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Synthesis of silica-PAMAM dendrimer nanoparticles as promising carriers in Neuro blastoma cells

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ABSTRACT

Mesoporous silica carriers are emerging as therapeutic drug delivery systems. The objective of this study was to develop a formulation for synthesizing silica-PAMAM dendrimer hybrid nanoparticles with sol-gel technique. Subsequently, black carrot anthocyanins were encapsulated and investigated for their capability in terms of inhibiting the proliferative effects of neuroblastoma (Neuro 2A). In this context, particle size distributions were ascertained followed by thermal analysis (DSC), scanning electron microscopy and encapsulation efficiency. Subsequently, *in vitro* release kinetics was determined along with cytotoxicity of empty and anthocyanin doped hybrid nanoparticles. The lowest particle size was 134.8 nm with a zeta potential of +19.78 mV which enhanced electrostatic interaction with the cell membrane in the cytotoxicity analyses. As the anthocyanin content was totally released at the end of 6 days, the cytotoxicity was observed for 134 h, reaching an inhibition of 87.9%. On the other hand, Neuro 2A cells incubated with empty nanoparticles exhibited a high proliferation indicating that hybrid nanoparticles were not toxic to the cells and the inhibitory effect was associated with the anthocyanins.

1. Introduction

The emergence of mesoporous silica as a carrier in drug delivery systems has led to the development of novel therapeutics particularly owing to the properties such as high surface area, pore volume and uniform particle size [1,2]. Mesoporous silica nanoparticles can be synthesized by sol-gel technology which involves the hydrolysis and condensation of alkoxysilane precursors under acidic or basic environments, in which the structure depends on many parameters such as type and concentration of precursors, water-to-precursor ratio, nature and concentration of a catalyst agent, solvent, temperature, as well as aging and drying processes [3,4]. Synthesis of mesoporous silica nanoparticles by sol-gel technology allows better control over drug loading and release. Furthermore, these nanoparticles can be targeted at specific sites through functionalization of the silica surface providing anchoring points for the molecule bonding, which in turn could enhance the adsorption of the biomolecules and release in the desired target [5], thereby reduce the potential side effects of anticancer drugs [6]. Indeed, mesoporous silica nanoparticles have been utilized as drug delivery vehicles for various anticancer compounds such as doxorubicin [7], camptothecin [8,9] and paclitaxel [10].

In regards to anticancer compounds, anthocyanins are reported to display anticancer and chemopreventive activities apart from antioxidant and anti-inflammatory effects [11–14]. Therefore, it has been proposed that anthocyanins may exert therapeutic activities on human diseases such as cancer [15,16], coronary heart [17,18] and neurodegenerative diseases [19] associated with oxidative stress [20] based on experimental and epidemiological evidence. However, these compounds are sensitive to various factors such as heat, light, metal ions, pH, glucose and ascorbic acid present in the environment [21,22]. Additionally, bioavailabilities are very low due to limited absorption and fast metabolization in the body [23,24]. Once absorbed, the systemic effects of circulating

Abbreviations: TEOS, tetraethoxysilane; PAMAM, polyamidoamine; DSC, Differential Scanning Calorimeter; SEM, Scanning Electron Microscopy; FTIR, Fourier Transform Infrared; HPLC, High Pressure Liquid Chromatography; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide; DMEM, Modified Eagle Medium; HCl, hydrochloric acid; NaH_2PO_4, sodium phosphate; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide; CY-3-GLY-EQUIVALENT, cyanidin-3-glycoside-equivalents.

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anthocyanins released in a time dependent manner might enhance its therapeutic activity.

In a previous study conducted by our group, anthocyanin rich black carrot extract exhibited a selective cytotoxic activity against Neuro 2A cells, whereas was not toxic to normal Vero cells [25]. Therefore, it was of great interest to formulate and synthesize anthocyanin doped biomimetic silica nanoparticles with sol-gel technique and investigate whether these nanoparticles are capable of inhibiting the proliferative effects of neuroblastoma (Neuro 2A) cancer cells. In this context, particle size distributions of synthesized nanoparticles were ascertained followed by thermal analysis (DSC), scanning electron microscopy for characterization purposes and assessment of encapsulation efficiency. Subsequently, *in vitro* release kinetics determined at pH 7.4 and cytotoxicity of empty and anthocyanin loaded hybrid nanoparticles against Neuro 2-A cancer cells were investigated.

2. Materials and methods

2.1. Materials

Black carrots (*Daucus carota* L. spp.) obtained from Izmir, Turkey were washed in water, crushed and then dried at 30 °C. The dried parts were homogenized in blender and transferred into soxhlet apparatus containing proper amount of ethanol. The extraction was carried out on a magnetic stirrer (IKA C-MAG HS7) at 40 °C for 3.2 h to obtain anthocyanins [25]. Subsequently, the mixture was centrifuged (Hettich Zentrifugen Universal 32 R) at 4000 rpm for 5 min and filtered through a 0.45 μm syringe filter. The anthocyanin rich extracts were kept at −20 °C before use. PAMAM dendrimer, ethylenediamine core, generation 2.0 solution (20 wt % in methanol) was purchased from Sigma Aldrich. The silane precursor tetraethoxysilane (TEOS) was supplied from Across Organic (New Jersey, United States). All other chemicals were of analytical grade purity.

2.2. Sol-gel synthesis of nanoparticles

Tetraethyl orthosilicate $Si(OC_2H_5)_4$ (TEOS) was hydrolyzed by hydrochloric acid (HCl) and stirred for 15 min using vortex at high speed. Hydrolyzed silica (0.0250 and 0.0125 M), PAMAM (Polyamidoamine) dendrimer and sodium phosphate (NaH_2PO_4) buffer at pH 8.0 were stirred for 1 h with a magnetic stirrer at high speed to synthesize nanoparticles. Subsequently, nanoparticles were centrifuged for 15 min at 9000 rpm, washed twice with ultrapure water and centrifuged at 9000 rpm once again for 15 min. Finally, nanoparticles were freeze-dried for further analysis.

2.3. Particle size and zeta potential analyses

The particle size distribution and zeta potential of the anthocyanin loaded nanoparticles were determined using dynamic light scattering by Malvern Zetasizer Nano-ZS (Malvern Inst. Ltd., UK). All experiments were done in triplicate.

2.4. Differential scanning calorimeter (DSC) analysis

Thermal properties of the nanoparticles and black carrot extract were studied by differential scanning calorimetry DSC (Perkin Elmer, model DSC 8000). Approximately 5–10 mg of nanoparticles was weighted for analysis. The samples were purged with pure dry nitrogen at a flow rate of 20 ml/min. The analysis was carried out at a linear heating rate of 10 °C/min and a temperature range of 30–200 °C.

2.5. Fourier transform infrared (FTIR) analysis

FTIR spectra of anthocyanin, loaded and empty SiO_2–PAMAM dendrimer hybrids were recorded between wave lengths of 650–4000 cm^{-1} on a Perkin-Elmer Spectrum 100 instrument.

2.6. Scanning electron microscopy (SEM) analysis

Scanning electron microscopy was used to investigate the morphology of anthocyanin encapsulated particles. SEM photographs were taken using a scanning electron microscope, FEI Quanta 250 FEG at the required magnification at room temperature. Prior to observation samples were mounted on metal grids using double sided adhesive tape and coated with gold under vacuum for 1 min at Emitech K550X sputter coater. The images were captured on SEM mode at desired magnifications.

2.7. Determination of encapsulation efficiency by HPLC analysis

Encapsulation efficiency was determined by ultracentrifugation method. The freshly prepared nanoparticles were centrifuged at 20000 rpm for 30 min, the supernatant was separated from the pellet and quantified by HPLC (Thermo Scientific system) in order to determine the unloaded anthocyanin amount. The samples were analyzed on Ace C-18 150 mm × 4.0 mm × 5 μm column (Advanced Chromatography Technologies Ltd, Aberdeen, Scotland) using Alliance 2695 Separations Module, equipped with a model 2996 PDA (Waters Instruments, Inc., Milford, MA). Chromatography was carried out using a mobile phase consisting of 0.01% TFA in water (solvent A) and 0.01% TFA in acetonitrile (solvent B). The injection volume was 10 μl, a flow-rate 750 μl/min was applied during the analysis and peaks were detected at 520 nm. Calibration curves were established by dissolving 400 μg Cyanidine-3-o-glycoside in 2 ml HPLC grade methanol and 6 different concentrations were prepared by serially diluting this stock solution 2-fold with methanol (200–3.125 μg/ml). The quantities of the black carrot extracts are expressed as "cyanidin-3-glycoside-equivalents" (cy-3-gly-equivalent). The latter yielding the entrapped anthocyanin when subtracted from the total anthocyanin amount. Encapsulation efficiency was expressed as the ratio of actual and theoretical black carrot extract loaded. The analyses were carried out in duplicate.

2.8. In vitro release studies

The dialysis membrane method was used to determine the amount of extract diffused from the encapsulated particles. Nanoparticle samples were enclosed in a dialysis membrane with a molecular weight cut-off value of 12000–14000, which acted as a donor compartment and incubated at 37 ± 0.5 °C in 50 ml of phosphate buffer saline of pH 7.4, which acted as a receptor compartment. Aliquots of dialysate were taken at predetermined time intervals and analyzed for anthocyanin release by HPLC. The release medium was replenished immediately with the same volume of fresh phosphate buffer.

2.9. Cell culture and in vitro cytotoxicity assay

Neuro-2A brain neuroblastoma from mouse and Vero (African green monkey kidney) normal cell lines were obtained from American Cell Culture Collection (ATCC Mannassas, VA). The cells were cultures in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 unit/ml bovine insulin, 100 units/ml penicillin and 100 μg/ml streptomycin and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The time-