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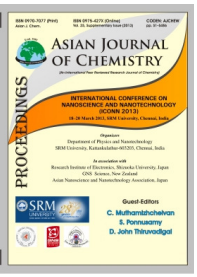

Synthesis and Characterization of Major Green Tea Catechin Nanoparticle†

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Epigallocatechin-3-gallate (EGCG), a principal polyphenol, which is most abundant and active component in green tea which has wide therapeutic effects against various diseases. However, EGCG used in clinical application has some downsides due to its low bioavailability and half-life. Hence increasing the bioavailability of EGCG without losing its potential therapeutic effects is of great challenge. In this study, we have prepared EGCG-loaded chitosan nanoparticles by ionic gelation method using sodium tripolyphosphate as ionic cross linking agent. The size, surface morphology, drug entrapment efficiency, drug releasing property and free radical scavenging activity of EGCG nanoparticle has been studied. The size of nanoparticle as determined by SEM ranged from 88-399 nm. FTIR results confirmed the drug entrapment in nanoparticles. The antioxidant property of nano-particulated EGCG and drug releasing assay showed sustained release of EGCG in the acid medium, which helps in increasing bioavailability of drug. Moreover the antioxidant activity of the drug was not found to be hindered by conversion into nanoparticles. However, further *in vivo* studies are warranted to explicate its therapeutic efficacy.

Key Words: Epigallocatechin-3-gallate, Nanoparticle, Chitosan, FTIR, SEM.

INTRODUCTION

Epigallocatechin-3-gallate (EGCG) is the main constituent of green tea extract[1] which is antioxidant, antiinflammatory and antiatherogenicproperties[2]. However, some studies showed that EGCG and other catechins were unstable under high temperature and neutral or alkaline conditions (pH > 6)[3,4]. Therefore, the stability of EGCG is dependent on pH. Absorption of EGCG takes place in the small intestine where the pH lies between 7-8, hence, substantial quantities of the unabsorbed and partially degraded EGCG pass from the small to the large intestine where it undergoes further degradation by the action of local microbiota[2]. Hence, if EGCG is provided in the alkaline resistant form, its biovailability might be increased.

The chitosan is a straight-chain copolymer composed of D-glucosamine and N-acetyl-D-glucosamine being obtained by the partial deacetylation of chitin. Chitosan is the most abundant basic biopolymer and is structurally similar to cellulose, which is a homopolysaccharide of glucose. Chitosan is considered one of the most valuable polymer for biomedical and pharmaceutical applications due to its biodegradability, biocompatibility, antimicrobial, non-toxicity, adsorption properties *etc*.[5]. Chitosan is insoluble in distilled water, but soluble in diluted acetic acid solution. Hence, chitosan was selected for the preparation of EGCG nanoparticles.

Recently, a new approach for the preparation of nanoparticles made solely of hydrophilic polymer has been described by Majeti and Kumar[5]. The preparation technique, based on an ionic gelation process, is extremely mild and involves a mixture of two aqueous phases at room temperature. One phase contains chitosan (CS) and the other contains sodium tripolyphosphate. Most of nanoparticles synthesis from water-insoluble polymers involves either heat, organic solvent or high shear force that can be harmful to the drug stability. Moreover, some preparation methods such as emulsion polymerization and solvent evaporation are complex and require a number of preparation steps that are more time and energy consuming. In contrast, water-soluble polymers offer mild and simple preparation methods without the use of organic solvent and high shear force. Therefore chitosan is a best choice for making drug loaded nanoparticle[6].

In order to fully maximize the therapeutic utility of EGCG, there is a need to enhance its oral absorption. The idea that nanoparticles might protect labile drugs from enzymatic degradation in the gastrointestinal tract (GIT) leads to the development of nanoparticles as oral delivery systems. Mecha-

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nism of chitosan nanoparticle transport across GIT is most probably through adsorptive endocytosis. Electrostatic interaction between positively charged chitosan and negatively charged sialic acid of mucin causes association of chitosan nanoparticle to the mucus layer and subsequently internalization *via* endocytosis[7]. With this notion, the current study was targeted to synthesize and characterize EGCG nanoparticles that are acid and alkali resistant, so that their bioavailability might be increased. Based on this idea, the approach in this study is designed to synthesize chitosan nanoparticle loaded with EGCG, characterize the nanoparticle property by SEM and FTIR and evaluate the antioxidant activity of EGCG, drug encapsulation efficacy and drug releasing property of EGCG nanoparticle.

EXPERIMENTAL

Epigallocatechin-3-gallate (EGCG) was purchased from Sigma Chemical Company (St. Louis, USA). Chitosan, Ferric chloride, 2,4,6-tripyridyl-s-triazine, Folin's phenol reagent, sodium carbonate, glacial acetic acid, were purchased from Sisco Research Laboratory Pvt., Ltd. India. Tripolyphosphate was purchased fromCentral Drug House Pvt., Ltd., potassium bromide IR grade formSisco Research Lab. Pvt. Ltd. India, Deionized water was obtained from a Millipore®filtration system.

Encasement of EGCG in chitosan nanoparticle: Epigallocatechin-3-gallate (EGCG) encased inside the chitosan nanoparticle were prepared as previously described by Dube *et al*.[8] with a slight modification. Briefly, nanoparticles were prepared by drop-wise addition of tripolyphosphate (TPP) solution (0.1 %, w/v; pH 3) to a chitosan solution (0.1 % w/v in 0.175 % v/v acetic acid; pH 3) containing EGCG (0.05 % w/v), under magnetic stirring for 2 h (800 rpm) at ambient temperature. Nanoparticles were freeze-dried at -80 ºC for 16 h.

Scanning electron microscopic analysis: The chitosan nanoparticle sample was coated with thin gold layer by a sputter coater unit (Cressington 108 Auto sputter coater). The surface topography was analyzed with a S-3400 N Fully Automated VP-scanning electron microscope (SEM; Hitachi, America) analysis, operated at an acceleration volt of 10,000 V, with raster scanning.

Fourier transform infrared spectroscopy: The IR studies have been followed by the method described by Jagmohan[9]. The lyophilized samples were mixed with dry potassium bromide (KBr pellet) and subjected to a pressure of about 5×10^6 Pa in an evacuated die to produce a clear transparent disc of diameter 2 cm and thickness 0.2 cm. IR spectra in frequency region 4000-400 cm^{-1}, ere recorded at room temperature on a Perkin-Elmer Fourier transform spectrometer.

Analysis of amount of EGCG in the nanoformulation: The Folin-Ciocalteu method[10] was used to determine the EGCG concentration in nanoparticle preparation in different condition.Folin-Ciocalteau reagent was diluted by 2 times using deionized water. In brief, to 100 µL of test sample was made up to 1 mL with distilled water. To this, 0.5 mL of folinciocalteu reagent was added. After 5 min, 2 mL of 20 % sodium carbonate was added and again incubated for 15 min to stop reaction and read at 765 nm. A calibration curve was constructed using EGCG (20-100 µg).

The drug loading efficiency was studied by taking same volume of EGCG and nanoparticle EGCG 100 µL solution. The EGCG solution prepared in a way that it has EGCG concentration of 500 µg/mL, the same concentration that was used in the preparation of nanoparticle EGCG.

Encapsulation efficiency

$$= \frac{\text{Total amount EGCG} - \text{Free amount EGCG}}{\text{Total amount EGCG}}$$

***In vitro* release study:** The particular amount of EGCG-loaded chitosan nanoparticles was suspended in separate tubes containing equal volumes of appropriate buffers (pH 3.5, 7, 8) and incubated by shaking at 37 ºC. At appropriate time intervals (0, 1, 2, 3 and 4 h) one tube was removed and the amount of EGCG released in the buffer was measured by Folin Ciocalteu method.

FRAP assay: The FRAP reagent was prepared by adding 300 mM acetated buffer, 10 mM TPTZ and 20 mM $FeCl_3$ in10:1:1 respective ratio. 900 µL of freshly prepared FRAP reagent was mixed with 100 µL of test sample. The reaction mixture was then incubated at 37 ºC for 10 min and absorbance was recorded at 593 nm, using a spectrometer (UV-VIS 1700 Shimadzu, Japan). The concentration of $FeSO_4$ was in turn plotted against concentrations of the standard antioxidant EGCG[12].

RESULTS AND DISCUSSION

Surface characterization of EGCG nanoparticle: The size and morphological characteristics of the prepared EGCG nanoparticle formulations were studied by scanning electron microscopy. SEM photographs showed semi-spherical nanoparticles with a size distribution ranging from 80-394 nm (Fig. 1). Therefore, it confirms that linear polymer of chitosan was converted to nanoparticle by drop wise addition of tripolyphosphate (TPP). The absorption enhancement property was mainly attributed to the nanoparticles of size below 500 nm[11].

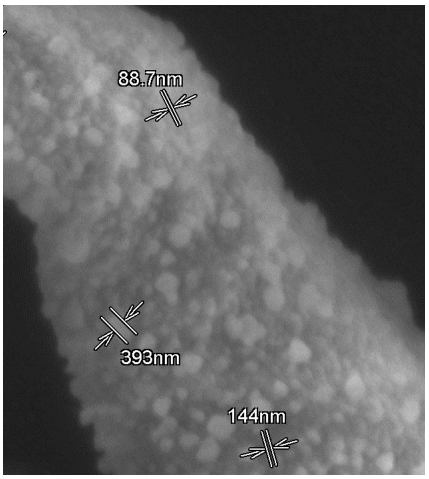

Fig. 1. Scanning electron micrograph of chitosan EGCG nanoparticle

FTIR analysis: The FTIR spectra of chitosan tripolyphosphate nanoparticles and EGCG loaded chitosan nanoparticles are shown in Fig. 2. In chitosan-tripolyphosphate nanoparticles

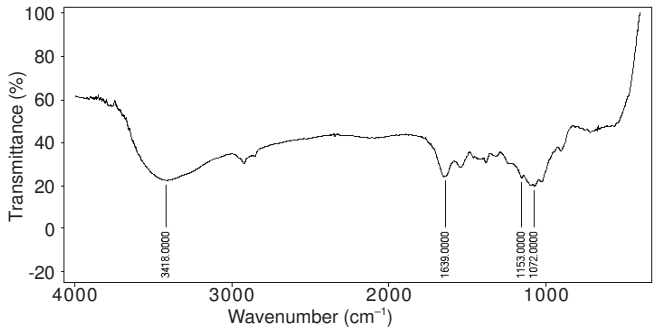

Fig. 2. FTIR of control nanoparticle

the tip of the peak 3418 cm^{-1} shows the presence of poly-hydroxyl group of chitosan, a peak at 1639 cm^{-1} indicates the presence of amino group and the 1153 cm^{-1} wavelength peak indicates the presence of phosphate group. Whereas in the FITR spectra of EGCG loaded nanoparticle (Fig. 3), the peak at 3418 cm^{-1} (which represents the poly hydroxyl group in control nanoparticle) have moved to 3429 cm^{-1} in drug loaded nanoparticle which indicates the hydrogen bonding between EGCG and hydroxyl group of chitosan. In contrast to control nanoparticle a unique peak for EGCG aromatic ring was found in drug loaded nanoparticle IR graph (1458 cm^{-1}). So we conclude that the hydroxyl groups of EGCG are hydrogen bonded with hydroxyl groups of chitosan and chitosan has entrapped EGCG along with it during nanoparticle preparation.

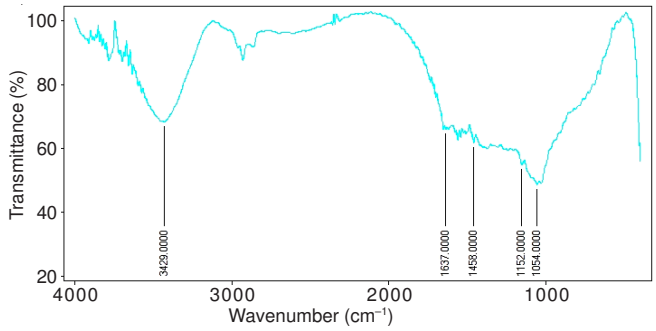

Fig. 3. FTIR spectra of drug encapsulated nanoparticle

Encapsulation efficiency of nanoparticle: In most nanoparticle delivery systems, the drug carrying capacity is defined as encapsulation efficiency. In the present study, EGCG was carried on the nanoparticles *via* the hydrogen bond interaction with chitosan. The hydrophilic hydroxyl group of EGCG is shown to have interactions with the hydroxyl hydrophilic chains of chitosan (as revealed by the hydrogen bonding in the FITR spectra), indicated that the EGCG was not only on the surface of the nanoparticles but also was distributed in the inner hydrophilic area. In this study, EGCG encapsulation efficiency was determined by the Folin Ciocalteau method and was computed to be 56.5-60 % with the average encapsulation efficiency of 58.38 % (n = 6).

***In vitro* drug release study:** The preliminary release test of EGCG from EGCG loaded chitosan nanoparticles *in vitro* proves that they have a sustained release and it dies not differ significantly with pH of the medium form as shown in Fig. 4 shows the stability of EGCG nanoparticles in both acidic and alkaline pH. The *in vitro* drug release profiles generally show

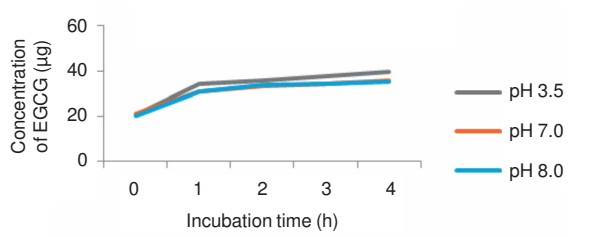

Fig. 4. Nanoparticulated EGCG release (WSC 1 mg/mL, BSA 0.5 mg/mL, TPP 1 mg/mL)

three phases[12,13], firstan initial burst release of drug is due to the drug desorbed from the particles surface, second a plateau for the following 3 h, resulting from the onlydiffusion of the drug dispersed in the polymer matrix, third a constant sustained release of the drug, resulting from the diffusion of the drug through the polymer wall as well as its erosion.

In the current scenario, the release of EGCG showed a prominent burst phase followed by a slow controlled release phase, which shows it's potentially to enhance bio-availability. Fig. 4 shows that the drug release is slightly higher in acidic medium than at neutral or alkaline medium.

Antioxidant capacity of EGCG: The study was designed for evaluating the antioxidant activity of fresh preparations of EGCG and 1 month old samples of EGCG nanoparticles as shown in Fig. 5. The results indicate that the loss of antioxidant property of EGCG was minimal in nanoparticle preparation than in EGCG solution alone. This may be due the encapsulation activity of chitosan nanoparticle.

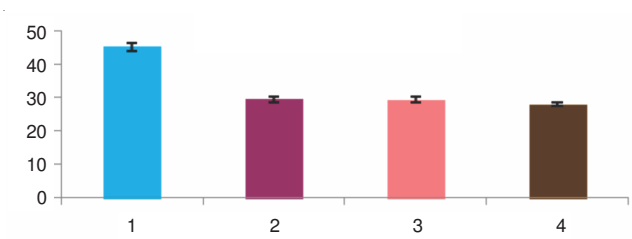

Fig. 5. Antioxidant activity of EGCG and nanoparticulated EGCG with and without time duration

Conclusion

This preliminary study shows that EGCG nanoparticles can be prepared by ionic gelation method with chitosan as the polymer. The size of the nanoparticles was found to be 88- 393 nm by SEM studies. Encapsulation was confirmed by FITR studies and the encapsulation efficiency was found to be optimum. The release kinetics and FRAP assay shows stability of the EGCG nanoparticle. Hence, these nanoparticles assumed to increase the bio-availability of EGCG. However further *in vivo* studies warranted to prove the pharmacokinetics and pharmacodynamics of EGCG nanoparticle.

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