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Original Article

Immobilization and *In vitro* Evaluation of Soyasapogenol B onto Functionalized Multi-Walled Carbon Nanotubes

A.A. Haroun [a,*], H.A. Amin [b], S.H. Abd El-Alim [c]

[a] *Chemical Ind. Res. Div., National Research Centre, 33 Bohouth Str., Dokki, Giza, Egypt*
[b] *Chem. of Natural & Microbial Products Dept., National Research Centre, 33 Bohouth Str., Dokki, Giza, Egypt*
[c] *Pharmaceutical Technology Dept., National Research Centre, 33 Bohouth Str., Dokki, Giza, Egypt*

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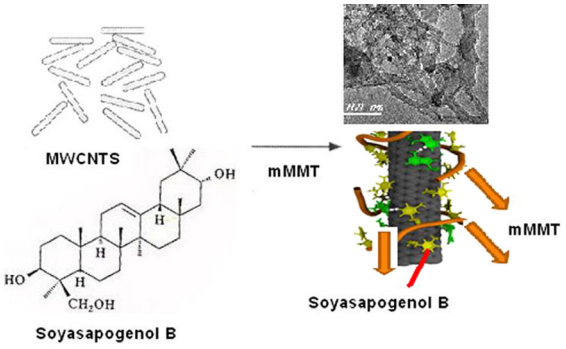
Highlights

- SSB production from soybean saponin by *A. flavus* whole-cells is investigated.
- SSB was immobilized onto functionalized MWCNTs using simple adsorption technique.
- *In vitro* SSB release profile, cellular uptake and cytotoxicity were carried out.
- SSB loaded particles exhibited low *in vitro* cytotoxic with different carcinoma cell lines.

Graphical abstract



Abstract

Context: Soyasapogenol B (SSB) has been shown to possess hepatoprotective, antiviral, anti-inflammatory, antimutagenic and anticancer activities. The goal of this work is to study the influence of functionalized multi-walled carbon nanotubes (MWCNTs) on the biological activity of the loaded soyasapogenol B.

Methods: SSB was prepared by enzymatic hydrolysis of soybean saponin using *Aspergillus flavus* whole cells. While, the functionalization of MWCNTs was conducted using the adsorption technique in the presence of the modified montmorillonite (mMMT) with cetyltrimethyl ammonium bromide (CTAB). *In vitro* drug release profile, kinetics of release, cellular uptake and cytotoxicity were also investigated. The prepared materials were characterized using: FTIR, particle size distribution analysis and TEM.

Results: The *in vitro* release and cytotoxicity of the SSB loaded and unloaded samples were carried out using the dialysis bag diffusion technique and sulphorhodamine B (SRB) assay, respectively. The results showed that SSB loaded MWCNTs, mMMT and MWCNTs/mMMT had particle size of 414, 1121 and 412 nm, respectively, and 338, 1071 and 268 nm, for the unloaded ones, respectively. FTIR proved that SSB was successful immobilized onto functionalized MWCNTs.

Conclusions: Successful loading of SSB, as a bioactive material, onto functionalized MWCNTs with modified clay (montmorillonite) using CTAB exhibited very low cytotoxic behavior with human normal melanocyte (HFB-4), breast (MCF-7) and liver (Hep-G2) carcinoma cell lines relative to SSB and doxorubicin standard.

* Corresponding author.
E-mail address: haroun68_2000@yahoo.com (A.A. Haroun).

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Keywords: Multi-walled carbon nanotubes; Montmorillonite; Soyasapogenol B; *In vitro* release kinetics; Cytotoxicity

1. Introduction

Saponins are a family of steroid or tri-terpeniod glycosides present in plants. Soyasaponins are oleanene-type triterpeniod saponins and can be divided into two groups: A and B according to their respective aglycones (soyasapogenol A and soyasapogenol B, respectively). Also, soyasapogenols can be obtained by enzymatic hydrolysis using some microorganisms [1,2]. They could help to lower blood cholesterol level [3], and inhibit growth of cancer cells [4–7]. Moreover, they have antiviral [8] and anti-inflammatory activities [9,10]. Immobilization of biologically active compounds on nanosized surfaces is a key process in bionanofabrication. It carried out using different techniques such as: adsorption, sol–gel and covalent approaches. Also, multi-walled carbon nanotubes (MWCNTs) with their high surface areas, serve as important carrier in the fabrication of novel functional materials [11–17]. Montmorillonite is a natural clay mineral which is used as metal nanoparticles catalyst supports. It has swelling capacity, high surface areas and strong adsorption capacities [18]. In this paper, we report the preparation, characterization of functionalized MWCNTs with mMMT and loading with SSB *via* adsorption technique. Furthermore, FTIR, TEM, particle sized analysis and *in vitro* kinetic SSB release were carried out. Additionally, *in vitro* cytotoxic examination using SRB assay against different cell lines (HFB-4, MCF-7 and Hep-G2) was investigated.

2. Materials and methods

2.1. Materials

Cetyltrimethyl ammonium bromide (CTAB) and montmorillonite 99% (MMT, pH 2.5–3.5, and particle size around 40 μm) were obtained from Fluka. Multi-walled carbon nanotubes (MWCNTs), carbon content 95%, diameters 6–9 nm × 5 μm was obtained by Aldrich. PGPR 90 Kosher surfactant (polyglycerol polyricinoleate) was obtained from Danisco, Denmark. All chemicals and other reagents were used as received without further purification. Cell lines were obtained from National Institute of Cancers, Cairo University, Egypt.

2.2. Methods

2.2.1. Oxidation and purification of MWCNTs

Pristine MWCNT (3.0 g) was dispersed in mixed concentrated sulphuric and nitric acids (3:1, v/v) at ratio of 50 mL of acid mixture per 10 mg of MWCNTs [19]. The resulted mixture was then refluxed at 110 °C overnight with continuous stirring to produce oxidized carbon nanotubes (ox-MCNTs). The sample was washed with deionized water until the filtrate was neutral (pH 7.0). The collected solid was dried under vacuum at 70 °C for 12 h. The obtained material was kept for further functionalization and analysis.

2.2.2. Modification of MMT

100 mg of MMT powder was dispersed in 100 mL of deionized water. Aqueous solution of CTAB (100 mg/10 mL) was slowly added to above solution at 70 °C. The resulting solution was ultrasonic treated for 4 h, followed by heating overnight at 70 °C. The resulting material (mMMT) was filtered and washed several times with hot water. The obtained modified clay was dried, ground, and kept for further investigation.

2.2.3. SSB preparation from soybean saponin by Aspergillus flavus whole-cells

SSB was prepared from soybean saponin using *Aspergillus flavus*, a fungus isolated from peanut pods, whole cells producing saponin hydrolase enzyme according to a method previously described by Amin et al. [20] as shown in Scheme 1. *A. flavus* was cultivated in an enzyme production medium containing (%, w/v), 2 soybean saponin, 4 malt extract, 2 yeast extract, 0.2 KH_2PO_4, 0.2 $(NH_4)_2SO_4$, 0.03 $MgSO_4{\cdot}7H_2O$ and 0.03 $CaCl_2{\cdot}2H_2O$ with pH adjusted initially to 9.0. After 48 h cultivation, cells were removed and washed with normal saline. Then, cells (100 g cell wet weight) were resuspended in acetate buffer (1L, pH 5.5) containing 2% soybean saponin and incubated for 48 h at 45 °C with stirring. The mixture was extracted by ethyl acetate (2000 mL) and the solvent was distilled off. The dried mixture was applied to a silica gel column (2.5 × 120 cm) and eluted first with benzene/ethylacetate (95:5, 2 L), then with benzene/ethylacetate (90:10, 7 L). Fraction B eluted with 10% ethyl acetate was distilled off and product was re-crystallized from chloroform/methanol (1:1) to yield pure SSB.

2.2.4. Functionalization of MWCNTs and mMMT [21]

Appropriate amounts of ox-MWCNTs and mMMT (25 mg) were suspended in 5 mL deionized water for 20 min at 25 °C. Subsequently, SSB (10 mg) dissolved in ethyl acetate (5 mL) and 10 mL of PGPR in cyclohexane mixture was charged into ox-MWCNTs and/or mMMT aqueous solutions and homogenized for 20 min at 12,000 rpm. Then 20 μL of tolylene-2,4-diisocyanate was added and stirred overnight. After that, the obtained materials were centrifuged at 10,000 rpm, collected and dried. The prepared materials with different compositions were kept for further investigation (Table 1).

2.3. Characterization

2.3.1. The samples were examined using Perkin-Elmer Fourier transform infrared spectroscopy (FTIR) spectroscopy under certain condition such as: scan resolution: 4 cm^{-1},