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Calcium phosphate-quercetin nanocomposite (CPQN): A multi-functional nanoparticle having pH indicating, highly fluorescent and anti-oxidant properties

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ABSTRACT

Calcium phosphate quercetin nanocomposite (CPQN) i.e., quercetin entrapped in calcium phosphate nanoparticle was synthesized by a precipitation method at 80 °C, taking ammonium hydrogen phosphate, calcium nitrate and quercetin as precursors and sodium citrate as stabilizer. The nanocomposite suspension had different color at different pH values, a property that could render the nanoparticle a pH indicator. Besides color, the particles also had different size, shape, stability and quercetin content with change of pH. In addition, the CPQN was highly fluorescent having two sharp emission peaks at 460 and 497 nm, when excited at 370 nm; by this property it behaved as an effective fluorophore to label biological cell. Moreover, the nanocomposite had potential anti-oxidant property, for which mortality of mouse neuroblastoma cell N2A, by H_2O_2-induced oxidative stress, was found to be lowered by the pre-treatment of the cells with CPQN.

1. Introduction

Quercetin is a member of naturally occurring flavonoid compounds, present in fruits (apples, berries), vegetables (onion, broccoli), wine and tea [1]. Flavonoids are polyphenolic phytochemicals, which contribute to plants providing various colors from blue to red in flowers, fruits and leaves [2]. Flavonoids also have antioxidant, radical scavenging and metal chelating properties [3,4], for which they possess good anti-inflammatory [5], anti-microbial [6], anti-cancer [7], cardio- and neuro-protective [8,9] activities. However, therapeutic use of quercetin is limited due to its hydrophobic and unstable nature in physiological medium [10], resulting in poor bioavailability, poor permeability and rapid metabolism before reaching the systemic circulation [11]. Researchers have attempted to improve its solubility through complexation with liposomes [12], poly-D,L-lactide (PLA) [13], etc. Encapsulation of quercetin in suitable carrier like chitosan nanoparticles [14] is suggested to be a promising way to circumvent the limitations of using quercetin as a potential drug. Here, we report about a nano-formulation of quercetin (CPQN) by loading it in calcium phosphate nanoparticle (CPNP). CPNP was chosen because of its high membrane penetrating capacity to carry DNA [15–17], RNA [18,19], proteins [20,21] and therapeutic drugs [22–25] into different cells and since, calcium phosphate was a principal inorganic component of human bones and teeth, it was highly biocompatible and biodegradable with non-toxic degradation products [26,27]. Although, our long-term goal behind the synthesis of CPQN is development of a potential drug against oxidative stress-induced diseases, however we communicate here about the interesting pH-dependent characteristics like color, size, shape, stability, etc. of the nanocomposite particles. Such pH-sensitive characteristics were not reported earlier for nano-structured quercetin, synthesized by other methods [12–14,28]. This study also revealed that CPQN might have potential applications as a good pH indicator, fluorophore and antioxidant.

2. Experimental

2.1. Method of preparation of CPQN

Ammonium hydrogen phosphate (11.94 mM) of volume 3.8 ml was added drop-wise with stirring to calcium nitrate (19.51 mM)

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solution of equal volume (3.8 ml) at 80° C. pH of this mixture was adjusted to 8.5 by adding 4–5 drops of 30% ammonia solution. To this mixture, 800 μl of quercetin (Sigma), dissolved in DMSO as a stock of 10 mg/ml, was added drop-wise and the temperature of the stirrer was lowered down to room temperature (30 °C). The stirring was allowed for about an hour until the color of the suspension was changed from light yellow to orange. A volume of 7.6 ml of 10 mM sodium citrate was then added, as a result of which the pH of the suspension decreased to approximately 7.5 and the final concentration of quercetin became 500 μg/ml. The suspension was placed in ice and finally sonicated (20 KHz, 70% amplitude) in a probe sonicator (Cole-Parmer, CPX 130) for 15 min in discontinuous mode (pulse on for 59 s and pulse off for 20 s), to obtain stable CPQN. The prepared NPs were centrifuged at 55,000 rpm for 1 h in an ultracentrifuge (Sorval, WX Ultra 90); the NP pellet was finally suspended in Milli-Q water. The prepared NP suspension was stored at 4 °C for analysis and use.

2.2. Characterization of the synthesized CPQN

2.2.1. Methodology to determine optical properties

The absorbance of CPQN suspension was scanned in the wavelength region of 220–800 nm by a UV–Vis spectrophotometer (Shimadzu, UV-1800). A solution of the precursors, except quercetin and ammonia, mixed in the same proportions as in the synthesis of CPQN, was taken as reference. The fluorescence emission from the nano-composite was scanned in the wavelength region of 450–700 nm by exciting its suspension at 370 nm. The fluorescence was measured in a spectrofluorimeter (Carrey Eclipse, Agilent), keeping the excitation and emission slit width fixed at 5.0 nm each.

2.2.2. Methodology to determine size, shape and stability

Hydrodynamic size and poly-dispersity index (which signifies homogeneity in size) of the NPs were regularly measured by a dynamic light scattering (DLS) instrument (Malvern, Nano-ZS). The exact shape and size of the core particles were determined by field emission scanning electron microscope (FESEM) and atomic force microscope (AFM). FESEM sample was prepared by drop-casting CPQN suspension on a cleaned cover-slip and subsequently drying overnight in a desiccator. The completely dried sample was coated with gold by low voltage sputtering and finally analyzed by an FESEM (FEI, Inspect F50), at an accelerating voltage of 20 kV. For AFM study, dried NP film was prepared on a cleaned cover slip as described in [29] and scanned by an atomic force microscope (Veeco, di-Innova) in contact mode. The zeta potential of the particles, which generally gives an idea regarding the stability of NP, was determined by the DLS instrument.

2.2.3. Methodology to determine the percentage of quercetin loaded in CPQN

The percentage of the precursor quercetin (500 μg/ml) loaded in CPNP was determined by DPPH assay, using UV–Vis spectrophotometry. The principle of the assay is that the free radical DPPH [1,1-diphenyl-2-picryl hydrazyl], with purple color, is reduced by any anti-oxidant (like quercetin) to 1,1-diphenyl-2-picryl hydrazine of yellow color having absorption maxima at 517 nm [30]. This assay, therefore, determined the amount of quercetin as, it was proportional to the absorbance of DPPH at 517 nm. In the assay process, a volume of the precursor mix [$(NH_4)_2HPO_4$, $Ca(NO_3)_2$, quercetin and Na-citrate] was added to 300 μl of DPPH of strength 200 μM, so that the final concentration of DPPH became 60 μM. The mixture was then incubated for 15 min at room temperature, followed by the measurement of the $(\text{absorbance})_{517\text{nm}}$, which gave a measure of total quercetin in the mixture. On the other hand, to get a measure of un-used quercetin, the synthesized CPQN suspension was centrifuged at 55,000 rpm for 1 h and the supernatant was subjected to DPPH assay as above. The percentage of quercetin loaded in CPQN was finally determined as,

$$\text{Loading percentage} = \left[\frac{(\text{Abs})_{\text{Total}} - (\text{Abs})_{\text{sup}}}{(\text{Abs})_{\text{Total}}}\right] \times 100.$$

2.2.4. Methodology to study the release of quercetin from CPQN

The release rate of quercetin from the nano-conjugate was determined by dialysis method, as described by Mukherjee et al. [31]. A volume of 2 ml purified NP-suspension was taken in a dialysis bag with 10,000 Da cut-off membrane and the bag was dipped in a beaker containing 10 ml phosphate buffer saline (PBS–NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, pH – 7.4). The dialysis was continued under gentle stirring at 37 °C, for seven days. At intervals of time, 1 ml aliquot was withdrawn from the beaker and 1 ml fresh PBS was added there. The amount of dialyzed quercetin in the withdrawn aliquot was estimated by measuring the $[(\text{Abs})_{372\text{nm}}]$.

2.2.5. Methodology to determine the chemical bonds in CPQN

Fourier transformed infra-red (FTIR) spectroscopic study was performed to investigate the different chemical bonds, present in CPQN. The purified NP suspension was dried in lyophilizer (Heto, DW-3). From the lyophilized powder, FTIR sample was prepared as described in [32] and the spectroscopy was done in FTIR machine (Perkin Elmer L 120-000A) in transmission mode, in wave-number region 400–4000 cm^{-1} with resolution of 1.0 cm^{-1}. The spectrum was analyzed with respect to the standard IR series data.

2.2.6. Preparation of CPQN-coated blotting paper strip

Strips of normal blotting paper were dipped in synthesized CPQN suspension of pH 7.5 and dried. The process of dipping and drying was continued for six times so that six coats of the nano-composite were formed on each strip.

2.2.7. Confocal microscopy of CPQN-treated neural cells

Grown cells of mouse neuroblastoma cell line Neuro-2A were seeded at a density of $(1–1.5) \times 10^5$ cells/ml on glass cover-slips in six-well plate and left overnight for cell attachment. Attached cells were treated with the NP (5 μg/ml) for different times. Cells were then washed twice with PBS, fixed with 4% para-formaldehyde for 20 min and again washed twice with PBS. Cover-slips were finally put upside down on glass slides with a drop of mounting solution and the slides were examined by a confocal microscope (Olympus, Fluoview FV-1000) to observe the cells in bright field, blue panel fluorescence and their overlay modes.

2.2.8. Antioxidant activity of CPQN on neural cells

The antioxidant activity of the nano-composite was determined by measuring its neutralizing capacity of the H_2O_2-induced oxidative toxicity in mouse neuroblastoma cells. The toxicity at the level of cell viability was determined by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [33]. In principle, mitochondrial dehydrogenases of metabolically active cells cleaved the tetrazolium ring of MTT, yielding purple colored and insoluble formazan crystals, which were subsequently solubilized in DMSO; the absorbance of the resulting purple solution was measured at 570 nm. The extent of cytotoxicity was directly linked to the decrease in absorbance, as less tetrazolium cleavage occurred with rising cell death. For this study, cells were grown in 24-well plate in DMEM (Dulbecco's modified eagle's medium, Himedia-India) supplemented with 10% FBS (Fetal Bovine Serum, Himedia) and 100 units/ml of each of penicillin (Sigma) and streptomycin (Sigma) at 37 °C. Healthy cells of 1 ml, at a concentration