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Supporting Information

"One-pot synthesis of template-free hollow anisotropic $CaCO_3$ structures: towards inorganic shape-mimicking drug delivery systems"

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1. Materials.

Anhydrous calcium chloride, anhydrous potassium carbonate, magnesium nitrate hexahydrate, cadmium nitrate tetrahydrate, manganese chloride tetrahydrate, hydrochloric acid (37%), anhydrous sodium hydroxide (98%), alizarin red s, fluorescein isothiocyanate (FITC), anhydrous ethylene glycol (99.8% pure), polyethylene glycol 8000 Da (PEG8000), sodium dodecyl sulfate (SDS), thiazolyl blue tetrazolium bromide (MTT, 97.5%), ampicillin and Ringer's solution ¼ strength tablets were obtained from Sigma-Aldrich. SYTO 9 was purchased from Thermo Fisher Scientific. Fetal bovine serum and PBS Tablets were purchased from Gibco. Luria-Bertani medium was purchased from Helicon. Anhydrous ethylenediaminetetraacetic acid (EDTA, 99% pure) was purchased from Sigma-Aldrich, and its 0.003 M (pH=7.5) solution was used. 5'-FAM-GACCTTCCAGGGACTGTATGCGCTGATC-GTAGAGACATGACC was purchased from Integrated DNA Technologies (IDT). Cell cultures E. coli Nova Blue Turbo and E. coli K12 were purchased from NEB and Sigma-Aldrich, respectively.

2. Syntheses.

2.1. General synthesis procedure of $CaCO_3$ capsules with bacteria-like shape. 80 µL of ethylene glycol (EG) was mixed and stirred for 5 minutes with 2 µl of 4M water solution of $CaCl_2$, 8 µL of 1M water solution of $Mg(NO_3)_2$ and 10 µL of distilled H_2O (solution 1) in 2 ml eppendorf. Following the same principle, 80 µL of EG was mixed with 20 µL of 0.1 M Na_2CO_3 (solution 2) in the separate eppendorf. While stirring on 1500 rpm, solution 2 was added all at once to the solution 1. The reaction proceeded at room temperature with constant stirring for 1 hour followed by centrifugation at 14000 rpm for 5 minutes. After that, the supernatant was carefully removed, followed by re-suspension of "wet" precipitate in 200 µL of distilled water and centrifugation under the same conditions, and this procedure was repeated 3 times. Then the precipitate was dried under the vacuum, freezed at -20^{o}C and lyophilized at -50^{o}C for 24 hours. The yield of the particles was calculated by upscaling the synthesis protocol to the final volume of 200 ml, thus all the volumes were multiplied by 1000. Theoretical yield is 200.18 mg, where the obtained precipitant mass was 77.6 mg, which is c.a. 38.8% total yield due to the partial precipitation inhibition by Mg^{2+} ions and other losses. The synthesis results mainly in 1D-, 2D-, 3D-like (Fig. 2i) and shapeless particles with the yield of 81, 9, 5 and 5%, respectively. Zeta-potential of the particles equals to -7.27 ± 0.16 mV. To study time-dependent morphological changes of as-made $CaCO_3$ capsules (hereinafter, capsules aging), they were dissolved in 200 µl of distilled water and stirred (500 rpm) at room temperature for 24 hours. Then, precipitant was centrifuged and examined using SEM (Fig. S1). Capsules aging is shown on Fig. S2.

2.2. Study of the $CaCO_3$ synthesis parameters on the capsule morphology.

2.2.1. Effect of (Mg^{2+}/Ca^{2+})-ratio. $Mg(NO_3)_2$ concentration in the reference synthesis 2.1 was varied when all the other parameters being constant. In particular, preparation of solution 1 was changed. To achieve Mg^{2+}/Ca^{2+}-ratios of 0.1:1, 0.25:1, 0.5:1, 0.75:1, 1:1, 1.25:1, 1.50:1, 1.75:1 and 2.00:1 volumes of 1M $Mg(NO_3)_2$ solution and distilled water were varied as follows: 0.8 and 17.2 (0.1:1), 2

and 16 (0.25:1), 4 and 14 (0.5:1), 6 and 12 (0.75:1), 8 and 10 (1:1), 10 and 8 (1.25:1), 12 and 6 (1.5:1), 14 and 4 (1.75:1), 16 and 2 µL (2:1), respectively. All the samples were studied using SEM (Fig. S3).

2.2.2. Effect of Mg^{2+} substitution by Mn^{2+} or Cd^{2+}. $Mg(NO_3)_2$ solution in the protocol 2.1 was substituted by solutions of other salts ($Cd(NO_3)_2$ or $MnCl_2$) with concentrations being varied. In particular, preparation of solution 1 was changed. To achieve Mn^{2+}/Ca^{2+} or ratios Cd^{2+}/Ca^{2+} ratios of 0.1:1, 0.2:1 and 1:1 volumes of 1M $MnCl_2$ or $CdCl_2$ solution and distilled water were varied as follows: 0.8 and 17.2 (0.1:1), 1.6 and 16.4 (0.2:1), 8 and 10 µL (1:1), respectively. All the samples were studied using SEM (Fig. S4).

2.2.3. Effect of EG/H_2O ratio. EG/H_2O ratio in the synthesis 2.1 was varied. In particular, the amount of EG and distilled water in both solution 1 and 2 were changed. To achieve EG/H_2O ratios of 1:1, 2:1, 3:1, 4:1, 5:1, 6:1 and 7:1 volume of EG and distilled water were varied 1) in solution 1 as follows: 50 and 40 (1:1), 66.7 and 23.3 (2:1), 75 and 15 (3:1), 80 and 10 (4:1), 83.3 and 6.7 (5:1), 85.7 and 4.3 (6:1), 87.5 and 2.5 µL (7:1), respectively; 2) in solution 2 as follows: 50 and 48 (1:1), 66.7 and 31.3 (2:1), 75 and 23 (3:1), 80 and 18 (4:1), 83.3 and 14.7 (5:1), 85.7 and 12.3 (6:1), 87.5 and 10.5 µL (7:1), respectively. All the samples were studied using SEM (Fig. S5). Samples with EG/H_2O ratios of 1:1, 4:1 and 7:1 were studied using X-ray diffractometer (Fig. S6).

2.2.4. Effect of the stirring time and rate. Synthesis protocol 2.1 was modified, in particular, stirring times of 5, 10, 30 and 60 minutes were probed. Data is presented on Fig. S7. To study stirring rate influence, synthesis protocol 2.1 was modified, in particular, stirring rates of 0, 250, 500, 750, 1000, 1250, 1750 and 2000 rpm were examined. Resulting data is presented on Fig. S8.

2.3. Hollow $CaCO_3$ microspheres synthesis (for comparative studies). At first, 1.0 mg of PEG8000 and 2.0 mg of SDS were dissolved in 1 ml of distilled water under stirring at 1000 rpm. 0.5 ml of 0.2M $CaCl_2$ was added to the solution and stirred for 1 minute until solution will become cloudy. Then, 0.5 ml of 0.2M Na_2CO_3 was added at once to the resulting solution. The reaction was proceeded at constant stirring (1000 rpm) and room temperature for 1 hour followed by centrifugation at 14000 rpm for 5 minutes. After that, the supernatant was carefully removed, followed by re-suspension of precipitate in 1 mL of distilled water and centrifugation under the same conditions, and this procedure was repeated 3 times. Then the precipitate was dried under the vacuum, freezed at -20°C and lyophilized at -50°C for 24 hours. All the samples were studied using SEM (Fig. S9).

2.4. Vaterite $CaCO_3$ microspheres synthesis (for comparative studies). 0.5 ml of 0.33 M Na_2CO_3 solution was rapidly poured into 0.5 ml of 0.33 M solution of $CaCl_2$ at room temperature, reaction is proceeded under constant stirring (1000 rpm) for 10 minutes followed by centrifugation at 14000 rpm for 5 minutes. After that, the supernatant was carefully removed, followed by re-suspension of precipitate in 1 mL of distilled water and centrifugation under the same conditions, and this procedure was repeated 3 times. Then the precipitate was dried under the vacuum, freezed at -20°C and lyophilized at -50°C for 24 hours.

3. Characterization.

The particle morphology and size were investigated by scanning electron microscopy (SEM) using a VEGA3 TESCAN scanning electron microscope equipped with an X-Act EDX detector. The particles were applied to carbon tape, dried in vacuum or in freeze drying, a conductive layer of metals (Au/Pd) was sprayed. The sample was prepared on the carbon/silicon wafer without any Au/Pd coating in order to perform energy dispersive X-ray spectroscopy. The X-ray powder diffraction studies were performed on Rigaku SmartLab 3 diffractometer of the Saint-Petersburg State University of Technology. The crystalline phase and crystallinity of the samples were measured by X-ray diffraction using Cu-Kα radiation (λ = 1.54 Å); the samples were scanned at 2θ at a rate of 0.5 degrees per minute. The surface area, pore volume and pore size (Fig. S10) distribution were investigated using Quantachrome Nova 1200e by nitrogen adsorption at 77 K and analyzed using the BET and BJH equations. Prior to analysis, all of the samples were degassed at 110°C for 4 hours. Zeta-potential of the particles was measured using Photocor Compact-Z (laser power is 15 mW, correlator is linear, number of channels is 128, room temperature, viscosity correction for temperature, baseline shift is 0.01, spike tolerance is 50%). In single-particle ion beam etching particles were cut by an ion beam on a device TESCAN LYRA utilizing two following protocols: a) deposition of a protective platinum mask. Beam current 200 pA 2. Ion beam etching. Current 1nA; b) without deposition of the mask. Only etching. Current 200 pA. Cavity volume has been estimated by ion beam etching (Fig. S11) image analysis as follows: rotation body of Cassini oval giving dumbbell shape was simplified to two ellipsoids and one cylinder in order to maintain much more convenient approximate volume estimation. Cavity and overall particle squares were measured with 2 ellipses and 1 rectangle, then rotation bodies were calculated and summarized resulting in volumes of the particle and cavity. Then, cavity percentage was approximately evaluated to be c.a. 40±10%. For confocal investigation particles synthesized according to the protocol 4.1. were placed onto cover glass slips and dried immediately. Cover glass slips were then removed, washed with distilled water, and placed on glass slides with a drop of glycerol. Slides were stored in the dark at +4°C prior to examination. All the confocal studies were performed on Leica TCS SP5.

4. Drug loading and release study.

4.1. Drug loading studies. For bacteria-like hollow $CaCO_3$ microparticles**:** protocol 2.1 was modified, namely, composition of solutions 1 and 2 was changed. 80 µL of ethylene glycol (EG) was mixed and stirred for 5 minutes with 2 µl of 4M water solution of $CaCl_2$, 4 µL of 2M water solution of $MgCl_2$ and 14 µL of the drug solution (solution 1) - where the concentration of the molecule to be loaded is selected depending on the objective - in 2 ml eppendorf. Following the same principle, 80 µL of EG was mixed with 2 µL of 1 M Na_2CO_3 and 18 µL of the drug solution (solution 2) in the separate eppendorf. For spherical hollow $CaCO_3$ microparticles**:** protocol 2.3 was modified, namely, SDS and PEG8000 were dissolved in 1 ml of 3.5 g/L water solution of alizarin red S.

4.1.1. Alizarin Red S. Quantitative alizarin red S loading for bacteria-like and spherical hollow $CaCO_3$ microparticles was evaluated as follows. For bacteria-like hollow $CaCO_3$ microparticles**:** alizarin-bearing bacteria-like $CaCO_3$ capsules were synthesized following the protocol 4.1, where the

concentration of added alizarin red S was 1 g/L (0.16 g/L in reaction mixture). For spherical hollow $CaCO_3$ microparticles**:** alizarin-bearing spherical $CaCO_3$ capsules were synthesized, where the concentration of added alizarin red S was 3.5 g/L (1.75 g/L in reaction mixture). All the supernatants were collected in separate cuvettes. Then, all the cuvettes were studied spectrophotometrically. Load percentage of alizarin red S was calculated as the mass of loaded alizarin per total mass of the alizarin in reaction. Total mass of loaded alizarin was calculated as its total amount in reaction minus its amount lost during the washing procedures, which was measured through calibration curves for alizarin in the reaction medium and in distilled water: 100% x mass (loaded alizarin) / mass (all alizarin). Loading efficacy for bacteria-like and spherical $CaCO_3$ capsules are 15,1 and 16,8%, respectively (given $CaCO_3$/alizarin molar ratio of 2.4/1).

4.1.2. BSA-FITC. BSA-FITC was obtained according to the following protocol: 1 mg of BSA was dissolved in 100 mM carbonate buffer with pH=10, containing 6.27 mg FITC (molar ratio FITC/BSA=100:1), and left in dark for 2 hours. The resulting mixture was dialyzed against 100 mM PBS for 4 hours and then stored at -23°C. BSA-FITC-bearing bacteria-like $CaCO_3$ capsules were synthesized following the protocol 4.1, where the concentration of added BSA-FITC was 1 g/L (0.16 g/L in reaction mixture).

4.1.3. FAM-DNA oligonucleotide. FAM-DNA-bearing bacteria-like $CaCO_3$ capsules were synthesized following the protocol 4.1, where the concentration of added FAM-DNA was 100 µm (16 µm in reaction mixture).

4.2. Drug release studies. Bacteria-like hollow $CaCO_3$ microparticles were synthesized according to the protocol 4.1.1, then the precipitate was diluted in 200 µl of either 1) pH=5.0 HCl solution, 2) water, 3) pH=9.0 NaOH solution, 4) PBS 1x, 5) Ringer 1x or 6) LB medium pH=7.0, resuspended, incubated for 1 hour (37°C) under constant stirring (500 rpm), and then – after 1 hour of incubation without any stirring in order for microparticles to precipitate - UV/Vis spectra were obtained. Then, 100 µl of 0.1 M EDTA (pH=7.5) was added, and new spectra were measured. Turbidity of the particles and dilution of particles solution in the media by addition of EDTA were considered by implementation of the same experiments without alizarin red S. Drug release was quantified colorimetrically using absorbance on both 335 and 505 nm. Then the amount of released dye was compared with the loaded one, thus release percentage was calculated.

5. Antibacterial activity and embedding into the biofilm.

5.1. Antibacterial studies. CFU 10^9 nightlife strain of E. coli Nova Blue (TrR) was diluted 5 times and 200 µL inoculated in the Cell Culture Plate for 24 hours to form biofilms. After a specified period of time, spherical vaterite / dumbbell-shaped calcite particles were added to the resulting biofilms, the concentrations of which in the final solution were 0.5 mg/mL. Incubation time was 24 hours. Next, the biofilms were stained by adding methyl violet to fixed PFA preparations and extraction of the absorbed dye with a mixture of acetone and alcohol (1:1). By intensity of the resulted solution

measured using a microplate reader (Infinite F50) cytotoxic properties of the particles were evaluated. A positive control was strain in a nutrient medium without the addition of particles. Negative control was a medium without addition of bacterial cells and particles. Comparison of vaterite and calcite microparticles antibacterial activity is presented (Fig. S12).

5.2. Capsules embedding into the biofilm. 1 mL of 10^9 CFU E. coli RFP Turbo AmpR TetR was added to 9 mL of LB medium (pH=7.0) containing ampicillin and tetracycline. Coverslip (2.5 cm) is placed in Petri dish (3.0 cm in diameter) and then 2 mL of diluted culture is added. Dye-bearing particles were added in the final concentration of 10 μg/ml per dye mass and incubated for 24 hours. Then, coverslips are carefully washed with saline solution and the culture is fixed by 5% paraformaldehyde water solution treatment for 30 minutes. Sample is washed with distilled water, dried, single drop of glycerol is added right on the dried sample, coverslip is rolled over and placed on the specimen slide, the edges are sealed. 1 μL of LB medium (pH=5.0) is added onto the similar sample to open the capsules and observe dye release.

5.3. Bacterial growth curves. 50 μL of 10^9 CFU E. coli K12 culture was diluted in 4 mL of LB medium (pH=7.0) and 1 mL of 10 μg/mL ampicillin-bearing capsules (per the antibiotic mass) was added. Control culture was prepared by addition of 50 μL of 10^9 CFU E. coli K12 culture in 5 mL of LB medium (pH=7.0). D-glucose was added to the final samples to reach concentration of 2 g/L. Once every 40 minutes pH and optical density (OD) values (wavelength is 600 nm) of the samples were measured. OD was converted into colony-forming units (CFU) using conversion factor for E. coli.

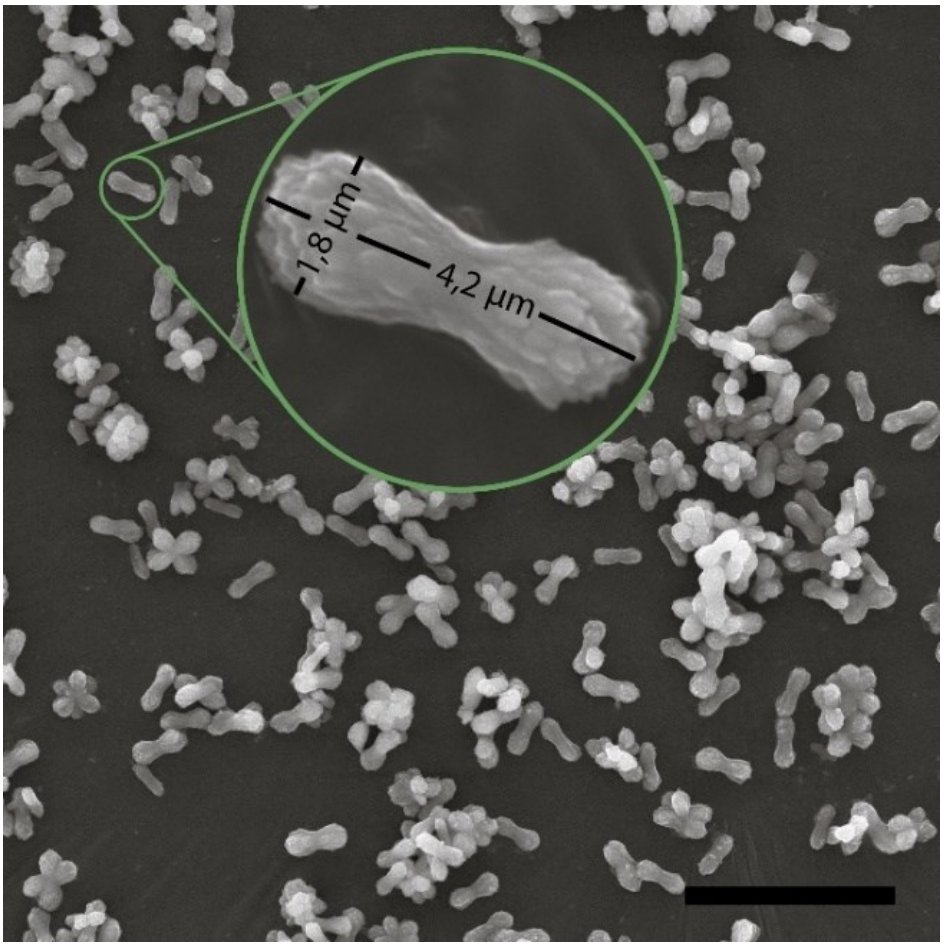


Figure S1. Scanning electron microscopy (SEM) image of bacteria-shaped $CaCO_3$ microparticles. HV = 20 kV.

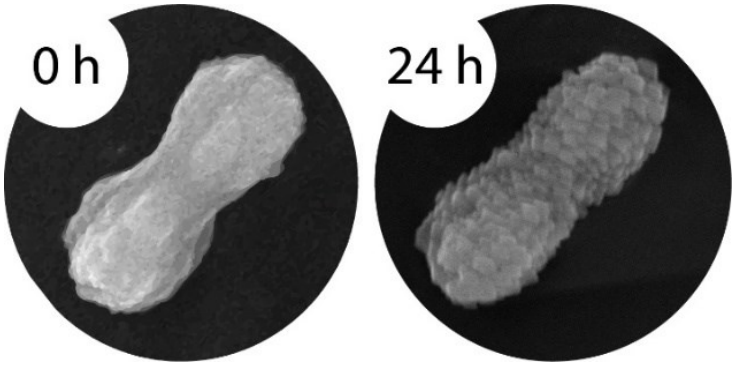


Figure S2. $CaCO_3$ capsules aging in distilled water under constant stirring (500 rpm) and room temperature (SEM image, HV = 20 kV). Morphological changes are clearly seen towards the formation of more crystalline structures, which is attributed to the slow dissolution-precipitation process of calcite phase in order to reduce its surface area contacting with polar water molecules.

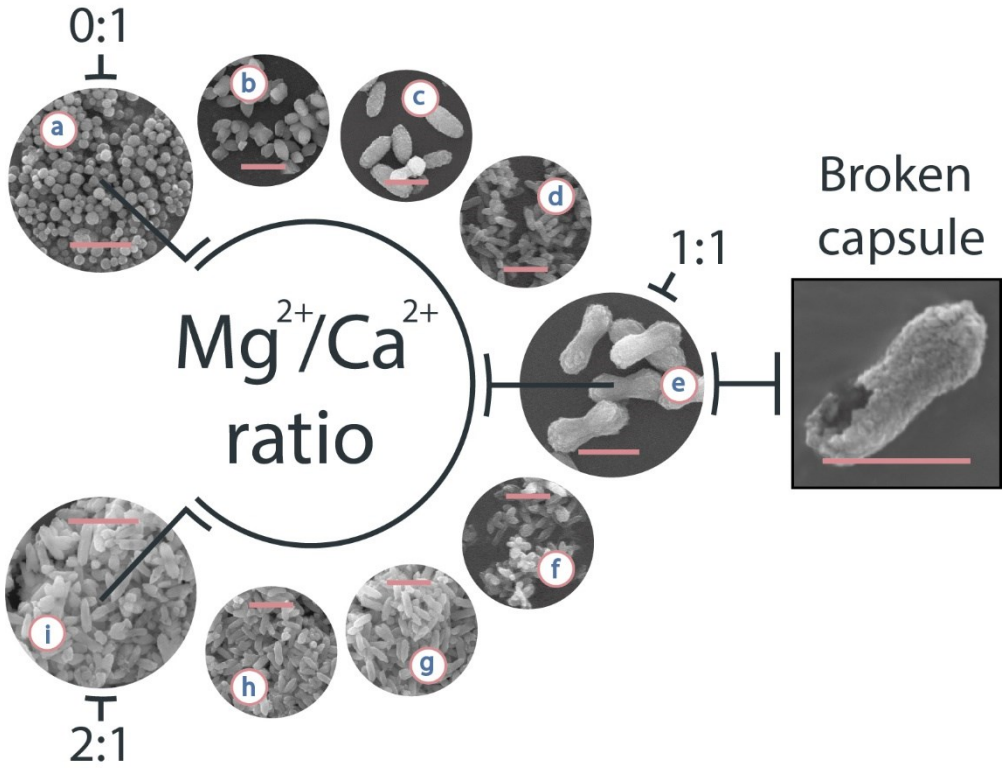


Figure S3. Effect of (Mg^{2+}/Ca^{2+})-ratio on particle morphology.

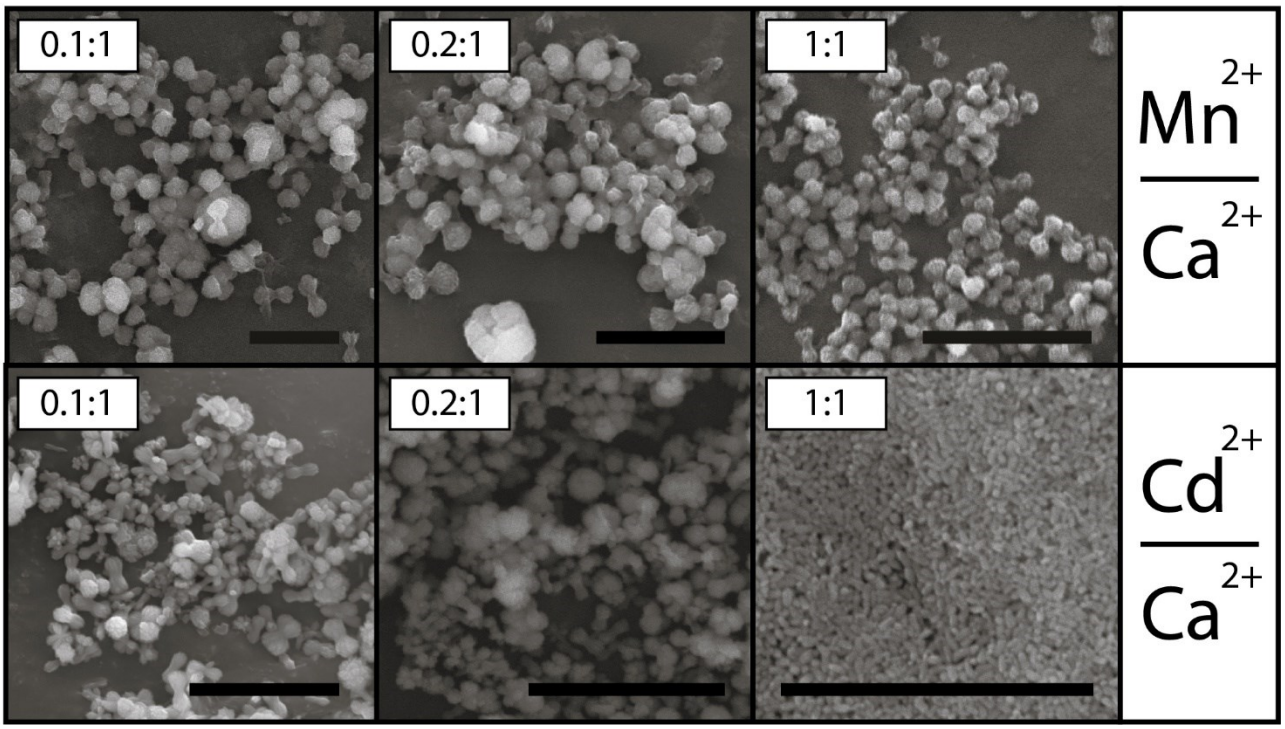


Figure S4. Effect of (Me^{2+}/Ca^{2+})-ratio on particle morphology. Scale bar is 5 µm.

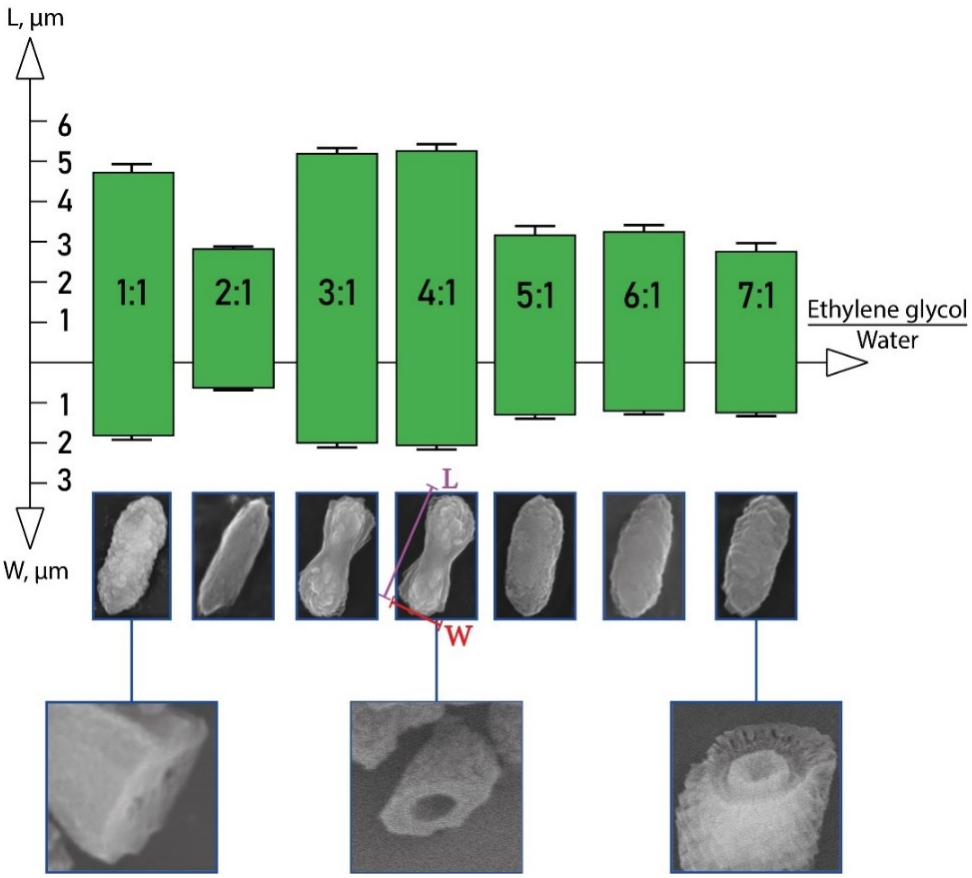


Figure S5. Effect of EG/H_2O ratio on particle morphology.

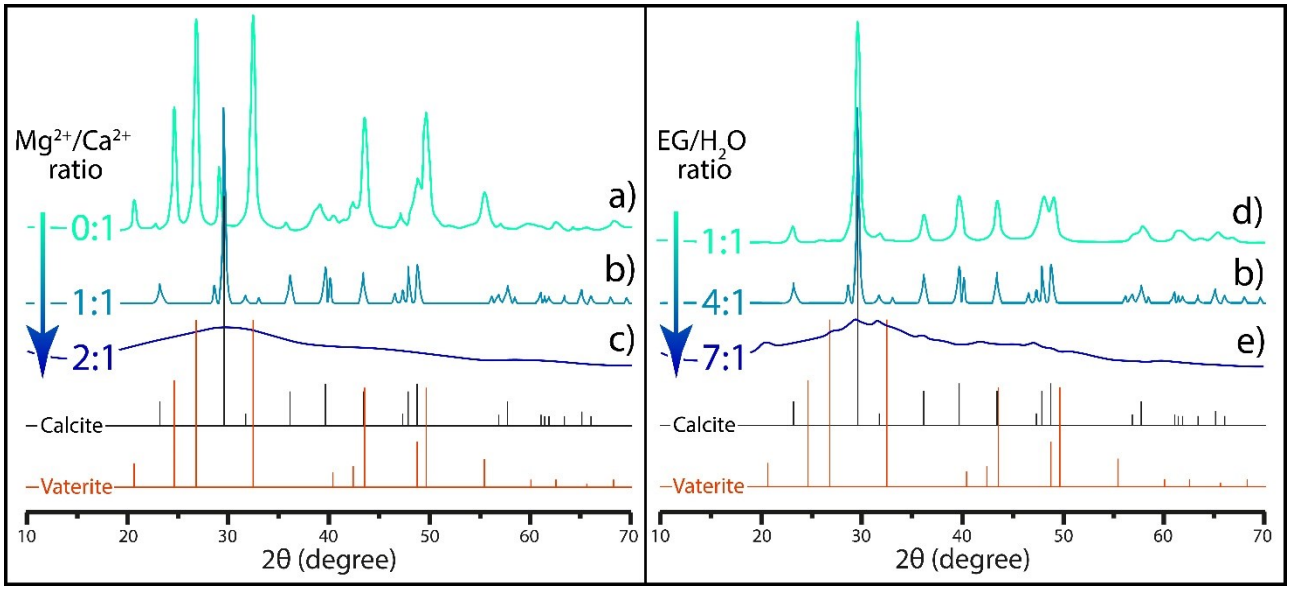


Figure S6. X-ray diffraction (XRD) patterns of the following samples: a) Mg^{2+}/Ca^{2+} is 0:1, EG/H_2O is 4:1; b) Mg^{2+}/Ca^{2+} is 1:1, EG/H_2O is 4:1; c) Mg^{2+}/Ca^{2+} is 2:1, EG/H_2O is 4:1; d) Mg^{2+}/Ca^{2+} is 1:1, EG/H_2O is 1:1; e) Mg^{2+}/Ca^{2+} is 1:1, EG/H_2O is 7:1.

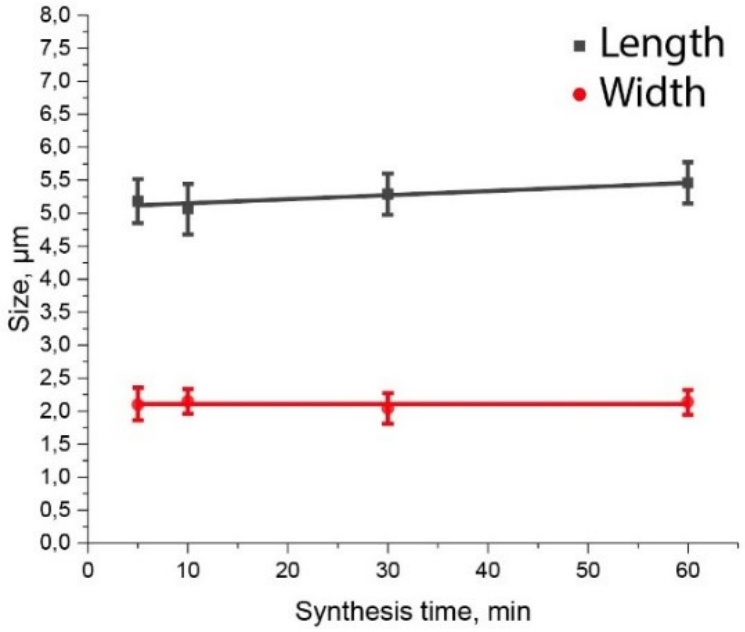


Figure S7. Synthesis time influence on size of resulting bacteria-shaped $CaCO_3$ microparticles. Presented data clearly shows no influence on particles size (morphology also stays the same), which means that these particles are already fully-formed at the point of 5 minutes, and further increase in synthesis time allows to increase the overall yield without losing the desired shape.

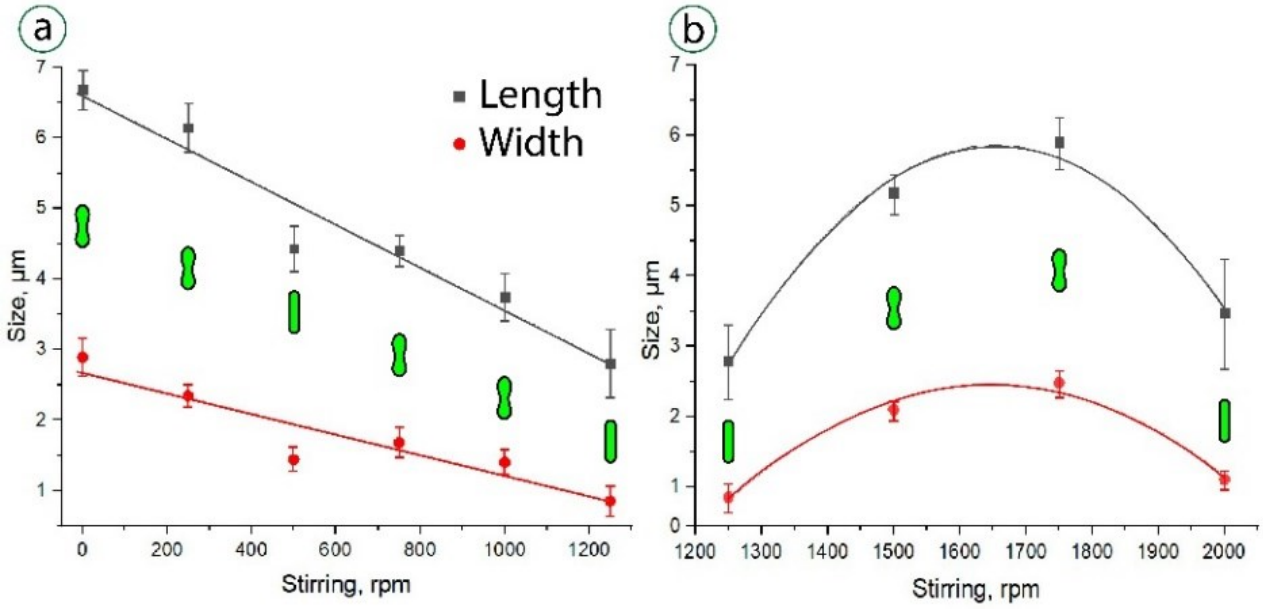


Figure S8. Stirring rate influence on size and shape of resulting microparticles. a) laminar and b) turbulent mode. Particle shape is represented schematically.

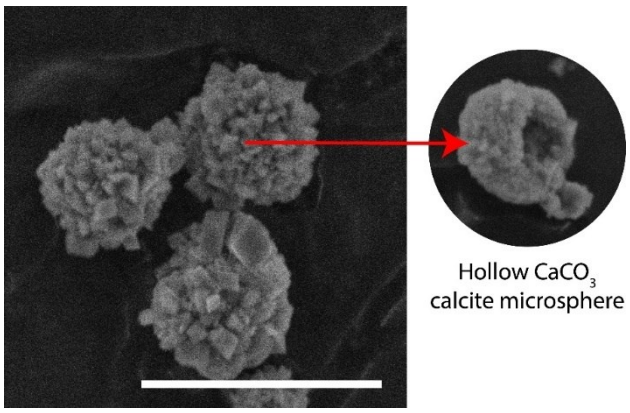


Figure S9. SEM image of hollow $CaCO_3$ calcite microspheres. HV = 5 kV.

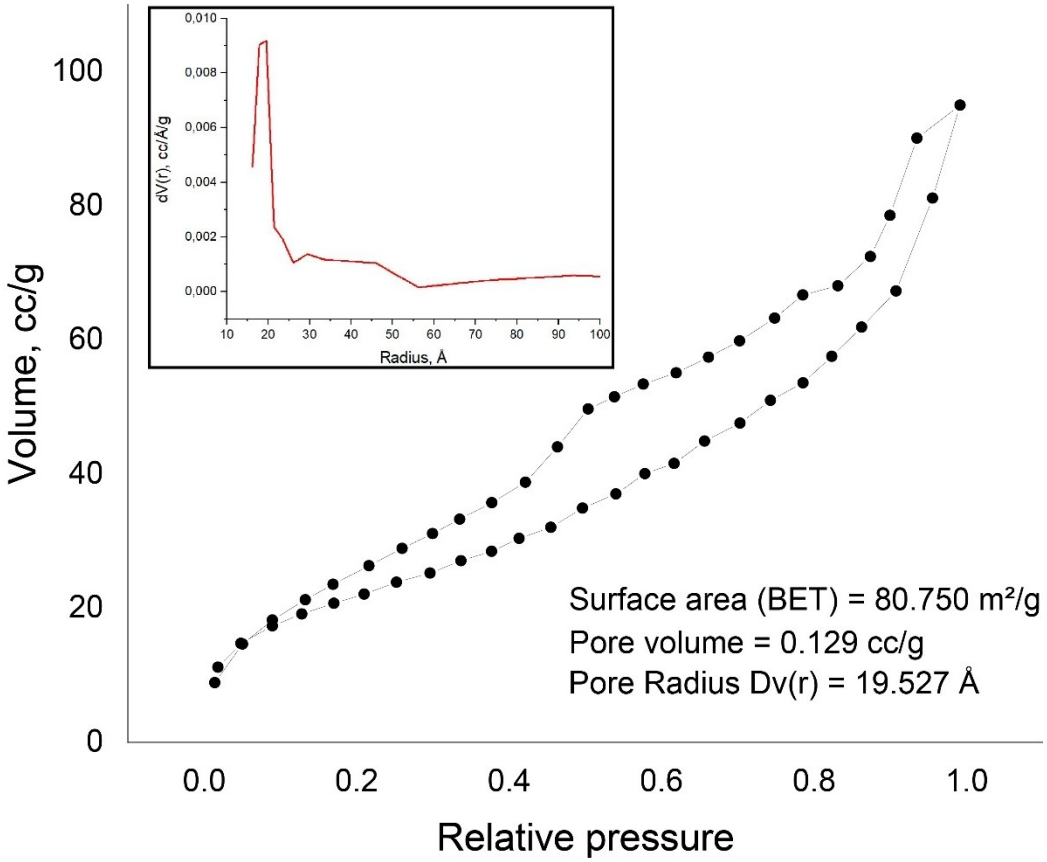


Figure S10. Nitrogen physisorption for $CaCO_3$ microcapsules with $EG|H_2O$ ratio of 4:1 and $Mg^{2+}|Ca^{2+}$ ratio of 1:1 (pore size distribution inset). Bacteria-shaped $CaCO_3$ microparticles demonstrate extremely high surface area of 80.75 m^2/g in comparison with other $CaCO_3$-based materials, which is profitable in the case of particle surface modification and wide range of environmental and analytical applications where high adsorption needs to be achieved. Pore radius is 1.95 nm, thus material can be classified as mesoporous. Slit-like structure of pores is consistent with proposed mechanism of needle-like substructure of bacteria-like microcapsules. Despite the fact that small molecules can diffuse through pores of this size, such a process is hindered by high shell thickness.

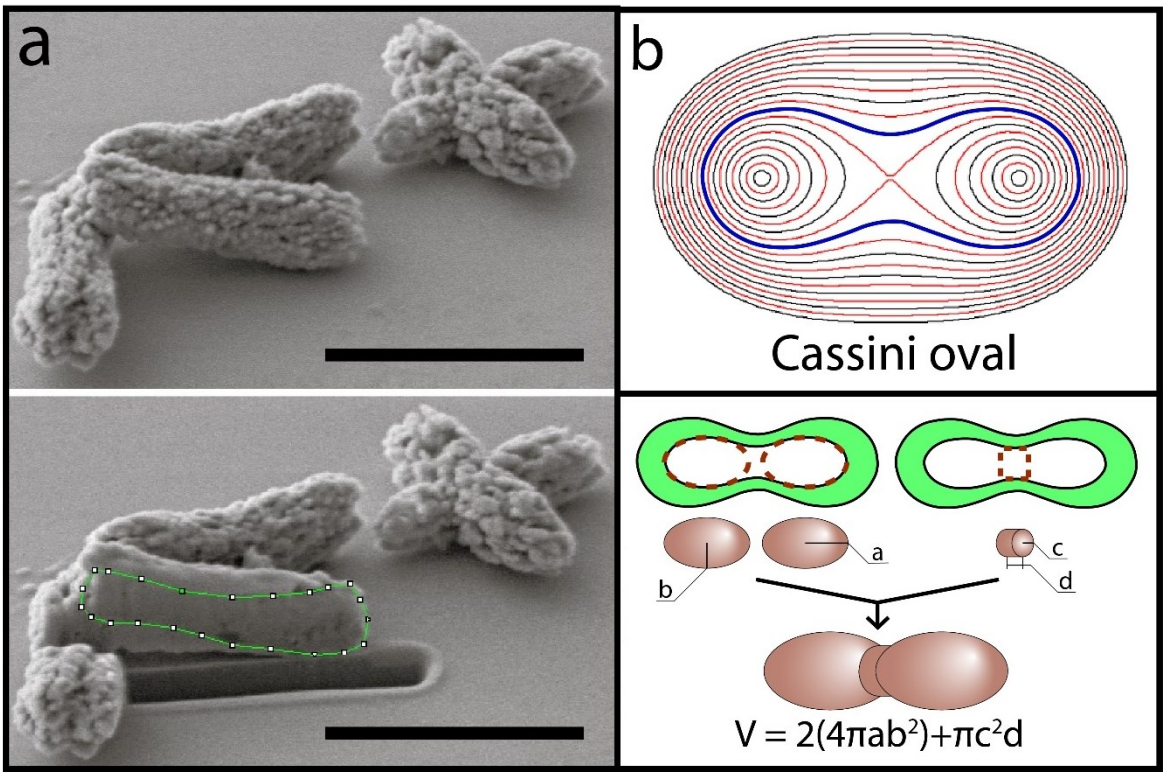


Figure S11. a) Ion beam etching of $CaCO_3$ bacteria-like microparticles and b) Cavity volume estimation.

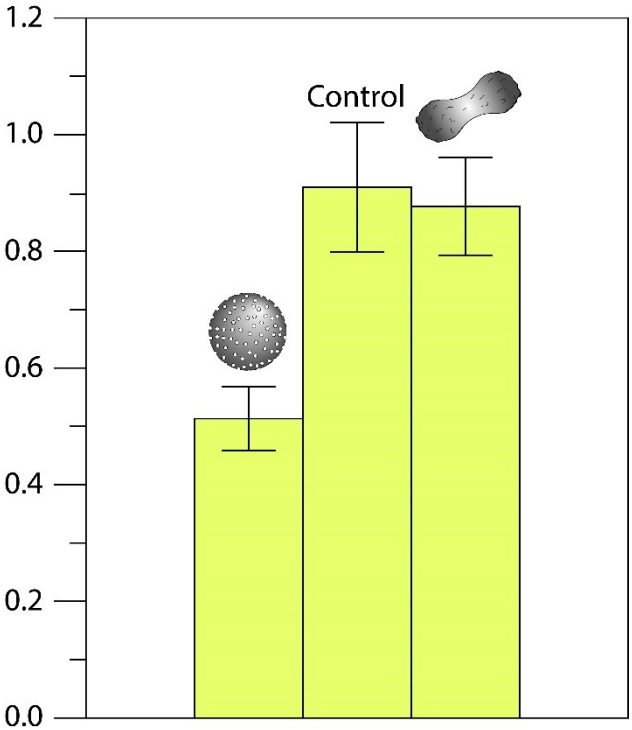
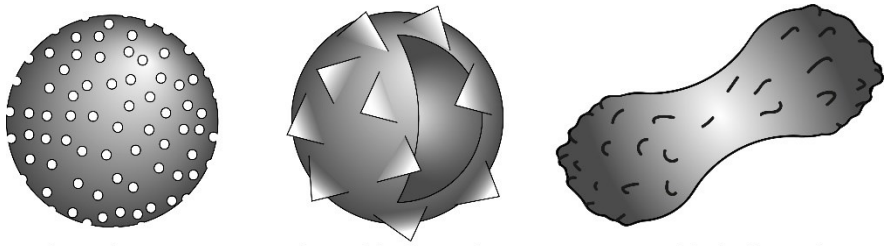


Figure S12. Antibacterial study on E. coli K12 bacteria with spherical vaterite and bacteria-shaped calcite $CaCO_3$ microparticles. Concentration of the particles equals to 10 µg/mL.



Surface area (BET), m^2/g	8.8	17.03	80.75
Mean pore size, nm	40	5.70	1.95
Zeta-potential, mV	-6.97	-13.25	-7.27
Shell thickness, nm	-	220	250
Cavity volume, %	-	48	39

Table S1. Comparison of physicochemical and DDS-specific parameters for spherical and bacteria-like microparticles.