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SUPPLEMENTARY MATERIAL FOR THE MANUSCRIPT:

Mode of antimicrobial action of curcumin depends on delivery system: monolithic nanoparticles vs. supramolecular inclusion complex

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Supplementary Materials and Methods

1.1 The CNP release profile

Release study was performed using a dialysis bag method. One millilitre of CNP dispersion with curcumin concentration of 100 mM was placed inside a Slide-a-Lyzer dialysis cassette with 10 kDa molecular weight cutoff (Thermo Fischer Scientific) and immersed in 200 ml phosphate buffer pH 7.4 containing 0.1% CHAPS with constant stirring (100 rpm). Samples (10 ml) were withdrawn at predetermined time intervals, snap frozen in liquid nitrogen and freeze-dried in a model FD5508 freeze dryer (ilShin Lab Co.). Upon each sampling, the same amount of fresh dissolution medium was added to maintain sink condition. Released curcumin concentration was determined by diluting the lyophilized residue with ethanol, measuring its absorbance with an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences, Piscataway NJ) at 425 nm in comparison with the calibration curve of curcumin in ethanol. The cumulative release of curcumin from the nanoparticles was calculated according to the formula: $Cumulative\ release\ (\%) = \frac{Volume\ withdrawn}{Bath\ volume} \times P(t-1) + P(t)$ where P(t-1) is a percentage release at time t-1 and P(t) is percentage release at time t.

2.1 Dose-response analysis

For the dose-response study, the LB broth was inoculated with ca. 10^5 CFU/ml of *E. coli* ATCC 25922 and the cultures were supplemented with increasing concentrations of either CNP or CCD. Bacterial growth was measured by dilution plating after 9 hours of incubation

at 37 °C. Growth inhibition was calculated as the percentage of viable cells compared with untreated cultures. Dose-response curves were calculated using GraphPad Prism software, version 7.0 (GraphPad, San Diego, CA, USA) by fitting to the variable slope Hill equation $Y = Bottom + (Top-Bottom)/(1 + 10^{((LogIC_{50}-X)*HillSlope)})$ where Y is the modelled response; Bottom is the lowest experimental growth inhibition value; Top is the highest experimental growth inhibition value; IC_{50} is the half-maximal inhibitory concentration, and X is the compound concentration.

2.2 Treatment of bacteria for toxicity studies

The bacterial inoculum was prepared as described in Materials and Methods. After treatment with CCD, cells were harvested by centrifugation at 10,000 g for 10 min. and washed three times with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) before proceeding to assays. In the case of the treatments with a CNP, a discontinuous sucrose gradient centrifugation has been used to separate the bacteria from the nanoparticles. The gradients were prepared by layering sucrose solutions of 0.6, 0.5, 0.4 and 0.3 g/mL at the bottom of the Falcon tube. The mixture of the cells and curcumin nanoparticles was layered at the top of the gradient. After centrifugation (10,000 g for 5 minutes), a brown band of cells was visible in the middle of the gradient, and curcumin nanoparticles localized at the bottom of the tube the bacterial layer was collected and washed three times with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) before proceeding to assays.

3. The toxicity screening

3.1 Membrane potential assay.

To study the influence of curcumin nanoformulations on membrane potential we used the BacLight bacterial membrane potential kit (Thermo Fischer Scientific) following the manufacturer's instruction with slight modifications. Briefly, the bacterial cells were normalised by optical density at a wavelength of 600 nanometers (OD600) and resuspended in 200 µl of PBS containing 30 µM of 3,30-diethyloxacarbocyanine iodide (DiOC2). Following the incubation at 37°C for 30 min, the cells were dispensed into a 96-well black plates (PerkinElmer), and fluorescence was measured using EnSpire^{TM} multilabel plate reader (PerkinElmer, Waltham MA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 535 nm (for green) or 645 nm (for red). Measurements were normalised by the emission from the DiOC2 blank well. As a positive control of membrane depolarization, the cells were treated with carbonyl cyanide m-chlorophenylhydrazone (CCCP) at working concentration of 5 µM.

3.2 Cellular ROS generation.

To determine the intracellular ROS, the bacteria were stained with oxidation-sensitive probe 2′,7′-dichlorofluorescein diacetate (H_2DCFDA), (Sigma-Aldrich, St. Lewis, MO). Cells were incubated for one hour min with PBS buffer containing 20 μM H_2DCFDA in the dark. After the incubation and washing in PBS buffer, cells were disrupted by sonication and fluorescence intensity was measured using EnSpire™ multilabel plate reader (PerkinElmer, Waltham MA, USA) (excitation 490 nm, emission 519 nm) and normalised to protein concentration measured by BCA kit (Thermo Scientific, Pierce, Rockford, USA). Superoxide-generating compound menadione (Sigma) in the working concentration of 100 μM has been used as a positive control. Significance was calculated with a two-tailed Student's t-test relative to the control.

3.3 Electron transport activity

The electron transport activity of bacterial cells exposed to curcumin treatments was measured using the BacLight™ RedoxSensor™ Green Vitality Kit (Molecular Probes) according to the manufacturer's instructions with slight modifications. To one millilitre of cell suspension, 1 μl of 1mM stock solution (in DMSO) of RedoxSensor™ Green reagent was added followed by incubation for 10 min at room temperature. After the incubation and washing in PBS buffer, cells were disrupted by sonication and fluorescence intensity was measured using EnSpire™ multilabel plate reader (PerkinElmer, Waltham MA, USA) (excitation 490 nm, emission 520 nm) and normalised to protein concentration measured by BCA kit (Thermo Scientific, Pierce, Rockford, USA).Bacterial electron transfer disruptor sodium azide at working concentration of 10 mM was used as a positive toxicity control.

3.4 Adenosine triphosphate levels

Adenosine triphosphate levels in bacteria were measured using the BacTiter-Glo™ Microbial Cell Viability Assay (Promega), a luciferase bioluminescence-based method, according to the manufacturer’s instructions. ATP disodium salt (Sigma) was used to generate a standard curve, according to the manufacturer’s instructions. Luminescence measurements were taken using an EnSpire™ multilabel plate reader (PerkinElmer). The 100 μl of bacterial cells were incubated with an equal volume of BacTiter-Glo reagent for 10 min, at 25°C, with shaking; simultaneously, samples were taken for cell density (CFU/ml) measurements. ATP values were calculated based on the standard curve and normalised by cell density.

4. Quantification of relative gene expression by RT-qPCR

4.1 RNA isolation and cDNA synthesis

Same biological treatments and controls were used as described in the toxicity study. Samples for qRT-PCR analysis were immediately introduced into tubes containing RNAlater (Ambion, Austin, TX) to protect and stabilise RNA, centrifuged at 4°C, frozen in liquid N_2 and stored at -

70 °C. Total RNA was isolated using SV Total RNA isolation system (Promega, Madison, WI) according to manufacturers' instructions. Extracted total RNA was treated with RNase-free DNase I (Takara Bio Inc., Kusatsu, Japan) to eliminate a genomic DNA contamination. RNA integrity was tested with electrophoresis in 1.2% agarose gels and its concentration measured by 260/280 nm absorbance ratio using Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The criteria for proceeding to cDNA synthesis included the presence of two sharp ribosomal RNA bands and an absence of visible RNA degradation products. Before proceeding with cDNA synthesis, complete degradation of genomic DNA in RNA preparations was confirmed by PCR analysis. cDNA was synthesised using Verso cDNA Synthesis kit (Thermo Scientific), using a mixture of random hexamer primers, according to manufacturers' instructions.

4.2 Real-time PCR conditions and analysis

RT-qPCR reactions were performed on a Step One Plus real-time PCR System (Applied Biosystems, Foster City, CA) using Fast SYBR Green master mix (Applied Biosystems) to monitor cDNA amplifications. Cycling conditions were: 20 s at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. For each reaction, amplicon specificity was examined by analysis of the melting curve (95°C for 15 s, 60°C for one min, followed by a slow incremental increase in temperature to 95°C with a ramp rate of 0.3°C/s and continual acquisition), finally samples were cooled to 40°C for 30 s. RT-PCR was performed in three independent biological replicates with at least two technical replicates. A control reaction without template was included for each amplicon. Both the PCR efficiency (E) and the crossing point cycle (Cp) were assessed using LinRegPCR software [1]. The efficiency and critical threshold values were used to calculate the relative amounts of cDNA according to the method described by Pfaffl [2]. Briefly, the expression (E) fold change for each gene was calculated using the following equation: ratio $= E_{target}^{\Delta Cp\ target}/E_{ref}^{\Delta Cp\ ref}$ (where "ref" refers to geometric mean of selected housekeeping genes, "target" refers to genes to be tested, and ΔCp equals control Cp minus treatment Cp) and expressed as a fold change relative to corresponding control. Dilution series were made for some genes, to confirm that recorded Cp values from the experiments were within the linear dynamic range and that efficiencies were consistent with the values calculated using the LinReg program.

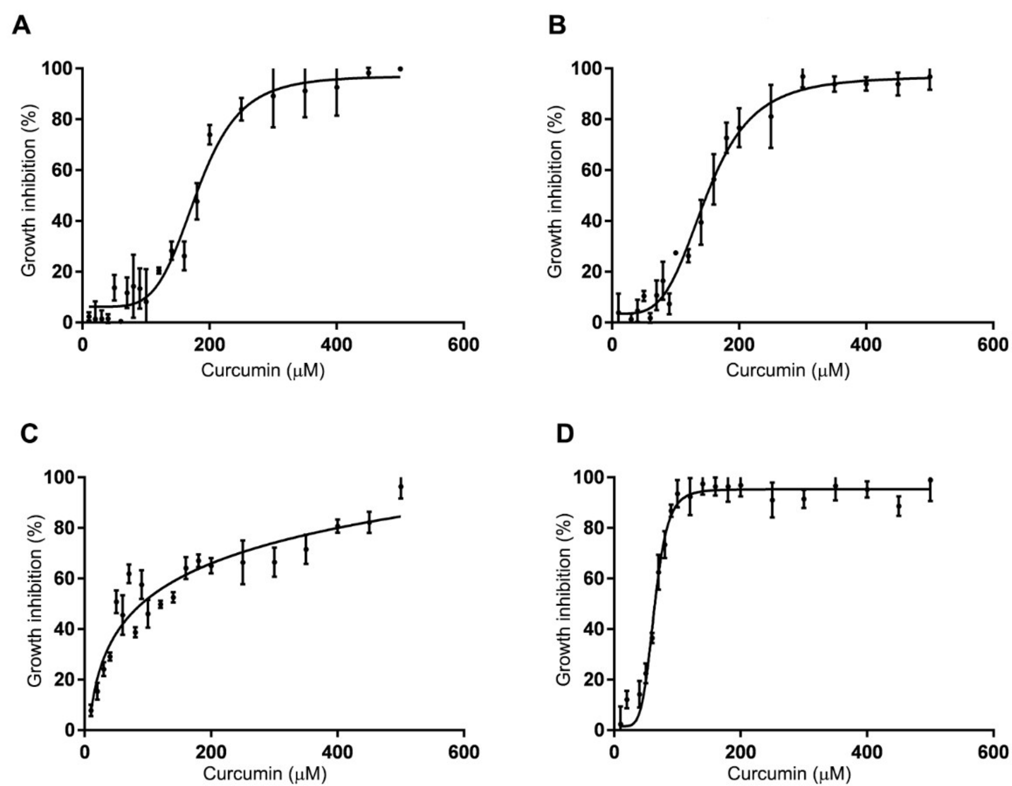

Figure S1.
Dose-response relationships of curcumin formulations

A- CNPs in the dark; B-CNPs upon illumination; C- CCD in the dark; D-CCD upon illumination

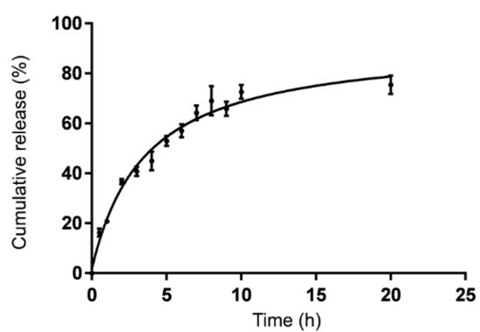

Figure S2.
Sustained curcumin release from the nanoparticles

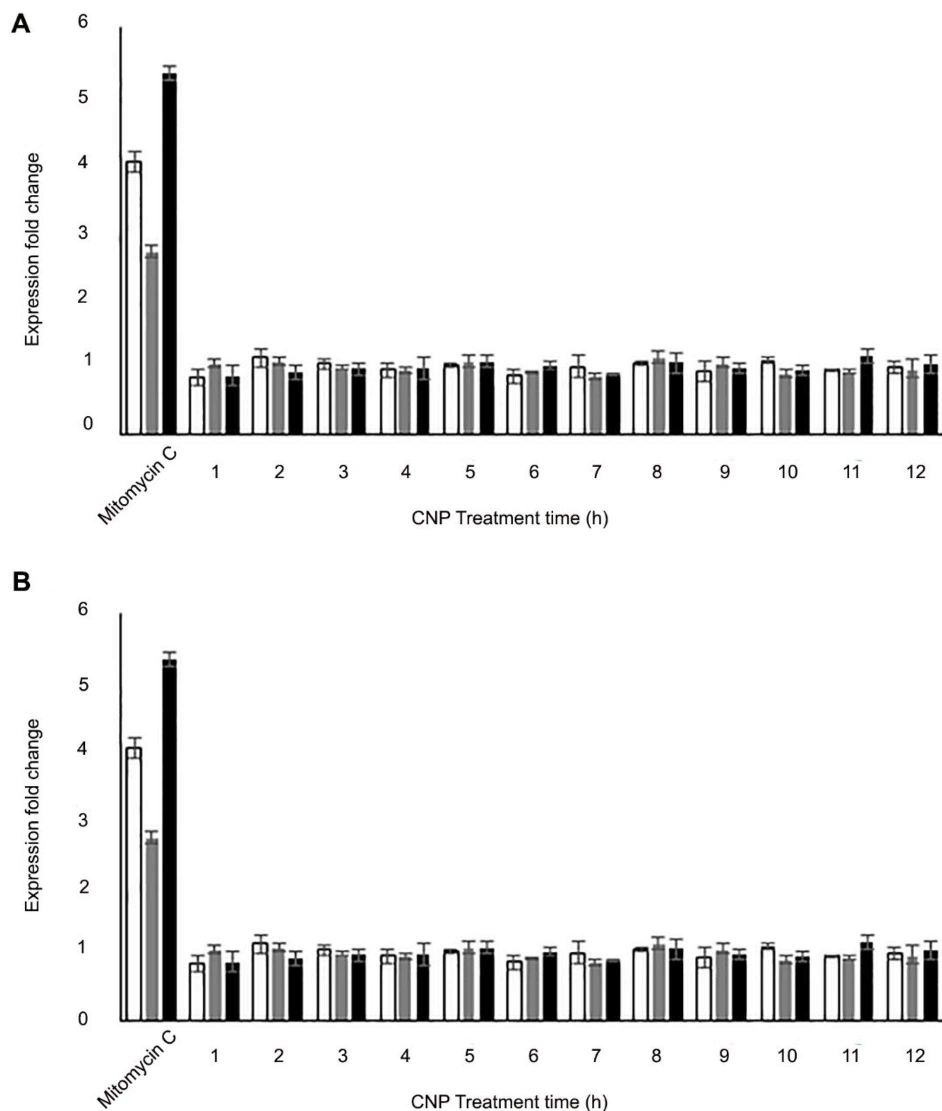

Figure S3.

Time-course RT-qPCR study of key SOS gene expression upon the treatment with CNP.

Expression determination by RT-qPCR of *recA* (white bars), *lexA* (grey bars) and *SulA* (black bars) in *E. coli* cells treated with mitomycin C (a SOS inducer used as a positive control), and in the cells treated with CNP in dark (A) and upon illumination (B). The error bars represent the standard errors of three independent biological repetitions.

Table S1 Primers used for qPCR assays

Gene	Forward Primer (5’-3’)	Reverse Primer (5’-3’)	Amplicon size (bp)	Reference
recA	GGAAATCGAAGGCGAAATC	CGGATCTGGTTGATGAAGA	129	This work
lexA	TTGCAGGAAGAGGAAGAAG	AGGAAGGATCGACCTGATA	106	This work
sulA	GCTACCCTTAACGAAAGTAATG	CCGATCACCACACTGTAAT	105	This work
opgB	ACCGTTAACCAGTATGTTCC	AGATCCATTCGCCATTACC	119	[3]
ihfB	CAGCAATCGCACATTCC	AGAGAAACTGCCGAAACC	120	[3]

References

1 J. M. Ruijter, C. Ramakers, W. M. H. Hoogaars, Y. Karlen, O. Bakker, M. J. B. van den Hoff and A. F. M. Moorman, *Nucleic Acids Res.* , 2009, **37**, e45–e45.

2 M. W. Pfaffl, *Nucleic Acids Res.*, 2001, **29**, e45–e45.

3 I. Shlar, S. Droby and V. Rodov, *J. Proteomics*, 2017, **160**, 8-20.