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Curcumin Nanoparticles: Preparation, Characterization, and Antimicrobial Study

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ABSTRACT: Curcumin is a highly potent, nontoxic, bioactive agent found in turmeric and has been known for centuries as a household remedy to many ailments. The only disadvantage that it suffers is of low aqueous solubility and poor bioavailability. The aim of the present study was to develop a method for the preparation of nanoparticles of curcumin with a view to improve its aqueous-phase solubility and examine the effect on its antimicrobial properties. Nanoparticles of curcumin (nanocurcumin) were prepared by a process based on a wet-milling technique and were found to have a narrow particle size distribution in the range of 2−40 nm. Unlike curcumin, nanocurcumin was found to be freely dispersible in water in the absence of any surfactants. The chemical structure of nanocurcumin was the same as that of curcumin, and there was no modification during nanoparticle preparation. A minimum inhibitory concentration of nanocurcumin was determined for a variety of bacterial and fungal strains and was compared to that of curcumin. It was found that the aqueous dispersion of nanocurcumin was much more effective than curcumin against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Penicillium notatum*, and *Aspergillus niger*. The results demonstrated that the water solubility and antimicrobial activity of curcumin markedly improved by particle size reduction up to the nano range. For the selected microorganisms, the activity of nanocurcumin was more pronounced against Gram-positive bacteria than Gram-negative bacteria. Furthermore, its antibacterial activity was much better than antifungal activity. The mechanism of antibacterial action of curcumin nanoparticles was investigated by transmission electron micrograph (TEM) analysis, which revealed that these particles entered inside the bacterial cell by completely breaking the cell wall, leading to cell death.

KEYWORDS: Curcumin, nanoparticle, nanocurcumin, antimicrobial activity

INTRODUCTION

Curcumin [(*E*,*E*)-1,7-bis(4-hydroxy-3-methoxy-phenyl)-1,6-heptadiene-3,5-ione] (Figure 1) is the main phenolic pigment extracted from turmeric, the powdered rhizome of *Curcuma longa*, along with demethoxy curcumin and bisdemethoxy curcumin.[1] It is commonly used as a spice, food preservative, and flavoring and coloring agent. Extensive research over the last 5 decades indicates that curcumin possesses potent antioxidant,[2,3] anti-inflammatory,[4,5] antitumor,[6] anti-HIV,[7] and antimicrobial properties.[8,9] It also inhibits lipid peroxidation and scavenges superoxide anion, singlet oxygen, nitric oxide, and hydroxyl radicals.[10,11] Despite having multiple medicinal benefits and extremely superior safety profile, the administration of curcumin to patients has a serious practical problem. Studies by Wahlstrom et al.[12] showed that, when rats were administered curcumin at a dose of 1 g/kg, about 75% of curcumin was excreted in the feces, while negligible amounts of curcumin appeared in the urine. Measurements of blood plasma levels and biliary excretion showed that curcumin was poorly absorbed from the gut and the quantity of curcumin that reached tissues outside the gut was pharmacologically insignificant. The studies indicated the insolubility of curcumin in water at physiological pH, limited absorption, poor bioavailability, rapid metabolism, and excretion.[13] Thus, for curcumin to exhibit its therapeutic effects in the human body, a person is required to swallow between 12 and 20 g of curcumin every day; otherwise, it is unlikely that substantial concentrations of curcumin occur in the body after ingestion. As a result, despite the inherent advantages of curcumin, it has never really made that journey from the kitchen shelf to the phamacist's counter. To overcome the problems of poor solubility and low bioavailability, nanoparticle-based drug delivery approaches,[14] in which curcumin is encapsulated in liposomes,[15] solid lipid microparticles, such as bovine serum albumin,[16] and chitosan,[17] or complexed with phospholipids[18] and cyclodextrin,[19] have been reported. Recently, synthesis of curcumin-encapsulated polymeric nanoparticles of *N*-isopropylacrylamide with *N*-vinyl-2-pyrrolidone and poly(ethyleneglycol)monoacryalte has been reported.[20,21] Although the colloidal nanocarriers hold considerable promise and are claimed to be biocompatible, the safety and toxicity issues cannot be ignored.[22,23] These materials have the capacity to penetrate cells and potentially translocate to other cells, tissues, and organs remote from the portal of entry to the body. This is considered to be a necessary step in the movement of particles deposited in the lung, entering the blood, acting upon cells in other tissues, and manifesting ultimately in a physiological response. Our cells may not detect these nanoparticles; therefore, they may linger on in our bodies, become activated later, and eventually, result in ailments.

Other techniques that are generally used to manufacture nano-sized particles are solvent-based processes, which include

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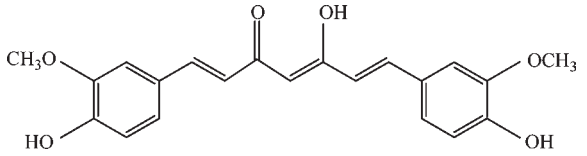

Figure 1. Chemical structure of curcumin.

emulsification−solvent evaporation, emulsification−solvent diffusion, and precipitation methods. The problem with these methods is that they require the addition of considerable amounts of surfactants to prevent coalescence during particle formation. Another approach for increasing the rate of dissolution of curcumin is by increasing its surface area. This can be achieved by decreasing the particle size by methods such as milling and grinding. In this study, we have used a wet-milling technique to reduce the particle size of curcumin to 2−40 nm. We found that nanocurcumin prepared by this method had good chemical and physical stability, could be stored in the powder form at room temperature, and was freely dispersible in water. Antibacterial assay and minimum inhibitory concentration (MIC) studies revealed that the therapeutic efficiency of curcumin significantly enhanced upon nanoparticle formation. It was quite an unusual finding that an aqueous dispersion of nanocurcumin had more effective antimicrobial activity than the solution of normal curcumin in dimethyl sulfoxide (DMSO). Our work shows for the first time how curcumin can achieve higher aqueous solubility, which could certainly help in extending its use in water-based food and pharmacological formulations.

MATERIALS AND METHODS

Apparatus and Materials. Dichloromethane used for the preparation of nanoparticles was of analytical grade. Curcumin was purchased from the Sigma Chemical Company, St. Louis, MO. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Advance 400 MHz spectrometer using $CDCl_3$ as the solvent for sample preparation. The ultrasound device used during preparation was an ultrasonic cleaner TPC-25 from Roop Telesonic. Buchi rotavapor (R-210) was used for removing the solvent. Thin-layer chromatography (TLC) analysis was performed on silica gel 60F254 (Merck, Germany) coated on an alumina sheet, and 1% methanol in chloroform was used as the developing solvent. All of the chemicals and Petri plates used for microbial studies were procured from HiMedia, Ltd., Mumbai, India.

Preparation and Characterization of Curcumin Nanoparticles. Curcumin (100 mg, 0.27 mmol) was taken in dichloromethane (20 mL), and 1 mL of this solution was sprayed into boiling water (50 mL) dropwise with a flow rate of 0.2 mL/min in 5 min under ultrasonic conditions, with an ultrasonic power of 100 W and a frequency of 30 kHz. After sonication for 10 min, the contents were stirred at 200−800 rpm at room temperature for about 20 min when a clear orange-colored solution was obtained. The solution was concentrated under reduced pressure at 50 °C and then freeze-dried to obtain an orange powder. A co-TLC of the powdered sample with standard curcumin showed both to have the same Rf values. Further, ^{1}H NMR and ultraviolet (UV) spectra of the lyophilized powder confirmed it to be curcumin. The choice of the solvent was crucial because spraying of curcumin solution prepared in other organic solvents, such as methanol or acetone, resulted in particle aggregation and nanoparticles could not be isolated. Further, maintaining the dropflow was significant for both the formation of nanoparticles and maintaining a uniformity in their size. It was seen that the addition of the entire curcumin solution to water in one lot led to particle aggregation.

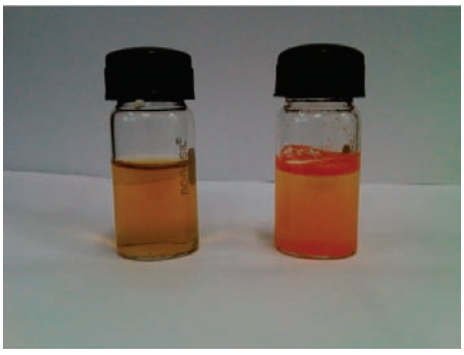

Figure 2. Solubility of nanocurcumin (left) and curcumin (right) in water.

Particle Size Analysis. The mean particle diameter of curcumin nanoparticles was measured by dynamic light scattering (DLS) performed on Malvern Zetasizer S90 series. The sample was prepared by taking 1 mg of the lyophilized nanocurcumin powder in 10 mL of distilled water. Transmission electron micrograph (TEM) analysis was performed on a Morgagni 268 D from FEI. The sample was prepared by placing a drop of the aqueous dispersion of curcumin nanoparticles on the copper grid and allowing it to air dry. A scanning electron micrograph (SEM) of the aqueous dispersion was recorded on a Jeol JSM 840 microscope by spreading the nanoparticle dispersion over a carbon tape and drying it under nitrogen stream. The sample was then coated in a sputter coater (EMITECH K 550 x) with a gold layer in a vacuum condition.

Preparation of Microorganism Suspension. The antimicrobial activity of curcumin was tested against *Staphylococcus aureus* (G+) MTCC 96, *Bacillus subtilis* (G+) MTCC 430, *Escherichia coli* (G−) MTCC 443, *Pseudomonas aeruginosa* (G−) MTCC 741, *Penicillium notatum*, and *Aspergillus niger* obtained from the Institute of Microbial Technology, Chandigarh, India. Nutrient agar (NA) and potato dextrose agar (PDA) were used to culture the test bacteria and fungi, respectively. Each strain was transferred from stored slants at 4 °C to 10 mL of nutrient broth (NB) or potato dextrose broth (PDB) tube and cultivated overnight at 37 °C. The bacterial cultures were then diluted in sterile 0.8% saline solution and adjusted to a cell suspension of 10^8 colony forming unit (cfu)/mL using a UV spectrophotometer and digital colony counter. Similarly, for fungi, an inoculum of viable spores or mycelial fragments was prepared.

Determination of MIC. MIC of curcumin and nanocurcumin was tested by the agar dilution method. A stock solution of nanocurcumin was prepared by taking 2 mg of the compound in 1 mL of distilled water, and an orange-colored clear nanodispersion was obtained. The stock solution was serially diluted to give concentrations in the range of 50−1000 μg/mL. For curcumin, a similar aqueous stock solution could not be prepared because curcumin is completely insoluble in water. Therefore, the stock solution was prepared by dissolving 2 mg of curcumin in 1 mL of DMSO. To flasks containing 20 mL of melted agar, different concentrations (50−1000 μg/mL) of curcumin (DMSO) and nanocurcumin (water) solutions were added separately. An equivalent amount of DMSO was used in the control plates, and they were then left to solidify. A total of 100 μL of culture was inoculated under aseptic conditions, and the plates were incubated at 37 °C for 24 h in the case of bacteria and at 25 °C for 5 days in the case of fungi. The inhibitory effect was calculated using the following formula

$$\text{percent inhibition} = (1 - T/C) \times 100$$

where T is cfu/mL of the test sample and C is cfu/mL of the control. Each experiment was performed in duplicate and repeated 3 times. The MIC was reported as the lowest concentration of curcumin capable of completely inhibiting the growth of each bacterial and fungal strain being tested.

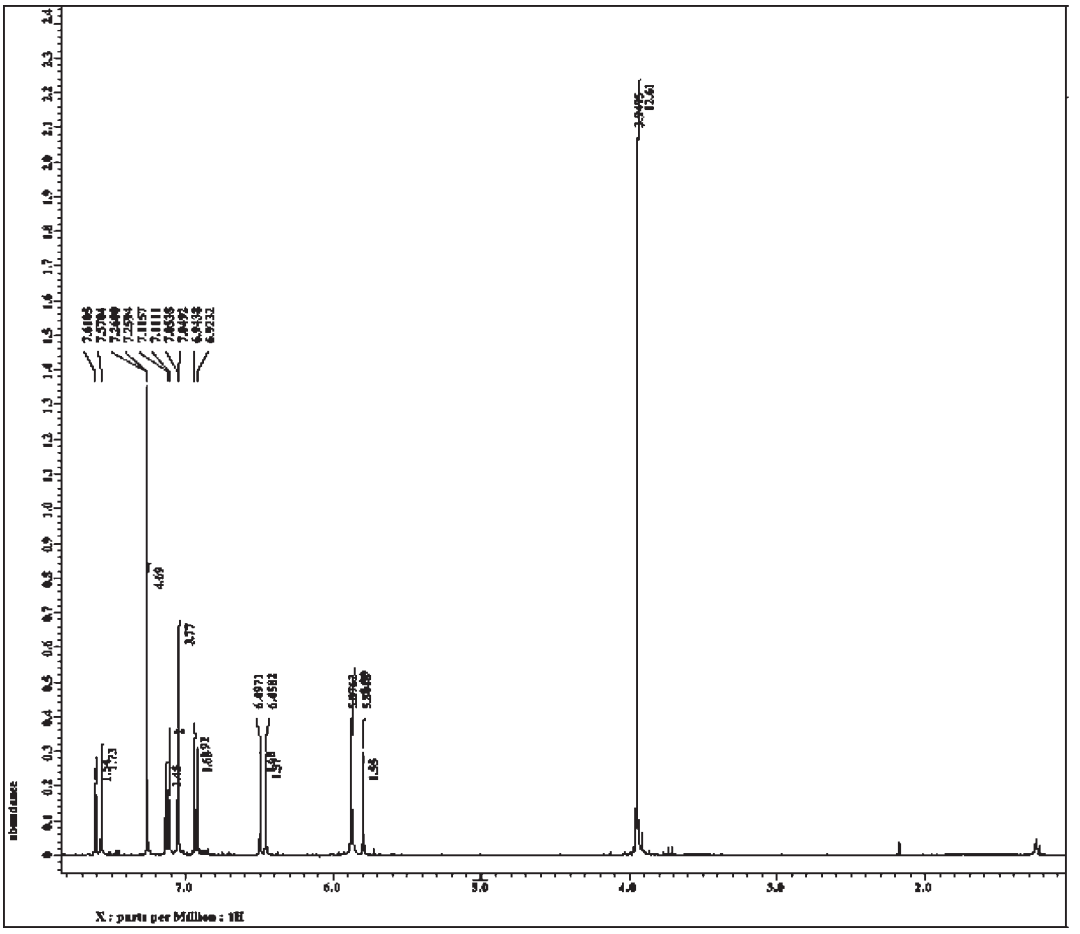

Figure 3. ^{1}H NMR spectra of curcumin nanoparticles in $CDCl_3$.

Determination of Zone of Inhibition. A well-diffusion method was used to assay the antibacterial and antifungal activity against test strains on Mueller-Henton agar and PDA plates, respectively. A total of 100 μL of diluted inoculum (10^8 cfu/mL) from organism suspensions was spread on the surface of the plates and allowed to solidify. Three wells were cut out with the help of a well borer under aseptic conditions on the agar medium. They were filled with 400 μg of nanocurcumin and curcumin solutions and DMSO as the control. The plates were incubated for 24 h at 37 °C for test bacteria and for 5 days at 25 °C for fungi. The antimicrobial activity was evaluated by measuring the diameter zone of transparent inhibition against test microorganisms.

Visualization of Morphology of Bacteria. *S. aureus* MTCC 96 was grown in the absence and presence of curcumin nanoparticles (100 μg/mL) for 4 h in nutrient broth. The cells were harvested by centrifugation, and the pellets obtained were fixed in 2.8% formaldehyde and 0.04% glutaraldehyde at 25 °C. The bacterial cells were finally collected, washed, and resuspended in phosphate-buffered saline (PBS) (pH 7.4). The cell morphology was viewed with a Morgagni 268 D electron microscope.

RESULTS AND DISCUSSION

Preparation and Characterization of Nanoparticles of Curcumin. The preparation based on a wet-milling technique[24] involved spraying the curcumin solution in a volatile organic solvent into hot water under ultrasonication, followed by concentrating the aqueous solution under reduced pressure, and then freeze-drying it to obtain a powder. When resuspended in water, the lyophilized powder formed a very fine dispersion and appeared to be soluble, unlike curcumin, which is completely insoluble in water, with undissolved flakes clearly visible in the suspension (Figure 2). Stabilizers or surfactants were not used, and the finished product entirely consisted of curcumin in the form of nanoparticles. To confirm that there is no chemical modification or degradation of curcumin during nanoparticle formation, a co-TLC of nanocurcumin with curcumin was performed and a ^{1}H NMR (Figure 3) spectrum was recorded. The appearance of a singlet at δ 3.94 and 5.80 ppm showed the presence of intact methoxy groups and an olefinic (C-4) proton. The peaks in the range of δ 6.93−7.11 ppm accounted for the aromatic protons. The data suggested that both the nanocurcumin and curcumin had an identical chemical structure. The particle size analysis and distribution of the nanoparticles was performed by TEM, SEM, and DLS analysis. DLS of an aqueous dispersion of nanocurcumin revealed the formation of nanoparticles with an average hydrodynamic diameter of 30 nm (Figure 4a). TEM of the aqueous dispersion showed the particle size to be in the range of 2−40 nm (Figure 4b), and SEM of the powdered sample showed the particles to be approximately 50 nm (Figure 4c). Dry, lyophilized powder of nanocurcumin was found to have good physical and chemical stability, was readily dispersible in water, and could be stored at room temperature for over 6 months without any decomposition or aggregation. The enhanced aqueous solubility of nano-sized curcumin particles could be attributed to their larger surface area, which promotes

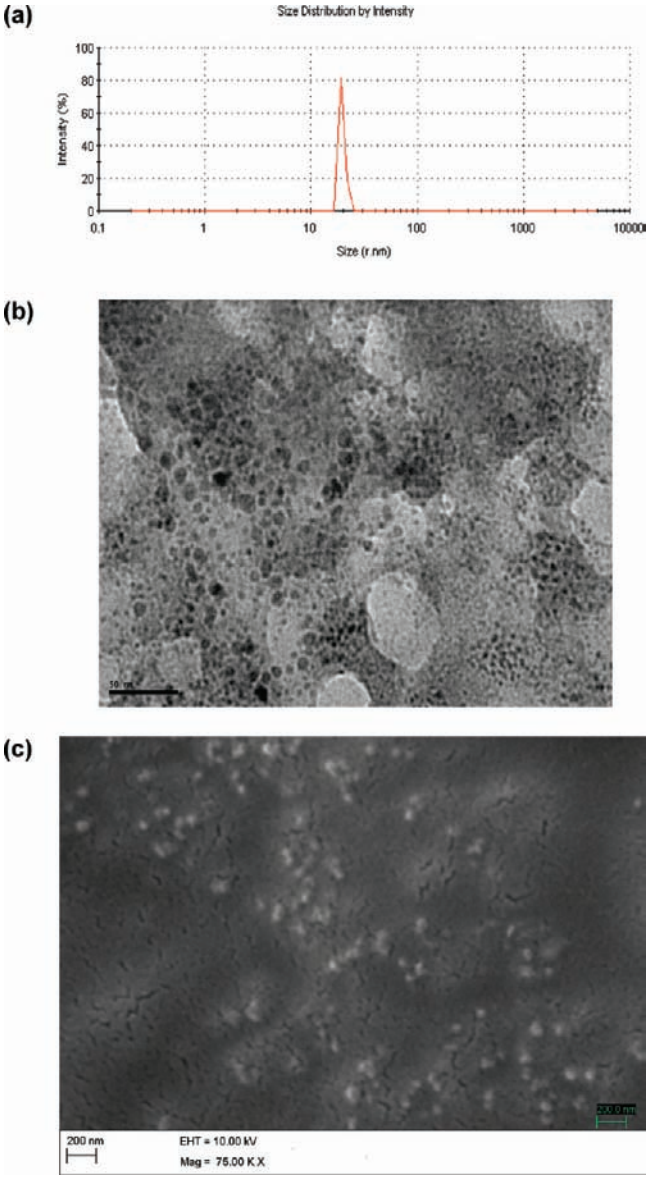

Figure 4. Size characterization of curcumin nanoparticles: (a) DLS, (b) TEM image, and (c) SEM image.

Table 1. MIC of Curcumin and Nanocurcumin against Different Microbes

	MIC (μg/mL)	
organism	curcumin (DMSO)	nanocurcumin (water)
S. aureus	150	100
B. subtilis	100	75
E. coli	300	250
P. aeruginosa	250	200
A. niger	400	350
P. notatum	ND	ND

dissolution.[25] Similar results have been demonstrated in previous studies also, where reduction in the particle size of active ingredients to nanoparticle size has shown improvement in its efficacy, solubility, and bioavailability.[26]

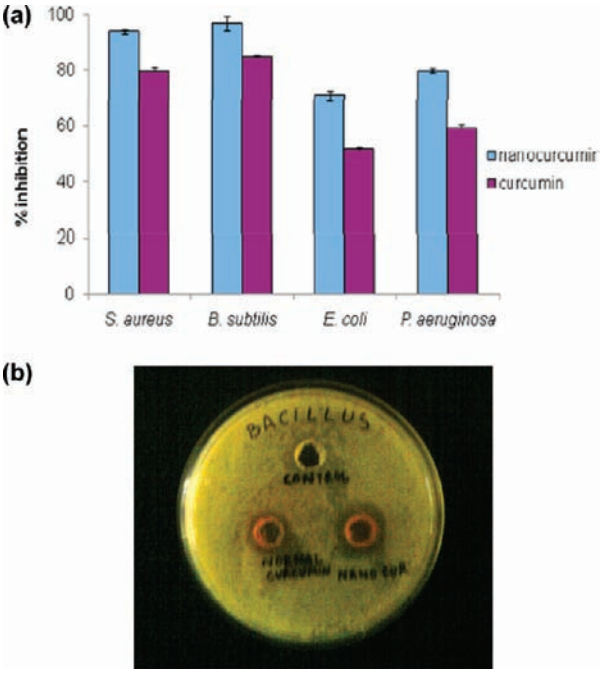

Figure 5. (a) Antibacterial activity of nanocurcumin (water) and curcumin (DMSO) solutions at a concentration of 200 μg/mL. (b) Zone of inhibition of *B. subtilis*.

Table 2. Zone of Inhibition of Curcumin and Nanocurcumin at a Concentration of 400 μg/mL

organism	zone of inhibition of curcumin (mm)	zone of inhibition of nanocurcumin (mm)
S. aureus	12	16
B. subtilis	15	20
E. coli	9	12
P. aeruginosa	10	14

Antibacterial and Antifungal Assay. MIC of curcumin and nanocurcumin was tested against two Gram-positive (*S. aureus* and *B. subtilis*), two Gram-negative (*E. coli* and *P. aeruginosa*) bacteria and two fungal strains (*P. notatum* and *A. niger*), and the results are presented in Table 1. The antibacterial activity of nanocurcumin against *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa* showed that it exhibits a broad spectrum inhibitory effect against all microorganisms. MIC of nanocurcumin for *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa* was 100, 75, 250, and 200 μg/mL, respectively, compared to 150, 100, 300, and 250 μg/mL for curcumin. The percent inhibition of different bacteria at 200 μg/mL concentration of nanocurcumin (water) and curcumin (DMSO) solutions followed the order: *B. subtilis* > *S. aureus* > *P. aeruginosa* > *E. coli*. (Figure 5a). The standard deviations were found to be in the range of 0.8−2.6. Further, the diameter of inhibition zones measured at the curcumin concentration of 400 μg/mL showed maximum efficiency for *B. subtilis* (Table 2 and Figure 5b). Taken together, the results indicated that the selected Gram-positive bacteria had higher sensitivity than the selected Gram-negative bacteria. This could be due to differences in their cell membrane constituents and structure. It is known that Gram-positive bacteria contain an outer peptidoglycan layer, while Gram-negative bacteria contain

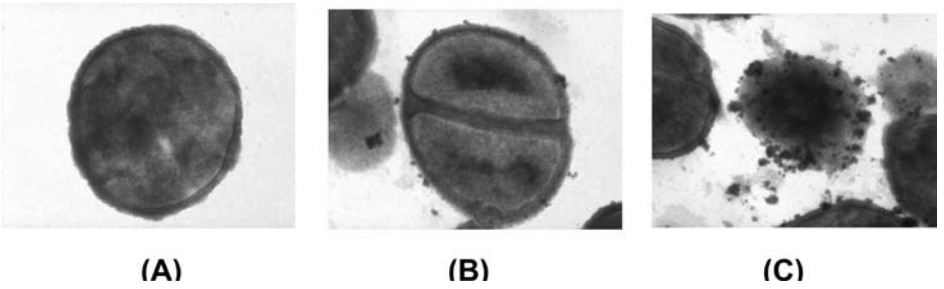

Figure 6. TEM images of (A) an unexposed (control) cell of *S. aureus*, (B) *S. aureus* treated with curcumin nanoparticles, with anchoring of curcumin nanoparticles at the cell wall, and (C) attack of curcumin nanoparticles, disruption of the cell wall (peptidoglycan layer), and penetration of nanocurcumin inside the cell.

an outer phospholipidic membrane, both of which undergo different types of interaction when encountered by curcumin. MIC of nanocurcumin and curcumin examined for the two fungal strains showed these compounds to be ineffective against *P. notatum* even at high concentrations up to 1000 μg/mL. They, however, showed some antifungal activity for *A. niger* (350 μg/mL for nanocurcumin), although the MIC value was higher than the MIC range (100−200 μg/mL) for the bacteria tested (Table 1). We point out that we have compared an aqueous solution of nanocurcumin to a DMSO solution of curcumin. This kind of a comparison was deliberately performed to examine the effect of particle reduction on solubility and bioefficacy of curcumin. It is expected that curcumin solution in DMSO would show the best antibacterial activity because curcumin has very high solubility in DMSO and that explains why all of the previous studies have been performed in DMSO. In none of the studies performed earlier has water been chosen as the solvent because curcumin is completely insoluble in water.

For the first time, we show that curcumin can be solubilized in water when it is in the nano form and that it is as much or even more effective than curcumin in DMSO. The rationale behind the stronger activity of nanocurcumin than curcumin in DMSO is related to the particle size. The key here is that, once curcumin forms nanoparticles, the size reduces to 2−40 nm, which is much less than the size of curcumin particles dissolved in DMSO (500−800 nm), which is responsible for better penetration and higher uptake by the cells.

The antibacterial and antifungal assay of aqueous solution of nanocurcumin demonstrated comparable or better *in vitro* antimicrobial efficacy compared to DMSO solution of curcumin. The antibacterial activity was more pronounced against Gram-positive bacteria than Gram-negative bacteria and was much better than antifungal activity. The *in vitro* biological assays clearly demonstrated that transformation to the nano form greatly improves the water solubility and efficacy of curcumin as an antimicrobial agent. Studies establishing the entire toxicological profile of curcumin nanoparticles are in progress, and it shall be interesting to see the effect of particle reduction on toxicity to macroorganisms. However, literature evidence shows that curcumin nanoparticles encapsulated in different nanocarriers, such as Eudragit S100 and hydrogels, were safe for oral administration for a short as well as a prolonged duration.[27,28]

Mechanism of Antibacterial Activity of Curcumin Nanoparticles. Figure 6 shows the TEM image of an isolated cell of *S. aureus* with a diameter of 700−800 nm. It was seen that, during the growth of the bacteria in the presence of curcumin nanoparticles, these nanoparticles (2−40 nm), which can be seen as dark, electron-dense spheres anchored at the cell wall of the bacterial cell, broke the peptidoglycan layer and penetrated inside the cell, thereby causing disruption of the structure of cell organelles and killing the cell through lysis. Our results are in agreement with earlier reports where nano-sized particles of different chemistries have been shown to mobilize inside the cell.[29] Previous studies carried out on *B. subtilis* 168 have shown that the mechanism of antibacterial activity of curcumin involves perturbing the GTPase activity of FtsZ protofilaments, which are known to play a critical role in bacterial cytokinesis. This perturbation becomes lethal to the bacteria and inhibits bacterial cell proliferation by inhibiting the assembly dynamics of FtsZ in the Z ring. The authors however mentioned that the membrane structure of the bacteria is not perturbed by curcumin in any way.[30] In another study, it has been shown that curcumin inhibits bacterial surface protein sortase A and prevents cell adhesion to fibronectin, thereby acting as an antibacterial agent against *S. aureus*.[31] In the present scenario, the mechanism through which curcumin nanoparticles are believed to manifest antibacterial properties is by anchoring to the cell wall of the bacterial cell, breaking it, then penetrating inside the cell, and disrupting the structure of cell organelles.

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