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Utilization of crab shell-derived chitosan in nanoparticle synthesis for curcumin delivery

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Chitosan derived from crustaceans is biodegradable as well as biocompatible and can be made into nanoparticles when chelated with chelators, such as sodium tripolyphosphate and barium chloride. In this study, crab shells-derived chitosan was chelated using sodium trimetaphosphate to form nanoparticles. Curcumin was encapsulated into nanoparticles and characterized using Fourier transform infra-red spectroscopy, scanning electron microscopy, atomic force microscopy, and X-ray diffraction analysis. The particles were found to be 18 nm in size, while the curcumin-loaded particles were 25 nm in size. The particles were observed to encapsulate 90% of the drug used. The nanoparticles produced were analyzed for *in vitro* controlled drug release against *Pseudomonas aeruginosa, Bacillus subtilis,* and *Candida albicans.*

[**Keywords**: Sodium trimetaphosphate (STMP); Chitosan; Curcumin; Drug delivery]

Introduction

In the field of nanobiotechnology, there are enormous expectations from biomaterials-based nanoparticles as they are biodegradable, biocompatible, non/low immunogenic, and nontoxic. Thus, they are well exploited in drug and gene delivery[1,3] tissue engineering[4] food industries[5] etc. Chitosan, an abundant natural polysaccharide found mainly in the exoskeletons of marine organisms and certain fungi and algae[6,9] is biocompatible and biodegradable[10]. Chitosan and its derivatives have been reported as potential carriers for drug delivery systems[11,12].

Chitosan nanoparticles are produced by ionic gelation method[13] using sodium tripolyphosphate (TPP)[14] and barium chloride[15]. These cross-linking agents combine two components with opposite charges to form nanoparticles[16]. Size formation can be controlled by ionic gelation method as well as by encapsulation of protein, ions, and drugs[17,18]. Using an alternate and new cross-linking agent may lead to formation of smaller-sized nanoparticles which may be better than the commonly used cross-linkers such as sodium TPP and barium chloride. Curcumin is a hydrophobic drug with multiple bioactivities, thus it was chosen as a drug of choice to load into the nanocarriers in most studies[12,15]. In this study, chitosan was derived from crab shell and an attempt was made to use sodium trimetaphosphate (STMP) as chelator to produce curcumin-loaded chitosan nanoparticle for drug delivery.

Materials and Methods

Materials

Curcumin was purchased from Sisco Research Laboratories Pvt. Ltd; Acetic acid from Qualigens Fine Chemicals, India; STMP, Nutrient agar, and carboxy methyl cellulose (CMC) from LOBA Chemie, HiMedia and Micro Fine Chemicals, India, respectively. Millipore water was used in this study.

Preparation and characterization of crab shell-derived chitosan

Chitosan from crab shells was prepared following Samrot *et al.*[12] and Yen *et al.*[19], mixed with KBr pellets, and subjected to Fourier transform infra-red spectroscopy (FTIR) analysis (Shimadzu, Japan).

Synthesis of STMP-chelated chitosan nanoparticles

Chitosan 0.8% was prepared in 50 ml 0.1N acetic acid. The solution was filtered to remove the unsuspended particles. 0.2% STMP in 25 ml of distilled water was added dropwise to the chitosan

solution. 25 ml of 0.4% CMC in 25 ml distilled water was added dropwise to the above solution with constant manual stirring, kept undisturbed for an hour, and then centrifuged at 5000 rpm for 15 min. The pellets were collected and lyophilized to produce curcumin-encapsulated nanoparticles; 0.1% of curcumin in 25 ml ethanol was added to 0.8% chitosan solution and chelation was done as described earlier.

Characterization of chitosan nanoparticles

Chitosan nanoparticles (before and after loading curcumin) were subjected to FTIR analysis (Shimadzu, Japan). The morphology, topography, and size of chitosan nanoparticles were examined under a scanning electron microscope (SEM) (Zeiss Ultra Plus, Germany) and an atomic force microscope (AFM) (Bruker, Germany). X-ray diffraction (XRD) patterns of the chitosan nanoparticles were recorded using a smart lab X-ray diffractometer (Rigaku, Japan).

Percentage encapsulation efficiency

After the synthesis process was completed, a 5 ml solution was centrifuged at 3500 g for 10 min and 1 ml of the supernatant was withdrawn every 10 min over 2 h. Absorbance was taken at 425 nm[15] and the concentration of curcumin was determined in the supernatant. The drug encapsulation efficiency of curcumin-loaded chitosan nanoparticles was calculated as a percentage using the following formula[20]:

$$\text{Percentage drug loading efficiency} = \frac{\text{total drug added - free non-trapped drug}}{\text{Total drug added}}$$

In vitro controlled drug release

The releasing ability of curcumin from chitosan nanoparticles in the presence of different solvent systems such as water, ethanol (25%), PBS (pH 6), acetic acid (0.1N), and crude chitosanase enzyme was evaluated using agar well diffusion method[21] against gram positive *Bacillus subtilis*, gram negative *Pseudomonas aeruginosa,* and the fungus, *Candida albicans*.

Swarming motility

1.3 g of nutrient agar with 1.5% of agar was prepared, autoclaved, poured into a sterile petri plate and allowed to solidify. 1 mg/ml of chitosan nanoparticles (curcumin loaded or unloaded) in distilled water (1 mg/ml) was mixed with 1 ml of steam-sterilized 10% (w/v) D-glucose. The above solution was added to 5 ml of nutrient agar with 0.5% agar. Gram positive *B. subtilis* and Gram negative *P. aeruginosa* were point inoculated at the centre. The ability of the nanoparticles to inhibit the bacteria from swarming was determined by comparing with control[22,23].

Results and Discussion

Preparation and characterization of crab shell-derived chitosan

The broad O-H stretch at 3550–3200 cm^{-1} was seen in the crab shell-derived chitosan. The methyl group in $NHCOCH_3$ and the methylene group in CH_2OH were confirmed by the corresponding stretching vibrations present in the range 2921-2879 cm^{-1}. A peak at 1451 cm^{-1} proved the presence of δ (CH_2) of CH_2OH group (Fig. 1a). Similar results were reported by Samrot *et al*[12].

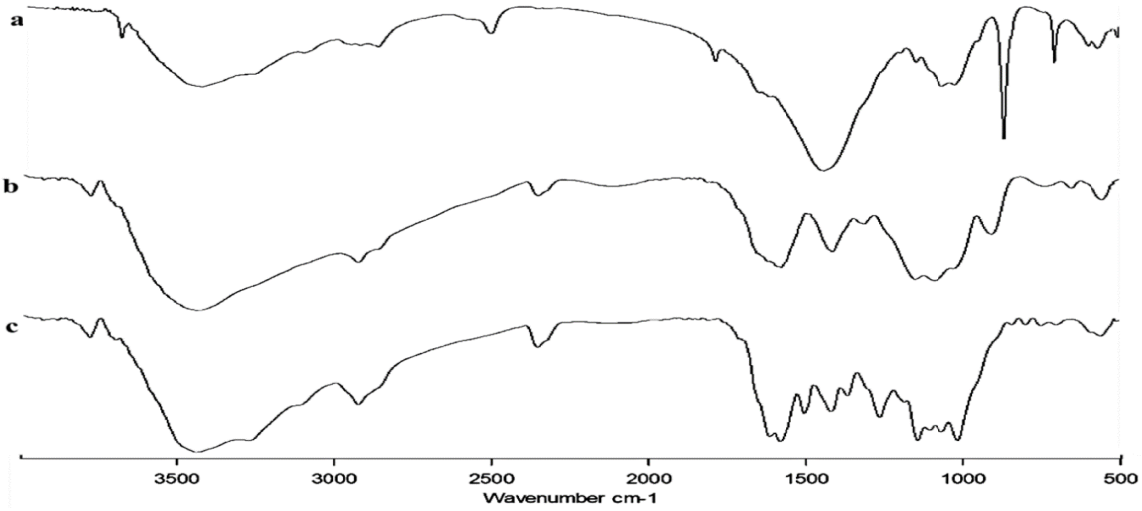

Fig. 1 — FTIR spectroscopy analysis of chitosan nanoparticles loaded with curcumin and chelated with STMP: (a) Chitosan, (b) Unloaded chitosan nanoparticles chelated with STMP, and (c) Loaded chitosan nanoparticles chelated with STMP

Characterization of chitosan nanoparticles

The characteristic absorption bands of the amides, N–H bending, and C–N stretching were confirmed by peaks at around 1700–1600 cm^{-1}, 1500–1550 cm^{-1}, and 2800–2950 cm^{-1}, respectively (Fig. 1a–c). As seen from Figures 1b and c, the peaks corresponding to amide band and N–H bending highly shifted to 1583, 1591 cm^{-1} (N–H stretching vibration of NH^{3+} group) and 1420, 1431 cm^{-1}, respectively, which may be due to strong ionic cross-linking of chitosan and STMP. Moreover, peaks at 1623 cm^{-1} (C=C symmetric aromatic ring stretching), 1591 cm^{-1} (C=O), and 1274 cm^{-1} (enol C-O) showed the encapsulation of curcumin in the chitosan nanoparticles.

The unloaded nanoparticles were found to be around 18 nm (Fig. 2a), whereas curcumin encapsulation increased the size to 25 nm (Fig. 2b). Spherical chitosan–carboxymethyl cellulose nanoparticles chelated with TPP and $BaCl_2$ were synthesized by Samrot *et al.*[12], where the size of the nanoparticles was found to be below 500 nm. Rejinold *et al.*[24] produced unloaded chitosan nanoparticles of size above 150 nm and loaded nanoparticles of size 180–200 nm. As STMP influenced the chitosan to form smaller-sized nanoparticles than the conventional chelators did, it is believed that STMP had cross-linked the chitosan and CMC effectively in this study.

Energy dispersive X-ray spectroscopy (EDX) showed the presence of carbon, oxygen, sodium, and phosphate in both drug-loaded and unloaded nanoparticles (Figs 2a and b), which indicated the involvement of STMP in nanoparticle formation.

The morphology and size of chitosan nanoparticles synthesized from crab shells were analyzed through AFM and are shown in Figures 3a and b. Both curcumin-loaded and unloaded chitosan nanoparticles exhibited spherical shape and their diameters were 25 nm (Fig. 3b) and 18 nm (Fig. 3a), respectively, which is on par with the SEM results. Samrot *et al.*[12] earlier reported the crab shell-derived chitosan nanoparticles chelated with barium chloride to be below 200 nm and were smooth and spherical in shape, whereas TPP-chelated nanoparticles were found to be below 100 nm and drug loading increased the size up to 250 nm, and the shape was smooth and spherical. Thus, STMP was found to produce smaller particles.

The XRD pattern of curcumin-loaded chitosan nanoparticles synthesized showed broad diffraction peaks at 2θ values ranging between 20° and 40° (Fig. 4b), which were typical fingerprints of chitosan

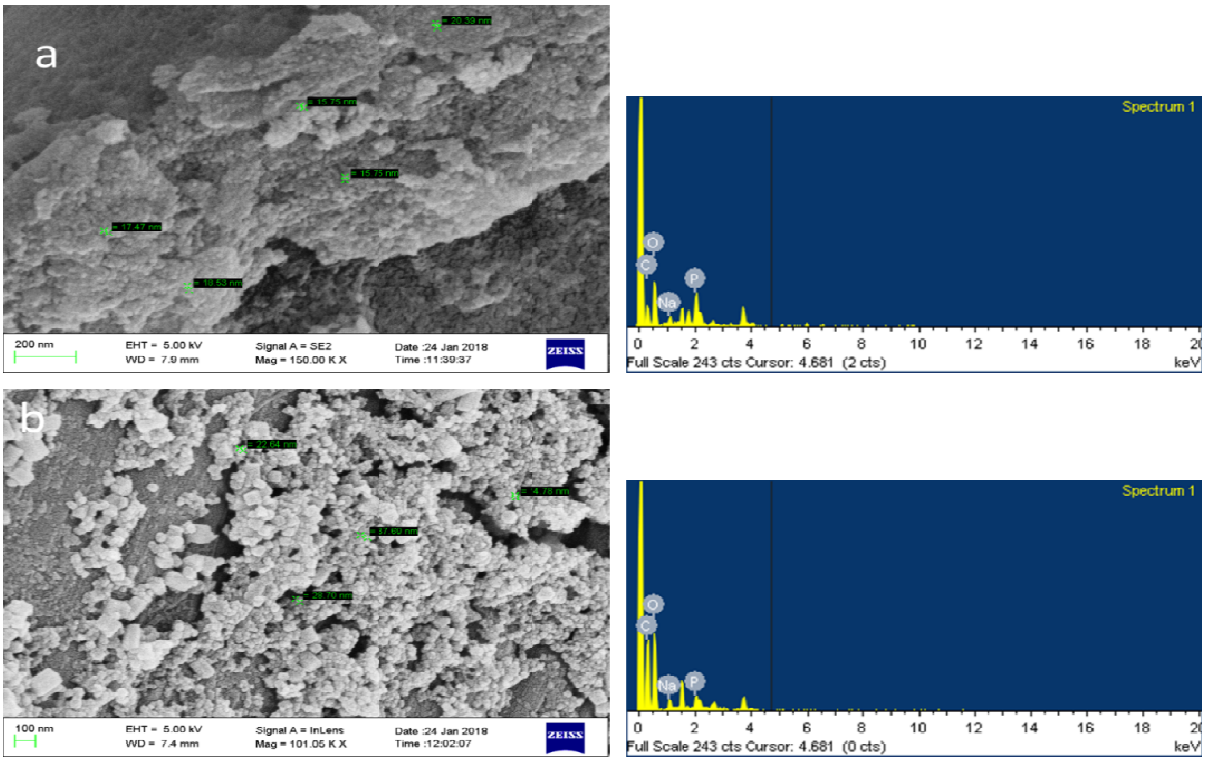

Fig. 2 — SEM and EDX analysis: (a) Unloaded chitosan nanoparticles chelated with STMP and (b) Loaded chitosan nanoparticles chelated with STMP

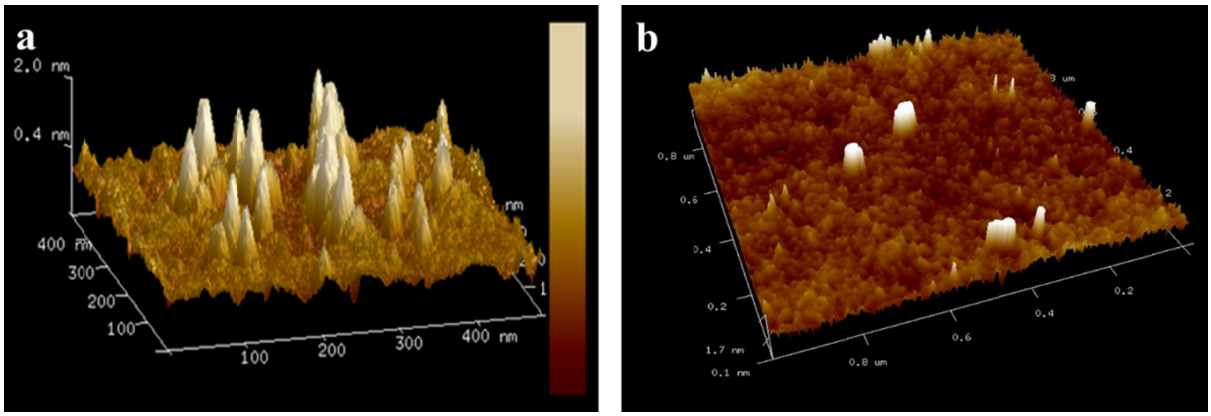

Fig. 3 — AFM analysis of chitosan nanoparticles chelated with STMP: (a) Unloaded chitosan nanoparticles chelated with STMP and (b) Loaded chitosan nanoparticles chelated with STMP

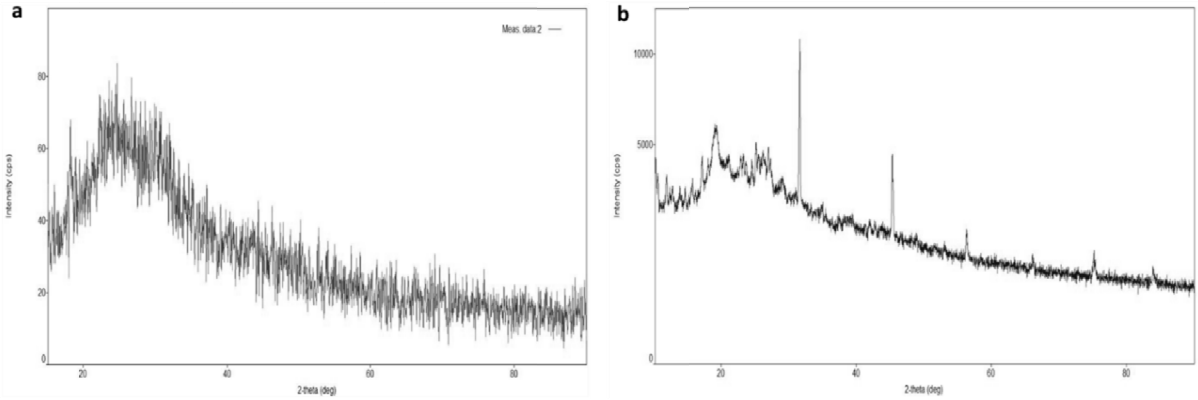

Fig. 4 — XRD of chitosan nanoparticles chelated with STMP: (a) Unloaded chitosan nanoparticles chelated with STMP and (b) Loaded chitosan nanoparticles chelated with STMP

and curcumin[25]. The lower intensity exhibited by the diffraction peaks of unloaded chitosan nanoparticles revealed that they were amorphic in nature (Fig. 4a).

Percentage encapsulation efficiency

The encapsulation efficiency of hydrophobic curcumin into STMP-chelated chitosan nanoparticles was found to be around 90% (Fig. 5). The percentage drug encapsulation efficiency was found to be increasing with time. Dounighi *et al.*[26] found TPP-chelated nanoparticles to encapsulate up to 90% of scorpion venom into the nanoparticles.

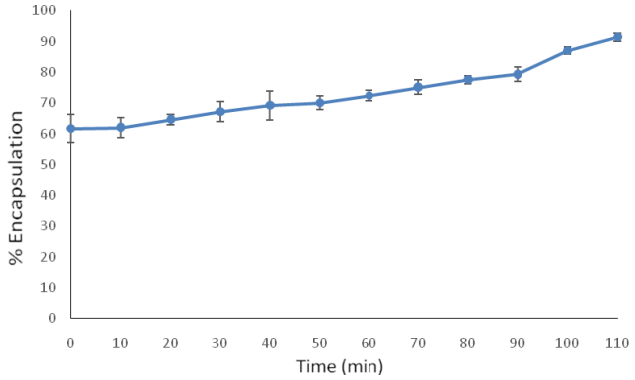

Fig. 5 — Percentage drug encapsulation efficiency of chitosan nanoparticles chelated with STMP

In vitro controlled drug release

The releasing ability of curcumin-encapsulated chitosan nanoparticles was studied by agar well diffusion assay using five different solvent systems, that is, water, ethanol, PBS (pH 6.8), acetic acid (0.1N), and crude chitosanase enzyme. No antibacterial activity against *B. subtilis, P. aeruginosa,* and *C. albicans* was observed when water and PBS were used as solvents. This might be due to the inability of water and PBS to dissolve the outer layer of chitosan to release curcumin[12,27]. The zone of inhibition at the highest concentration of curcumin-loaded chitosan nanoparticles was observed when ethanol, acetic acid, and crude chitosanase enzyme were used as solvents (Tables 1–3). Earlier reports also support the finding that the acidic environment favors the drug release out of chitosan nanoparticles[12,15].

Table 1 — Zone of inhibition of chitosan nanoparticles incorporated with curcumin against *B. subtilis* using various solvents

Solvents	Zone of inhibition (cm)					
	Positive control	Solvent	Unloaded	5 µg/ml	10 µg/ml	15 µg/ml
Water	1.5	Nil	Nil	Nil	Nil	Nil
PBS	1.5	Nil	Nil	Nil	Nil	Nil
Ethanol	1.6	Nil	Nil	Nil	Nil	0.3
Acetic acid	1.5	Nil	Nil	0.5	0.6	1.0
Chitosanase enzyme	1.5	Nil	Nil	0.3	0.7	1.5

Table 2 — Zone of inhibition of chitosan nanoparticles incorporated with curcumin against *P. aeruginosa* using various solvents

Solvents	Zone of inhibition (cm)					
	Positive control	Solvent	Unloaded	5 µg/ml	10 µg/ml	15 µg/ml
Water	1.3	Nil	Nil	Nil	Nil	Nil
PBS	1.3	Nil	Nil	Nil	Nil	Nil
Ethanol	1.3	Nil	Nil	Nil	Nil	1.3
Acetic acid	1.3	Nil	0.5	0.7	0.8	0.9
Chitosanase enzyme	1.3	Nil	Nil	Nil	Nil	1.2

Table 3 — Zone of inhibition of chitosan nanoparticles incorporated with curcumin against *C. albicans* using various solvents

Solvents	Zone of inhibition (cm)					
	Positive control	Solvent	Unloaded	5 µg/ml	10 µg/ml	15 µg/ml
Water	1.3	Nil	Nil	Nil	Nil	Nil
PBS	1.3	Nil	Nil	Nil	Nil	Nil
Ethanol	1.3	Nil	Nil	Nil	Nil	1.0
Acetic acid	1.3	Nil	Nil	Nil	Nil	1.4
Chitosanase enzyme	1.3	Nil	Nil	Nil	1.2	1.6

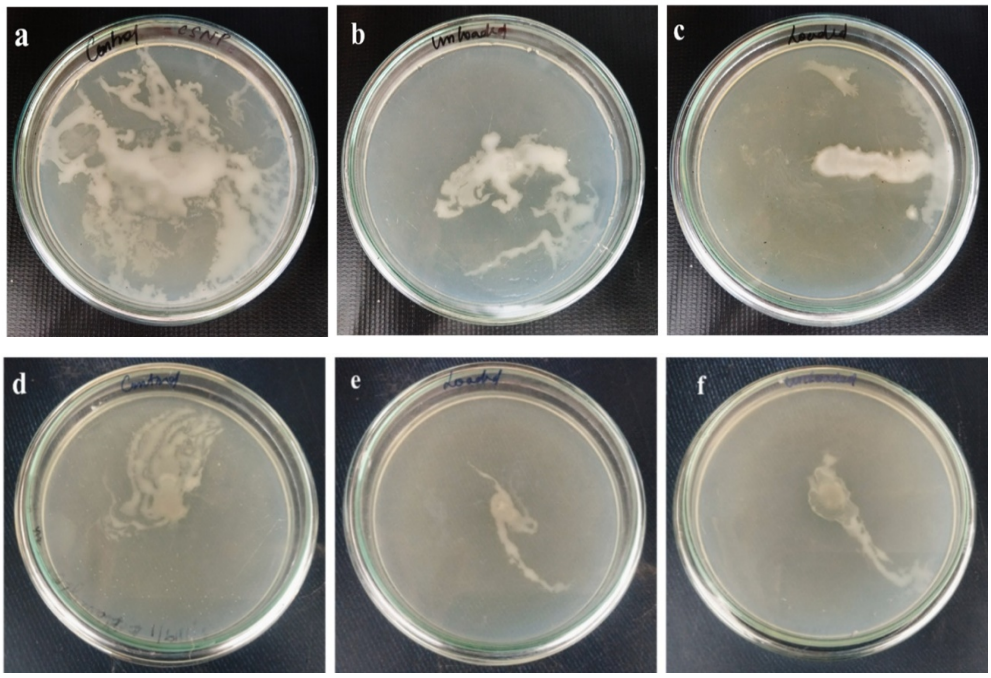

Fig. 6 — Swarming motility by different bacteria against chitosan nanoparticles chelated with STMP: (a) *B. subtilis* control, (b) Unloaded chitosan nanoparticles with *B. subtilis*, (c) Loaded chitosan nanoparticles with *B. subtilis*, (d) *P. aeruginosa* control, (e) Unloaded chitosan nanoparticles with *P. aeruginosa,* and (f) Loaded chitosan nanoparticles with *P. aeruginosa*

Swarming motility of bacteria

The swarming motility results showed that the curcumin-loaded and unloaded chitosan nanoparticles inhibit the motility of Gram positive *B. subtilis* (Figs 4b and c) and Gram negative bacterium *P. aeruginosa* (Figs 4e and f) to a certain extent. Thus, these nanoparticles can be used for biofilm inhibition.

Conclusion

In this study, chitosan was extracted from crab shell. The extracted chitosan was chelated with STMP and characterized as 18-25 nm sized spherical nanoparticles. The particles were found to encapsulate curcumin better, that is, with 90% encapsulation. Nanoparticles were found to inhibit the swarming motility to a certain extent. The curcumin-loaded nanoparticles were found to release curcumin in an acidic environment.

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