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Enhanced colon cancer chemoprevention of curcumin by nanoencapsulation with whey protein

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ABSTRACT

To improve bioavailability and enhance colon cancer prevention ability of curcumin, whey protein was used to nanoencapsulate at three different ratios such as 70:30, 50:50 and 35:65 for the first time. The drug loading, entrapment efficiency and structural changes of curcumin was confirmed by quantitative NMR spectroscopy. The nanoparticles prepared using the three ratios had an average diameters of 236.5 ± 8.8, 212 ± 3.4, and 187 ± 11.4 nm, as well as zeta (ζ) potentials of −13.1, −9.26, and −4.63 mV, respectively, at pH 7.0. The cytotoxicity assay was performed for human colon and prostate cancer (SW480 and LNCap) by MTT assay and results showed significantly higher cytotoxicity of nanoencapsulated curcumin (NEC) (equivalent to 30.91, 20.70 and 16.86 μM of NEC-1, 2 and 3 respectively), as compared to plain curcumin at 50 μM after 72 h of treatment. Cytotoxicity was also confirmed by microscopy of treated cells stained with acridine orange and propidium iodide. The cells treated with 50 μM of curcumin, 30.91 μM (NEC-1), 20.70 μM (NEC-2) and 16.86 μM (NEC-3) showed enhanced activation of p53 and elevated bax/Bcl2 expression (NEC-3), increased cytochrome-c in cytosol (NEC-2) confirming the enhanced cytotoxicity. To confirm the increased bioavailability, the intracellular curcumin was measured using fluorescence intensity. The fluorescent signal for intracellular curcumin was increased by 12, 30, and 21% for NEC-1, NEC-2, and NEC-3 respectively as compared to plain curcumin at 4 h. Based on these results, we conclude that nanoencapsulated curcumin with whey protein will have potential to be considered for clinical applications for future studies.

1. Introduction

In spite of promising health beneficial properties of curcumin in various *in vitro* and *in vivo* models, the pharmacokinetic and bioavailability studies indicates the poor absorption and rapid elimination from the body (Shehzad et al., 2010). Therefore, a number of strategies have been attempted to enhance the bioavailability of curcumin, including the use of therapeutic adjuvants, liposomal curcumin, curcumin nanoparticles, curcumin-phospholipid complexes, and structural analogs of curcumin (Yallapu et al., 2015). Piperine enhanced the bioavailability of curcumin up to 20-fold by acting on the major metabolic enzyme glucuronidase, which is responsible for rapid degradation of curcumin in liver and intestinal tissue (Shoba et al., 1998). More recently curcumin-β-cyclodextrin/cellulose nanocrystals tested for the inhibition of colorectal and prostate cancer cell lines. The curcumin nanocrystals exhibited IC_{50} less than curcumin (Ndong et al., 2016). However, the release rate seems to be very poor. The use of curcumin-phospholipid combination resulted in significant elevation of bioavailability compared to plain curcumin in plasma at different time points (Marczylo et al., 2007). Currently more than thousands of different nanoparticles are known but there are no well-defined guidelines to evaluate their potential toxicity and safety (Elzoghby et al., 2012). It is important to consider the safety of the polymeric material and other excipients used in alternative delivery system.

Protein serves as the natural counterpart to synthetic polymers for the development of nanoparticles due to their characteristic properties such as biodegradability and added nutritional value. Thus proteins were considered as generally regarded as safe drug delivery devices. Due to this reason, protein encapsulated nanoparticles were widely prepared for commercial applications (Elzoghby et al., 2012). Based on the existing literature, we hypothesized the use of whey protein as safe alternatives to synthetic polymer, which may improve pharmacokinetic properties of curcumin. The current study investigates the development of

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formulation process for the nanoencapsulation of curcumin with whey protein at different ratios to obtain stable complexes. These nanoencapsulated samples were characterized for entrapment, size, PDI, zeta potential, *in vitro* release, and morphological behavior, along with their cytotoxicity effect on human colon and prostate cancer cells. Novelty of the study lies in utilizing natural ingredient for encapsulation and formulation of nanosized molecules for enhancing health beneficial properties of curcumin.

2. Materials and methods

2.1. Chemicals

Whey protein (gold standard) was purchased from a local supermarket (College Station, TX, USA). All analytical and high-performance liquid chromatography (HPLC) grade solvents and molecular biology grade dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA). Nanopure water (NANOpure, Barnstead, Dubuque, IA, USA) was used for liquid chromatography. Pure curcumin was isolated and identified in our laboratory according to our recently published method (Jayaprakasha et al., 2013). Dulbecco's Modified Eagle Medium, RPMI-1640 and other chemicals used for cell culture were purchased from Hyclone (Logan, UT, USA); penicillin, streptomycin mixture and trypsin ethylene diamine tetra acetic acid were purchased from Mediatech Inc., (Herndon, VA, USA). Protein detection bicinchoninic acid kit, all primary and secondary antibodies used in the study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technologies (Danvers, MA, USA). The western blot images were captured using a LAS4000 mini imaging system (Fuji medical system, Woodbridge, CT, USA) under chemiluminescence mode.

2.2. Nanoencapsulation of curcumin by desolvation method

A 10% (w/v) solution of whey protein in nanopure water and 10% curcumin in acetone was prepared by stirring on a 500 rpm magnetic stirrer at 27 °C for 2 h and 30 min, respectively. Nanoencapsulated curcumin (NEC) was prepared by a desolvation method (Langer et al., 2003) with three different ratios of curcumin to whey protein such as 70:30 (NEC-1), 50:50 (NEC-2), and 35:65 (NEC-3). For instance, NEC-1 was prepared by slowly mixing dropwise 140 mg of curcumin in 1.5 ml of acetone to 2.5 ml aqueous whey protein (60 mg) under constant stirring on a magnetic stirrer at 500 rpm. The mixture was further kept on the magnetic stirrer at 25 °C for 8 h followed by homogenization at 5000 rpm for 1 min. The resulting emulsion was centrifuged at 7826 × *g* for 20 min at 4 °C to remove the unbound curcumin. The reside was lyophilized and stored at −80 °C until further use. This preparation was named as nanoencapsulated curcumin 1 (NEC-1). In similar fashion, the other two formulations (50:50 and 35:65) were prepared by drop wise mixing of 1.5 ml of acetone containing curcumin (100 and 70 mg) to 2.5 ml of whey protein (100 and 130 mg) separately and named as NEC-2 and NEC-3, respectively. The remaining steps were followed as mentioned above to obtain dried powder.

2.3. Particle size, polydispersity index (PDI), and zeta potential

The particle size, PDI, and zeta potential of encapsulated curcumin were measured with a Zetasizer Nano ZS (Malvern Instruments, Westborough, MA) using phosphate-buffered saline (PBS) buffer (pH 7.4) as dispersion medium at 25 °C. Two mg of sample was mixed with 2 ml of buffer solution and vortexed for 3 min; this sample was then used for particle size and zeta potential measurement. The sizes of the curcumin-loaded whey protein formulations were measured using dynamic light scattering (DLS) particle size. Sample aliquots in PBS buffer were loaded into disposable capillary cells for reading by the Zetasizer, which measures the diffusion of particles moving under Brownian motion, and converts this to size and a size distribution using the Stokes–Einstein relationship. The measured particle size was calculated by Zetasizer software (version 7.02) and includes inputs of the particle reflective index and the buffer system used. The dispersion pH was 7.0 for the control and the sample containing curcumin. An average of 10 measurements was used to calculate the size. PDI gives an indication of the particle size distribution, which was calculated as the weight average molecular weight divided by the number average molecular weight. The zeta potential of the particles was measured with the Zetasizer. The ζ-potential was calculated from the electrophoretic mobility based on the Helmholtz-Smoluchowski formula. All calculations were carried out with the Zetasizer software.

2.4. Drug-loading and entrapment efficiency of curcumin

The drug loading (DL) and entrapment efficiency (EE) of curcumin were analyzed with a quantitative proton NMR spectrometer (JEOL USA, Inc., Peabody, MA, USA) operating at 400 MHz and equipped with a 5-mm multinuclear inverse probe head and NM-ASC24 sample changer (Jayaprakasha and Patil, 2016). A known quantity (2–4 mg) of nanoencapsulated sample was dissolved in 525 μl DMSO-d_6, transferred to a 5-mm NMR tube, and spectra were recorded at 25 °C. An external coaxial glass tube (OD 2 mm) (Shigemi Inc, Allison Park, PA, USA) containing 65 μl 0.012% 3-(trimethylsilyl) propionic-(2, 2, 3, 3-d_4) acid sodium salt (TSP-d_4) (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) in D_2O was inserted into the NMR sample tube as a quantitative reference. The TSP-d_4 concentration in the tube was pre-calibrated using a separate standard solution. All spectra were acquired with a 15 ppm sweep width, 12 s relaxation delay, four prescans and 32 transients. Further, spectra were zero filled to 131 k data points and data were processed using Delta software version 4.3.6. The DL and EE of NEC-1, NEC-2, and NEC-3 were calculated according to the formula:

$$DL=\frac{\text{drug}}{\text{polymer + drug}} \text{ and}$$

$$EE = \frac{\text{experimental drug loading}}{\text{theoretical drug loading}} \times 100$$

According NMR results, the actual curcumin present in stock solution of 50 μM of NEC-1, NEC-2 and NEC-3 was found to be 30.91, 20.70 and 16.86 μM respectively.

2.5. Field emission scanning electron microscopy (FE-SEM)

The shape and surface morphology were determined with an ultra-high-resolution field emission JSM-7500F scanning electron microscope (JEOL-USA, Peabody, MA, USA) equipped with a high brightness conical field emission gun and a low aberration conical objective lens. For SEM, a thin film of aqueous well dispersed nanoemulsion was applied to double-stick tape over an aluminum stub and vacuum dried to get a uniform layer of particles. These particles were coated with platinum-palladium using a sputter gold coater. Samples were dried under vacuum and the morphology of the nanoparticles was observed at 15 kV.

2.6. In vitro release kinetics of curcumin

The *in vitro* release kinetics of curcumin was measured using PBS (10 mM, pH 7.4) buffer and the release rate was determined by