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Formulation and in vitro evaluation of quercetin loaded carbon nanotubes for Cancer Targeting

Zahra Alimohammadi[1], Asma Zamansani[2], Azadeh Abreshteh[3], Mehrsa Edalaat[4], Zohreh Ebrahimi[5]

[1]Faculty of Chemical Engineering, Naghshe Jahan University, Isfahan 8199874941, Iran;
[2] Faculty of Chemical Engineering, Naghshe Jahan University, Isfahan 8199874941, Iran
[3] Faculty of Chemical Engineering, Naghshe Jahan University, Isfahan 8199874941, Iran
[4]Faculty of Chemical Engineering, Naghshe Jahan University, Isfahan 8199874941, Iran
[5]Faculty of Chemical Engineering, Naghshe Jahan University, Isfahan 8199874941, Iran

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Abstract - *Quercetin has high level antioxidant and antiradical properties that are considered beneficial to cancer treatment. However, therapeutic applications of quercetin have been restricted due to its limited solubility and instability in physiological medium. The purpose of this study was to develop quercetin – Loaded carbon nanotube and to evaluate the potential of the carrier as a topical delivery system in cancer therapy. The drug delivery system has been designed based on functionalized carbon nanotubes by chitosan. Then, quercetin was conjugated to the carrier. The highest loading efficiency (38%) was achieved at 4°C and equal initial weight ratio of drug/carrier. The drug delivery system is stable under neutral pH conditions (pH 7.4), while effectively released quercetin at reduced pH conditions (pH 5.5). In vitro cytotoxicity assays showed that functionalized carbon nanotube did not exhibit notable toxicity against Hela cells; whereas, the cytotoxicity of quercetin conjugated carrier increased significantly in comparison with pure quercetin. Hence, such a targeting nanocarrier is a suitable candidate for targeted drug delivery in tumor therapy.*

Key Words: Carbon nanotube, Quercetin, Chitosan, targeting, Drug delivery, Cancer therapy

1.INTRODUCTION

Cancer is one of the main causes of death in the world and its victims increase every day [1]. Quercetin (3, 3′, 4′, 5′-7-penta-hydroxy flavone) (fig1) is one of the most abundant flavonoids in plants found in fruits, vegetables and herbs [2]. It has a broad extent of chemotherapeutic properties for many diseases such as anti-cancer, anti-inflammatory, anti-viral, antiradical and anti-oxidant [3, 4]. However, its superior anti-cancer activity is well demonstrated in colon, breast, ovarian and lung cancer cells [5,6]. In vivo reversal efficacy of quercetin is not satisfactory when it is administered systemically via free drug due to its low solubility in aqueous media (7.7 µg/mL in water), poor permeability, low bioavailability (about 1% in men) [7], biodegradation limits and high binding ratio of drug–plasma protein (99.4%) [8].

One way to overcome these drawbacks is to entrap/adsorb this molecule into carriers. Since nanoscale drug delivery carriers offer potential advantages, which include enhancement of quercetin solubility and bioavailability, improved tissue macrophages distribution, enhancement of pharmacological activity, sustained delivery, and protection from physical and chemical degradation [9,10], a number of carriers such as liposomes [11,12,13], lipids [14,15,16], micelles [17, 18], polymeric nanoparticles [19, 20], magnetic nanoparticles [21, 22] and graphene [23] have been applied for entrapment of quercetin. Recently, single walled carbon nanotubes (SWNTs) have been applied for targeting delivery of various anti-cancer drugs such as doxorubicin and paclitaxel [1, 24]. Since single walled carbon nanotubes (SWNT) provide unique properties such as high surface area (1300 m2/gr) allowing for higher drug loading and possibility for accompanying additional therapeutic ligands through surface functionalization, recently they have attracted many attentions in the treatment and diagnosis of cancer.

However, drug delivery systems based on SWNTs still face critical challenges. They are potentially toxic and extremely hydrophobic. Functionalization of carbon nanotubes by covalent and non-covalent interactions can improve their compatibility and cellular uptake, decrease their hydrophobicity, and, make them nontoxic. Surfactants [27], peptides and polymers [1,28] have been used to modify SWNT via non covalent interactions. Among the frequently used biological species, chitosan is much more attractive due to its biodegradability and biocompatibility which can endow the SWNTs excellent water solubility and good biological compatibility [29,30]. Moreover, presence of amine group in chitosan structure can facilitate attachment of targeting ligands to the drug delivery system [31,32]. In this paper, for the first time, to the best our knowledge, single walled carbon nanotube was used to targeting delivery of quercetin. Chitosan have been applied to functionalization of carbon nanotubes. First, the influence of the binding conditions during the drug loading step (e.g., temperature, initial weight ratio of drug/carrier and initial amount of chitosan applied for functionalization) on the drug

loading efficiency was investigated. Then pH dependent release of the drug was demonstrated which promoted its release inside tumour cells upon internalization. Finally, folic acid (fig 1b) was also applied to the nanotube surface to endow targeting ability to the carrier and the therapeutic efficacy of the drug delivery system was determined by in vitro cell viability assays.

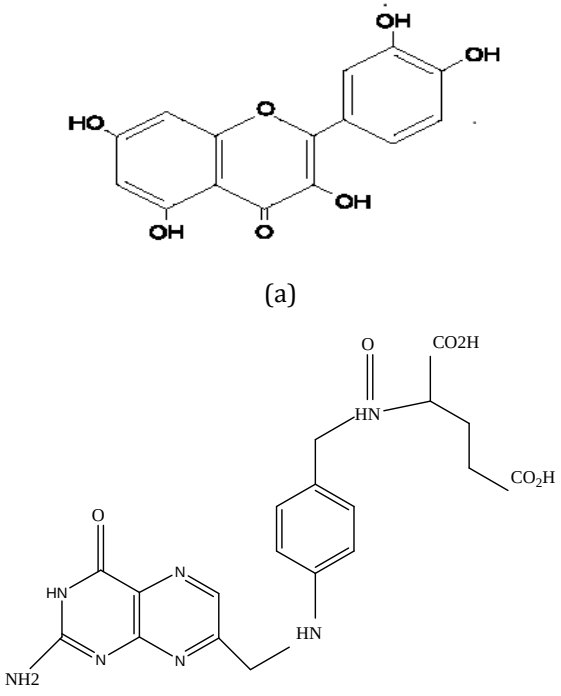


Figure 1. Molecular structures of (a) quercetin and (b) folic acid

2-Experimental procedure

2.1. Materials and Methods

Single walled carbon nanotubes were purchased from Neutrino Company (Tehran, Iran). (Purity >99%, length >5µm, diameter 1–2 nm, surface area >380 m^2/g). Low molecular weight chitosan (~50kDa) with 75-85% degree of deacetylation, folic acid, ethanol (≥ 99.5%), N, N′-dicyclohexyl carbodiimide (DCC), dimethylsulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and Quercetin (QU) were obtained from Sigma-Aldrich. Fetal bovine serum (FBS), RPMI 1640 and penicillin/streptomycin were obtained from Gibco Inc. Human cervical cancer HeLa cells were purchased from the Pasteur Institute of Iran, Tehran, Iran. Analytical reagent grade salts and chemicals were used without further purification. HPLC grade (> 99%) solvents were used for these studies.

UV-Visible spectroscopy (UV-2501, Shimadzu, Japan) was used for quercetin concentration measurement. Fourier transform infrared (FTIR) (Perkin–Elmer, Spectrum65) and CHNS elemental analyzer (LECO 932, USA) were used for characterization of functional groups. Infrared (IR) spectra of the samples were scanned in the range from 400 to4000 cm−1 and obtained at a resolution of 4 cm−1 with a minimum of 256 scan per spectrum.

2.2. Functionalization of single walled carbon nanotubes

As raw SWNTs are often too long with bundled or aggregated structures contaminated by heavy metal impurities, Single walled carbon nanotubes were cut and purified through oxidative acid treatment using the procedure documented in the literature [33]. Briefly, 1g of the SWNTs were suspended in 250 mL solution of nitric acid (65%) and sulphuric acid (98%) in a volume ratio of 2:3 then refluxed for 24 h at 50 °C. The resultant mixture was diluted with ultra-distillated water followed by centrifugation for several times to obtain neutral ph. Finally, the precipitated oxidized SWNTs (ox-SWNT) containing carboxylic acid groups were collected and dried at 60°C over night. For functionalization of ox-SWNTs with chitosan, 50 mg of ox-SWNT and predetermined amount of chitosan in different chitosan/ ox-SWNTs weight ratio (1/1, 1/2, 1/3, 1/4) were suspended in PBS (pH 7.4, 100ml); and sonicated for 1h. Then, mixture was stirred at room temperature for 16 h. The product was filtered and rinsed with distilled water to remove the unbound polymer; finally, the solid (CS-SWNT) was collected and freeze dried.

2.3. Folate targeting

Folic acid (FA) was conjugated to the carrier, and make it targetable to cancer cells. Folic acid (6 mg) was stirred with a mixture of 10 mg DDC and 10 ml DMSO for 1 h at room temperature. Then, CS-SWNTs (3 mg) were added to the mixture and stirred for 16 h at room temperature in darkness. The unreacted folic acid was removed by filtration and washing with distilled water. Finally, the resultant solid was dried at room temperature and named as FA-CS-SWNT.

2.4. Drug loading

Quercetin loading onto carriers was performed by mixing10 mg of carrier and pre-determined weight of quercetin in quercetin/CS-SWNTs weight ratio (1/1then both components were submerged in 50 ml methanol and stirred for 16 h. The suspension was centrifuged (13000 rpm, 30 min), filtrated, and washed thoroughly with methanol until the filtrate became colorless. Finally, the products (known as QU-ox-SWNTs and QU-CS-SWNTs) were collected and freeze dried at 20 °C for 24 h. The above-mentioned procedure was conducted in 4, 25 and 60°C. The amount of unbound quercetin in the filtrate measured by UV/vis absorption spectroscopy at 370 nm (the characteristic absorbance of quercetin in methanol) respective to a calibration curve accomplished under the same conditions [34]. The

spectroscopy results were used to calculate drug loading efficiency according to the following equations:

Drug loading efficiency (%) = 100× (wfeed QU-wfree QU)/ Wfeed QU (1)

Where Wfeed QC and wfree QC are the total amount of quercetin and the amount of unbound quercetin in the filtrate, respectively.

2.5. Drug release

1 mg drug loaded SWNT was dispersed in 10 ml of solutions of PBS and methanol (90:10 v/v) in a screw capped tube and placed in a shaker incubator at 37oC with constant horizontal shaking at 100 rpm. At specified time intervals, the nanotubes were separated from the buffer by ultracentrifugation, and the whole release medium was replaced with fresh one. These experiments were carried out on pH 7.4 (normal condition) and pH 5.5 (tumor condition).

2.6. Cell culture & cell viability experiments

Hela cells were cultured in T25 culture flasks in RPMI-1640 medium supplemented with10% fetal bovine serum and penicillin/streptomycin (100 units/mL penicillin and 100 µg/mL streptomycin) in an incubator at 37°C in which the CO2 level was maintained at 5%. In order to measure cell viability, cultured cells were seeded at a density of 104 cells per well into 96 well plate and incubated for 24 h, then the medium was replaced with fresh mediums containing 100 µg/mL CS-SWNTs, 100µg/mL FA-CS-SWNTs, 100 µg/mL FA-QU-CS-SWNTs (based on quercetin concentration) and 100 µg/mL free QU at 37°C. In different time intervals (24, 48, 72h), the in vitro cell cytotoxicity was assessed by the MTT assay through addition of 10µl solution of MTT reagent (0.5mg/ml) and incubation for 4 h at 37°C. Then, the medium was discarded and replaced by 100 µL DMSO to dissolve the formazan crystals of which the absorbance was calculated after 1 h of incubation at 570 nm using microplate reader.

The percentage viability of cells was calculated as the ratio of absorbance of triplicate readings with control wells.

Results and discussion

Functionalization of single walled carbon nanotubes

Fig 2 displays FTIR spectrums of chitosan and CS-SWNT. The basic peaks of chitosan are at 3200-3500 cm−1 (O-H and N-H stretch), 2850 cm-1 (C-H stretch), 1596 cm-1 (N-H bend), 1420 (C-H bend), and 1090 cm-1 (C-O-C stretch). The spectrum of CS-SWNT display all the characteristic absorption bands of both ox-SWNT and chitosan, indicating of successful adsorption of chitosan on the carbon nanotubes surface.

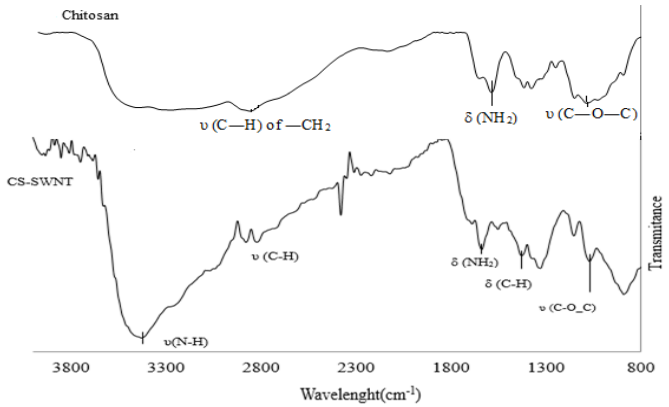


Figure 2. FTIR spectroscopy chitosan and CS-SWNT

Drug loading

Loading of quercetin on QC-CS-SWNT confirmed by FTIR spectroscopy (Fig 3). FTIR spectroscopies of quercetin (Fig 3a) shows broad peaks at 3290 and 1358 cm−1 which are assigned to stretching vibration and phenolic bending of hydroxyl (O-H) groups. Peaks at 1662 and 1093 cm−1 assigned to (C=O) stretching and C-O stretching vibrations from (C-O-C). Peaks at 1512 and 932 cm−1 represent the aromatic C=C stretching and C-H bending vibration of aromatic group [7]. The basic characteristic peaks of CS-SWNT (Fig 3b) are at 3200-3500 cm−1 (O-H and N-H stretch), 1640 cm-1 (N-H bend), 1152 and 1072cm-1 (C-O-C stretch). Loading of quercetin on CS-SWNT (Fig 3c) leads to presence of alkene and aromatic stretching vibration at 1630, 1427 and 1377 cm−1 confirm of quercetin loading on nanotubes. Moreover, peak at 932 cm−1 in quercetin (Fig 3a) related to bending vibration of C-H bond in aromatic ring, shifted to 891 cm−1 in QC-CS-SWNT which is due to the formation of hydrogen bonds between quercetin and carrier.

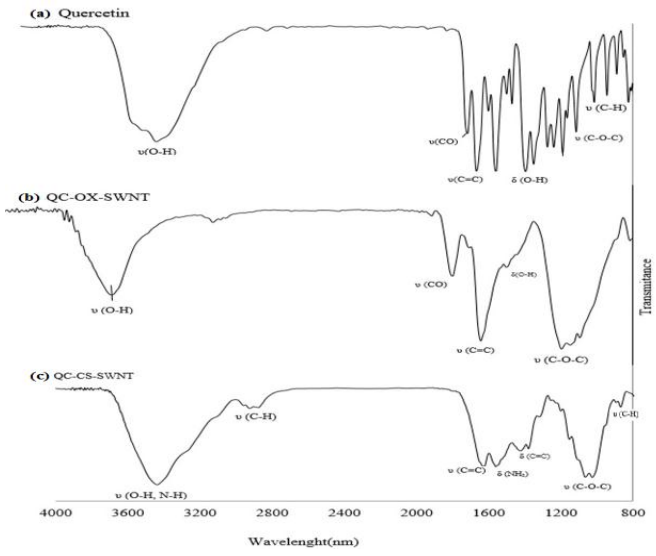
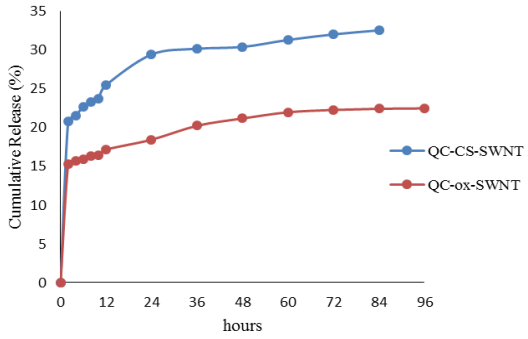


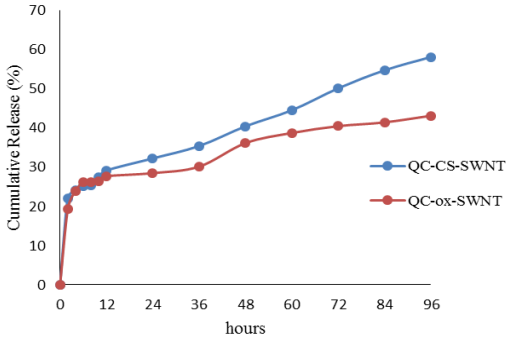
Figure 3. Fourier transform infrared (FTIR) spectra of (a) Quercetin, (b) CS-SWNT and (c) QU-CS-SWNT

In-vitro quercetin releasing

Fig 4a and 4b display cumulative drug release curves for a duration of 96 hr. at 37 °C, in PBS pH=5.5 and 7.4, respectively. The in vitro release kinetics shows initial burst release followed by a slow and sustained release. This burst release is normally attributed to the fraction of quercetin which was adsorbed in the surface of the nanotubes. As shown in Fig 4 quercetin was released slower from the nanocarrier at neutral conditions (pH 7.4). There was only about 32 % of the total drug released for QC-CS-SWNT after 96h While under the pH of tumor tissues (pH 5.5) nearly 61.5% was released. In pH 7.4, the hydrogen bonds between quercetin and SWNTs were tightly bound; while, by decreasing the pH, quercetin was quickly released due to competition of H+ ion with functional groups capable of forming hydrogen bonding with drug. A higher release rate of quercetin was achieved for QC-CS-SWNT. As, chitosan increase hydrophilicity of CNT surfaces, which leads to weakening of the π–π interactions of QC to the CNT surfaces, quercetin can be released easier and faster.



(a)



(b)

Figure 4. Drug release of modified SWNTs at (a) pH=7.4 and (b) pH=5.5

Cell viability studies

The control, CS-SWNT, pure quercetin, quercetin conjugated nanotubes QU-CS-SWNTs and FA-QU-CS-SWNTs were incubated individually with human cervical cancer HeLa cells to study the inhibition of the cell growth rate using MTT assay. Results are shown in figure 5. As shown in this fig, CS-SWNT displayed no toxic effect on cells, and cell viability was 94% even after 72 h. This clearly demonstrated that functionalized carbon nanotubes with chitosan have good biocompatibility. whereas quercetin-loaded SWNTs (QU-CS-SWNT) with quercetin concentration of 100 µg/ml caused significant Hella cancer cell death and the cell viability decreased to 44% after 72 h which could be higher compared to pure quercetin (57%) at the same concentration. The toxicity of the Hela cancer cells might be attributed to the strong binding between the Hela cells and quercetin molecules due to the partial release of quercetin from the nanotubes.

The FA-QU-CS-SWNTs exhibits a cell viability of 37% which demonstrate the targeting drug carrier, FA-QU-CS-SWNT had stronger cytotoxicity than did the non-targeting one (QU-CS-SWNT). Folic acid increased cellular uptake and internalization through receptor-mediated endocytosis mechanism, which lead killing of specific cancer cells for the targeting drug carrier [35].

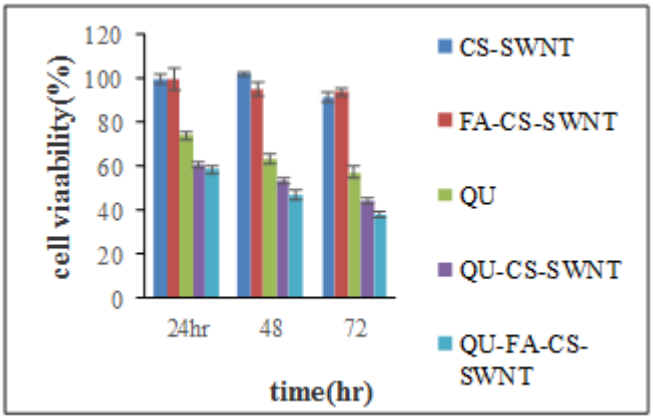


Figure 5. Cell viability of the modified SWNTs: Viability of HeLa cells treated with FA-CS-SWNTs, QU-CS-SWNTs, QU-FA-CS-SWNTs, CS-SWNTs and free quercetin for 24, 48 and 72 h.

3. CONCLUSIONS

An efficient drug delivery system based on modified SWNTs was established. Chitosan and folic acid were applied for functionalization of single walled carbon nanotubes. Then, an anticancer drug (quercetin) was loaded on the modified carrier by π-π interactions and hydrogen bonds. The FT-IR analysis demonstrated that quercetin formed intermolecular hydrogen bonding with carriers. The loading efficiency of quercetin on CS-SWNT was determined to be around 38% under best conditions (at 4°C, chitosan/ox-SWNT weight ratio 1/1 and drug / carrier weight ratio 1/1). This means that 1 gram of chitosan wrapped carbon nanotubes is able to deliver 0.38 g of quercetin, which an acceptable loading capacity compared with many other common nanocarriers.

The introduced drug delivery systems displayed stability under physiological conditions (pH = 7.4 and temperature of 37°C), while simulating the cancer cell condition at pH=5.5, the quercetin was efficiently released (61.5% after 96hfor QU-CS-SWNT). Based on the data gathered from in vitro studies, it can be concluded that, chitosan coating of carbon nanotubes enhances their biocompatibility and thus making them safe and efficient candidate for targeted delivery applications. Quercetin delivery by FA-CS-SWNT may be considered as an effective delivery system for improving pharmacokinetic and properties of quercetin.

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