chitosan chloride has been used as an effective gene carrier. [4,5] It has been found that this polymer can enhance absorption of peptide and protein drugs across nasal[6] and intestinal epithelium cells.[7] The chitosan-folate modified microcapsules with camptothecin have been reported for targeted therapy of tumor cells.[8]

Application of chitosan as drugs for treatment of diseases, however, hasn't been realized because of its low pharmaceutical activities. Limited progress regardinging utilisation of modified chitosan as potential drugs has been reported. For instance, chitosan and its quaterinized derivatives have been found to possess good antitumor activities, antimicrobial and antimycotic properties.[9] Formation of Schiff base could enhance the pharmaceutical activities associated with chitosan.[10] Indeed, the citral-chitosan Schiff base was demonstrated to show higher antimicrobial activities than chitosan. Furthermore, the copper complex of salicylaldehyde-chitosan schiff base has been found to be strongly antitumor active.[11]

Herein, we synthesized a new class of chitosan based antitumor drugs from a Schiff base formed by the coupling reaction between chitosan and xanthone. The combination of xanthone with chitosan improved the biological activity of the Schiff base while the cationic property of chitosan enhanced the drug permeability into tumor cells, providing additional advantages for the resulting antitumor drugs.

In this work, the unique 2D nanosheet graphene oxide (GO) was further incorporated into the chitosan-xanthone Schiff base (CS) to further improve tumor passive uptaking of the polymer compexes via enhanced permeability and retention (EPR) effects.[12] Graphene based materials have been widely studied for applications in biology, bioorganisms and biosensing.[13,14] Graphene-based nanomaterials have been reported to effectively inhibit the growth of *E. coli* bacteria while showing minimal cytotoxicity against normal cells.[15] Graphene based networks or scaffolds have been found to provide well biocompatible interface for live cells to support their growth and adhesion.[16,17] In particular, GO has been shown to be an effective carriers to deliver water-insoluble drugs into cells.[18,19]

It has been recognized that high biological effects of nanocomposites could be obtained from composites based on certain inorganic compounds and chitosan. For example, the chitosan-PVP-TiO_2 nanocomposite can improve the wound healing.[20] The gold nanoparticles are found to facilitate the chitosan-pluronic to effectively enter the cells.[21] The most striking GO-chitosan nanocomposite can stimulate the growth of osteoblasts whilst showing nontoxicity to normal cells.[22] Chitosan modified GO composites as nanocarries for anticancer drugs (e.g. camptohecin) and genes via π-π stacking and hydrophobic interactions have been realized though the release of drugs is uncontrollable.[14] Of particular interest, chitosan-graphene dispersions have been found to be pH-responsive.[23] Therefore, incorporation of GO with the chitosan Schiff base could provide

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additional pH sensitivity which is useful for controlled release of the as-designed drugs. In this study, we have prepared novel nanostructured antitumor drugs from GO-CONH-Schiff base (GCS) compounds, in which CS acted as the tumor inhibitor whilst GO worked as the drug delivery carrier. The resulting drug was further found to be pH-sensitive attributed to presence of amines in its molecular structure. The release of CS from GCS was found to be pH-dependent, which would be useful for pH-triggered release at specific points, and protection of drugs from degradation when they were passing through different organs and tissues. Our in vitro results suggested that the resulting GCS significantly inhibited the growth of hela cells. Successful fabrication of the resulting pH-sensitive antitumor drugs provides new insights into design of future nanostructured oral drugs.

2. Experimental

2.1 Materials

Graphite with an average particle size of 100 μm was obtained from Shanghai Reagent Co., Ltd. GO used in our experiments was prepared according to the Hummers' method.[24] Chitosan powders (with a deacetylation degree of 95%, and viscosity of 200–400 mPa.s), xanthenone (analytic grade), and acetic acid were all obtained from Aladdin-reagent Inc. Deionized water was collected from the Millipore purification system. All other chemicals were at the analytical grade and directly used without further purification.

2.2 Characterization

Fourier transform infrared spectra (FT-IR) were recorded on a PE Spectrum One spectrometer with KBr pellets in the 4000–450 cm^{-1} region. UV–vis spectra were measured on Perkin Elmer Lamda 35 Spectrometer. Fluorescence emission spectra were obtained using AB-series2 luminescence spectrometer. Atomic force microscopy (AFM) was carried out using a SPI3800N microscope operating in the tapping mode. Transmission electron microscopy (TEM) was characterised by a PHILIPS EM400ST microscope at an accelerating voltage of 150 kV. X-ray photoelectron spectra (XPS) were measured using ESCALB MK-II spectrometer. Thermogravimetric analysis was performed by a STA 409 PC/4/H Lux at a heating rate of 10 ^{o}C per minute under N_2.

2.3 Synthesis of the chitosan-xanthone Schiff base (CS)

Chitosan powders (0.5g) were dissolved in 100 mL aqueous solution of acetic acid (1wt %) for 24h, and then transferred into a three-necked flask. 50 mL hot xanthone/ethanol solution was dropped into the flask and 2 mL acetic acid was subsequently added. The mixture was refluxed at 70 ^{o}C for 18h, followed by cooling down to the room temperature. The product was neutralized by diluted NaOH, and separated, and subsequently washed with deionized water and ethanol, respectively. The yield of the final produce was found to be 80%.

2.4 Synthesis of the GO-CONH-Schiff base composite (GCS)

20 μL N-hydroxysuccinimide (NHS, 1.0 mM) and 20 μL of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimde (EDC, 1.0 mM) solutions were added into the GO dispersion and remained for 10 min. 5 mM CS aqueous solution was subsequently introduced. The resulting solution was heated at 50 °C for 2 h and then stirred at the room temperature for 12 h. Excess CS (precipitated as solid) was removed by centrifuge. The solution was then filtered using the Millipore filter (0.22-μm). GCS remained in the filter was washed 4-6 times and redispersed in water. The schematic illustration of CS and GCS base was shown in Fig. 1.

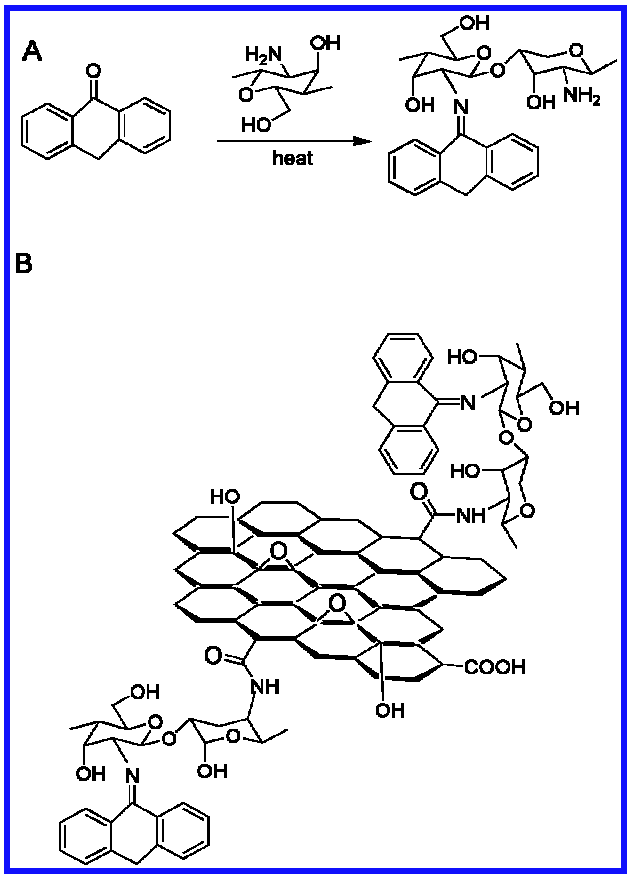

Fig. 1 (A) Schematic synthesis of CS, and (B) schematic representation of GCS nanocomposite.

2.4 The release of CS from GCS

To identify the contents of xanthone on CS and GCS, two samples were subjected to 3 M HCl solutions respectively. Xanthone was subsequently extracted by trichloromethane and determined by HPLC. For measurement of the CS release from GCS, a calibration curve was first obtained using the fluorescence emission spectra at various pHs. 50 mg GCS were then immersed in 10 mL solution with different pHs. At predetermined time points, 1 mL of this solution was taken out and analysed for the released CS using luminescence spectrometry. 1 mL additional solution with the same pH was added to keep the total volume constant. The percentage of released CS was calculated from standard calibration curves.

2.5 Cell culture

Hela cells were cultured in Dulbecco's modified Eagle's medium/F12 (12800-017, high glucose Gibco), supplemented with 10% fetal bovine serum (SV 30087.02, Hyclone) and 50μg/mL gentamicin, in a humidified 5% CO_2 balanced air incubator at 37 °C. Medium was changed every 2 days. Cells

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were passaged with 0.25% trypsin (Invitrogen) plus 0.02% EDTA (Sigma). Cell viability was measured using the CCK8 assay. All samples were sterilized at a high temperature (120 °C). 4000 cells in 100 μL medium were seeded into each well of the 96-well culture plate. Sterilized samples were incubated with cells for 72 h. 10 μL CCK8 solution was then added into each well and incubated for 3 h. Absorbance was measured at the wavelength of 450 nm with a microplate reader (Biorad 680). The polystyrene (PS) surface of the 96-well culture plate was adopted as a negative control. Three repeats were done for each group.

3. Results and Discussion

3.1 Characterization of CS and GCS.

The successful incorporation of chitosan, xanthone and GO was confirmed using FT-IR spectroscopy. The differences in FT-IR spectra of chitosan before and after the synthesis were shown in Fig. 2 (a) and (b). A broad peak at 3400 cm^{-1} from chitosan was associated with the coupled vibration of –OH and $–NH_2$. The peak at 3400 cm^{-1} became sharp in the spectrum of CS which might be attributed to the breaking of hydrogen binding and subsequently formation of C=N. A new peak at 3280 cm^{-1} in the spectrum of CS was arisen from the unreacted $–NH_2$. The residual $–NH_2$ of CS further reacted with GO to form the GCS. Another peak of $–NH_2$ presented at 1650 cm^{-1} were found both in chitosan and CS. Compared with the spectrum of chitosan, a new peak (1720 cm^{-1}) associated with C=N double bond was observed for CS. Four peaks at 1556, 1350, 1268, 953 cm^{-1} from the characteristic C=C vibration of aromatic rings were presented, confirming the successful synthesis of CS. Fig. 2(c) confirmed the formation of amide groups. It can be seen that the amide vibrations appearing at 1657 and 1607 cm^{-1},[25] differed from the $–NH_2$ vibration at 1650 cm^{-1} as observed both from chitosan and CS. The peak at 3400 cm^{-1} turned broad again, suggesting the hydrogen binding of GO with chitosan. However, the vibration at 1720 cm^{-1} seen from CS was not well identified. It might be shielded by amide vibrations.

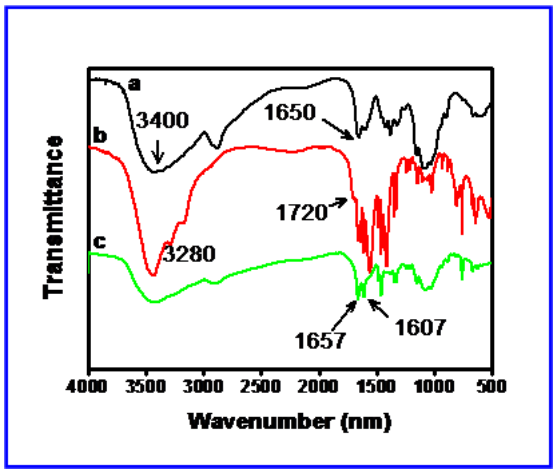

Fig. 2 FT-IR spectra of (a) chitosan; (b) CS; (c) GCS

Formation of GCS nanocomposite was also confirmed by XPS analysis (Fig. 3). As shown in Fig. 3a, there were only C and O peaks presented in the XPS survey spectrum of GO, but C, N and O peaks were found in the XPS spectrum of GCS. Three peaks related to C-C (285.0 eV), C-O (286.4 eV), C=O (287.5 eV) and C(O)O (289.1 eV) groups were identified in the high resolution C 1s spectrum of GO (Fig. 3b) while five bands associated with C-C (284.5 eV and 284.6 eV), C-N (285.8 eV), C-O (286.5 eV), C=N (287.9 eV) and N-C=O (288.7 eV) were fitted in the high resolution of C 1s spectrum of GCS (Fig. 3c). Two kinds of C-C bonds found at GCS were assigned to sp^3 carbons on the CS and GO, respectively. Another significant peak at 288.7 eV indicated the formation of amide from covalent linkage of CS and GO. The C=O and C-O of GCS were identified at 531.2 eV and 532.9 eV respectively in the high resolution O1s spectrum (Fig. S2, ESI). Typical C-N and C=N peaks at 400.1 and 402.2 were clearly observed in the high resolution N 1s spectrum of GCS (Fig. 3d), suggesting presence of CS in the resulting GCS. XPS results further confirmed the successful synthesis of GCS.

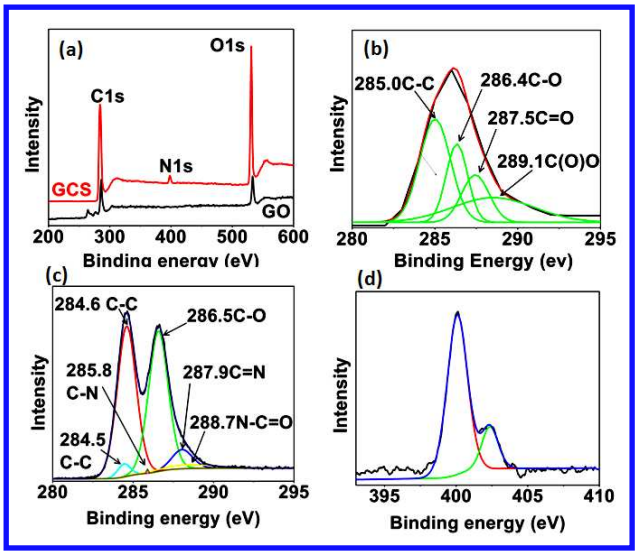

Fig. 3 (a) XPS survey spectra of GO and GCS; (b) high resolution XPS C 1s spectrum of GO; (c) high resolution XPS C 1s spectrum of GCS; and (d) high resolution XPS N 1s spectrum of GCS.

UV-vis spectra of samples were shown in Fig. 4. Three characteristic peaks of xanthone were found at 261 nm, 285 nm, and 340 nm, associated with the π-π* electron transitions of aromatic rings.[26] When chitosan was introduced and the C=O at xanthone was replaced by the C=N, the red shifts were observed. Three characteristic peaks of xanthone were found to shift to 265 nm, 288 nm, and 343 nm in the spectrum of CS. After GO was incorporated with the CS, the spectrum of GCS showed a very similar curve with that of the CS, but the absorption intensity significantly increased over a wide range of wavelength, presumably because the solubility of GCS has been remarkably increased by the newly-grafted GO moieties.

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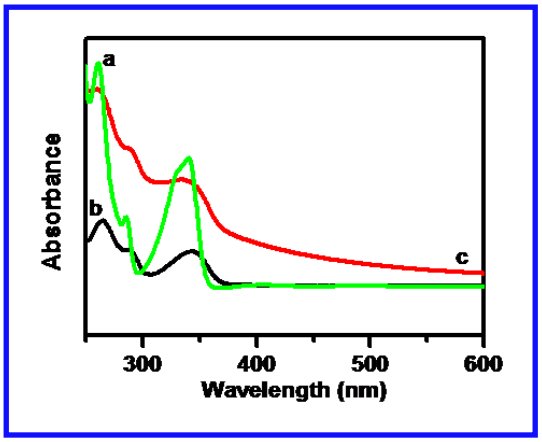

Fig.4 UV-vis absorption spectra of (a) xanthone; (b) CS; and (c) GCS.

Thermogravimetric analysis (TGA) was carried out to further confirm the successful synthesis of GCS. TGA is a very useful technique for identification of various ingredients of a composite.[27,28] As shown in Fig. 5 (a) and (b), the thermal stability of CS was decreased when compared to chitosan. The weight loss of CS in the 50-130 ^{o}C range was attributed to the release of adsorbed water. The continuous weight loss above 180 ^{o}C was assigned to the breakdown of –C=N bond and hydroxyl groups,[29] which was followed by glucose ring scissions and carbonization of the composite. After incorporation of GO into CS via amido bonds (Fig. 1), the TGA curve of GCS (Fig. 5c) showed a slower decomposition rate than that of CS, indicating more thermo-stability of the resultant GCS. Physical mixture (MC) of CS and GO was also characterized to confirm covalent linkage between CS and GO in the as-prepared GCS. Difference between the resulting GCS and CS/GO MC was evident from the TGA results. The MC exhibited lower weight loss rate and higher stability than GCS, since the MC did not have to break the amide groups. Compared to the TGA curves of CS and MC, the salient feature to be noticed on the TGA curve of GCS is the newly-appeared decomposition at ca. 470 °C attributable to the weight loss caused by the session of amide linkage between the GO and CS constituent components. These variations confirmed the successful chemical covalent linkage between CS and GO in the resulting GCS.

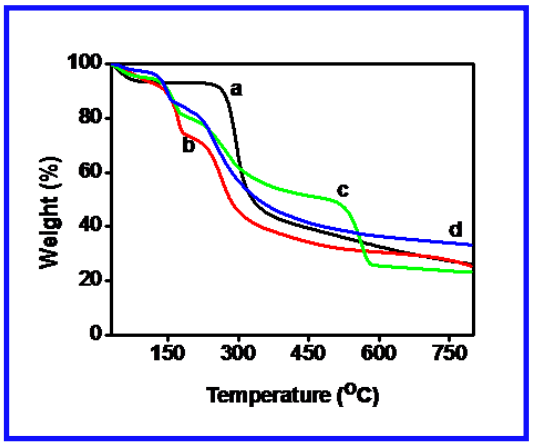

Fig. 5 TGA curves of (a) chitosan; (b) CS; (c) GCS; and (d) physical mixture of CS and GO.

3.2 pH-dependent drug release

We further studied fluorescence performance of CS, GCS and MC in aqueous solutions. Concentrations of xanthone groups in the three composites were remained the same since xanthone was the predominantly fluorescent group. A significant fluorescence quenching effect was observed at the MC, presumably due to the FL quenching induced through the π-π stacking between the xanthone group in CS and GO (Fig. 6c).[30] Conversely, obvious florescence signals were obtained from both CS (Fig. 6a) and GCS (Fig. 6b). A higher fluorescence intensity was found at the CS than that of the GCS under the identical conditions. This may be associated with formation of –CO-NH- bond at the GCS, which significantly reduced the FL quenching induced by the aforementioned π-π stacking, but still caused somewhat fluorescent quenching due to photoinduced electron transfer (PET) effect.[31]

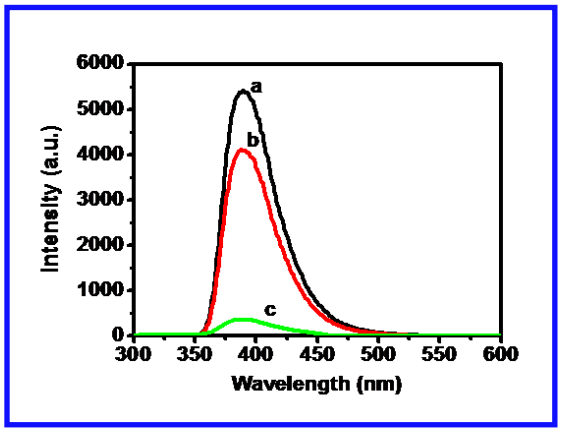

Fig.6 Fluorescence spectra of (a) CS; (b) GCS; and (c) MC with the same concentrations of xanthone, respectively (pH=6.0).

GCS further exhibited strong pH-dependent fluorescence performance as shown in Fig. 7(a), comparable to that of the CS precursor (Fig. 7(b)). Higher fluorescence intensity obtained at the higher pH value was probably attributed to the deprotonated amine atoms at the high pH. In the acidic solutions, protonation of the amine groups caused PET quenching of the fluorophore by the xanthone moiety since the energics of the fluorophore was changed during the protonation process. This might result in the quenched fluorescent intensity. [32]

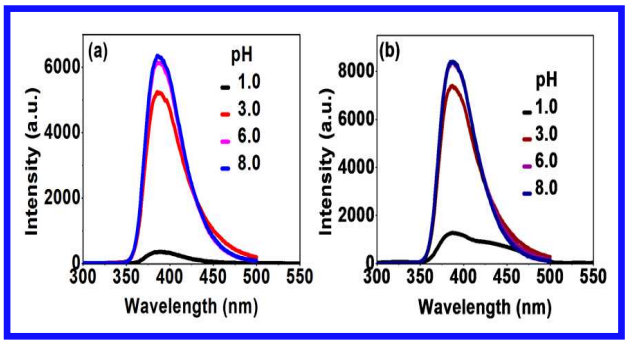

Fig. 7 pH-dependent fluorescence emission spectra of (a) GCS and (b) the pristine CS with the same concentration of xanthone.

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The stability of the amido bond is diverse in solutions with different pH values. We thus investigated the pH-dependent release of CS from GCS. As shown in Fig. 8, the amounts of CS released increased with decreasing pH values. There were totally 34.33%, 31.11%, 15.6%, and 12.4% CS was released from GCS in the 2nd day in solutions with pH=1, 3, 6 and 8. Released CS amounts gradually increased with increasing release time. It was attributed to that varied pH values influenced the breaking of amido bonds, resulting in the release of CS. For the purpose of comparison, physical mixture MC was measured for controlled release at the identified conditions. It was found that CS release amounts increased with increasing pH values at MC but the release equilibrium observed in the 2nd day, suggesting poor stained release capability and stability of the mixture without chemical covalent linkage. The CS release results suggested that the as-synthesized GCS exhibited superb pH-controlled and stained release capability. Particularly, lower pH value facilitated the release of GCS, suggesting the great possibility for the resulting GCS to be a good candidate for oral administration since the pH value of gastric juice is about 1.3.

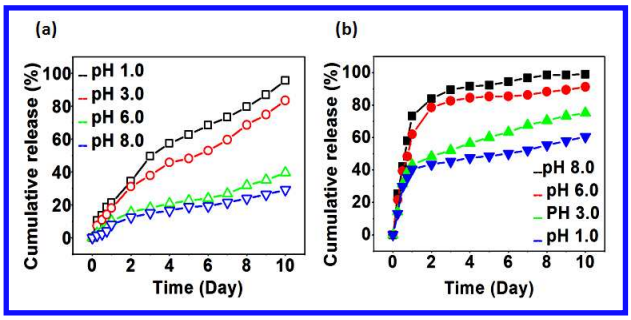

Fig. 8 (a) pH-dependent release of CS from the GCS; (b) pH-dependent release of CS from the physical mixture MC.

3.3 Cytotoxicity with hella cells

Antitumor ability of the resulting GCS against hela cells were measured by CCK8 assay. For the purpose of comparison, xanthone and paclitaxel were also evaluated for cytotoxicity. Concentrations of CS and GCS were determined by the amount of xanthone attached. It was found that xanthone and paclitaxel showed little toxicity with hela cells at relative high concentrations. As shown in Fig. 9(a), 2500 nM paclitaxel induced 35% cell viability while the same amount of xanthone induced more than 80% viability. Both CS and GCS, however, showed significantly antitumor effects even at the concentration of 10 nM, which induced nearly 50% hela cells apoptosis. When concentrations of both CS and GCS were increased to 1000 nM, only 35% and 25% hela cells alive were observed respectively. Particularly, stronger antitumor ability against hela cells was found for GCS compared to CS.

A better potential antitumor drug should possess highly toxicity against tumor cells at low concentration while remain low toxicity to human normal cells at high concentration. So we evaluated biocompatibility of the as-prepared GCS with human normal retina cells. As shown in Fig. 9(b), there were over 85% human retina cells survived when the concentration of GCS was 1000 nM. Furthermore, no obvious toxicity against retina cells was obtained for different concentrations of GO without loading drugs, suggesting good biocompatibility of GO with normal retina cells. The results suggested that incorporation of GO with CS enhanced the antitumor effect significantly while remained low toxicity to normal cells, indicating that GO was suitable to be used as nano carriers. When the concentration of GCS was lower than 10 nM, the relative cell viability was over 93%, suggesting that the resulting GCS induced low toxicity to human normal cells.

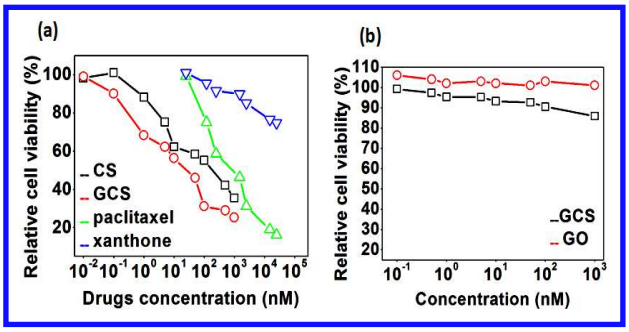

Fig.9. (a) Relative cell viability (versus untreated control) of Hela cells incubated with CS, GCS, paclitaxel and xanthone for 72h respectively; (b) relative cell viability data of human retina cells incubated with GCS and GO after incubation for 72h.

4 Conclusions

In this work, we have synthesized novel pH sensitive antitumor drugs from chitosan-xanthone-graphene oxide (GCS) nanocomposites. Release of antitumor parts CS from the as-synthesized GCS showed superb pH-dependent properties since changes in pH values resulted in the breakdown of amido bonds between GO and chitosan, leading to the controlled release of CS from GCS. CS was further found to be released stably and completely in the pH 1 solution which was pH value for gastric juice, suggesting possibility of the resulting GCS to be employed in oral administration. In addition, GCS exhibited excellent antitumor activity when compared with xanthone and paclitaxel. Our preliminary results present a simple but efficient way to prepare the pH-controllable GCS antitumor drugs, which might open up great possibilities for design of novel nanostructured oral drugs for various applications, such as detection and therapy of tumors.

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†Electronic Supplementary Information (ESI) available: AFM images of GCS and GO, High resolution XPS O1s spectra of GO and GCS, TEM images of hela cells incubated with GCS. See DOI: 10.1039/b000000x/

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