Supporting Information

Controllable Synthesis of Calcium Carbonate with Different Geometry: Comprehensive Analysis of Particles Formation, Cellular Uptake and Biocompatibility

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Number of tables: 2

1. Materials

All chemicals were provided by Sigma Aldrich and were used without any further purification. calcium chloride ($CaCl_2$), sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), poly(styrene sulfonate) sodium salt (PSS), ethylene glycol (EG), Ethanol (CH_3OH), dextrane sulphate (DS), tetrarhodamine isothiocyanate (TRITC), polyallylamine hydrochloride (PAH), sodium 4-vinylbenzenesulfonate (SV). Dulbecco's Modified Eagle Medium (DMEM) was obtained from Gibco. Fetal bovine serum (FBS) was obtained from HyClone. Penicillin and streptomycin (P/S) were purchased from Biolot, Russia. Trypsin-EDTA solution were obtained from Sigma Aldrich. Milli-Q water with a resistance greater than 18.2 MΩ cm^{-1} was used for all experiments.

2. Synthesis of $CaCO_3$ particles

2.1 Reactions (α1, α2, α3; β1, β2, β3) of $CaCl_2$ with Na_2CO_3

The synthesis of $CaCO_3$ particles was performed using conventional procedure in co-precipitation reaction. For this, salts $CaCl_2$ and Na_2CO_3 were dissolved in EG:H_2O at certain concentrations (enlisted in **Table S1**) in volumetric ratio of 85:15 for all the reactions to the final volume V= 20 mL. Then, 20 mL $CaCl_2$ and 20 mL Na_2CO_3 were mixed together under magnetic stirring at 400 rpm for 5 min (**reactions α1, α2, α3**) and 24 h (**reactions β1, β2, β3**). At defined time periods (5, 10, 15, 20, 25, 30, 45, 60, 90, 1440 min) samples were taken from the **reactions β1, β2, β3** for the SEM analysis. The reaction conditions (reaction time, salt concentration, and their ratio) were varied depending on the reaction and are enlisted in **Table S1**. Afterwards, particles were washed 2 times with water and 1 time with ethanol by centrifuging (10 000 rpm during 2 min). The resulted particles were dispersed in ethanol to prevent recrystallization of $CaCO_3$ particles.

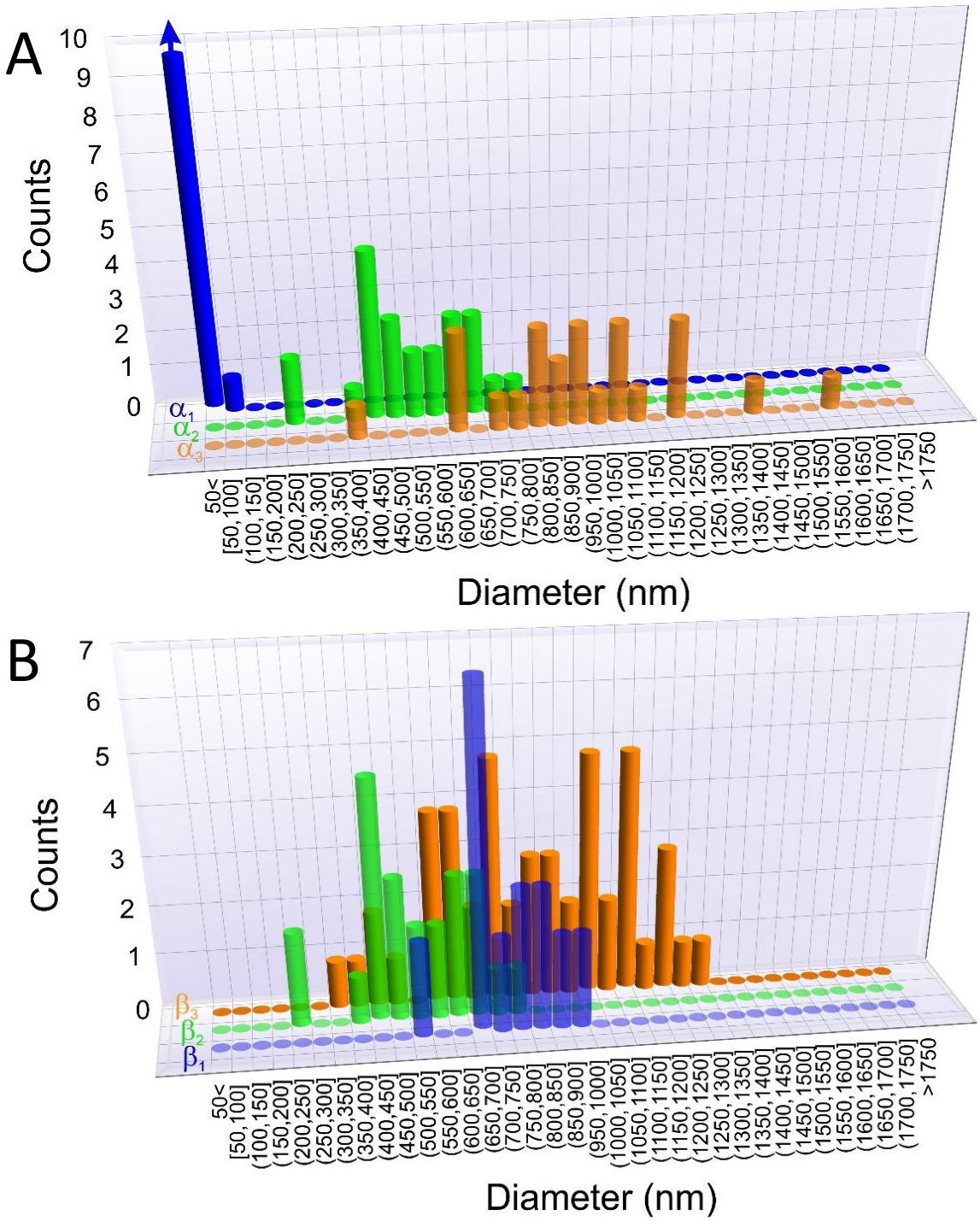

Figure S1. Size distributions *of $CaCO_3$ particles obtained from the reactions α1, α2, α3 (**A**) and β1, β2, β3 (**B**)*

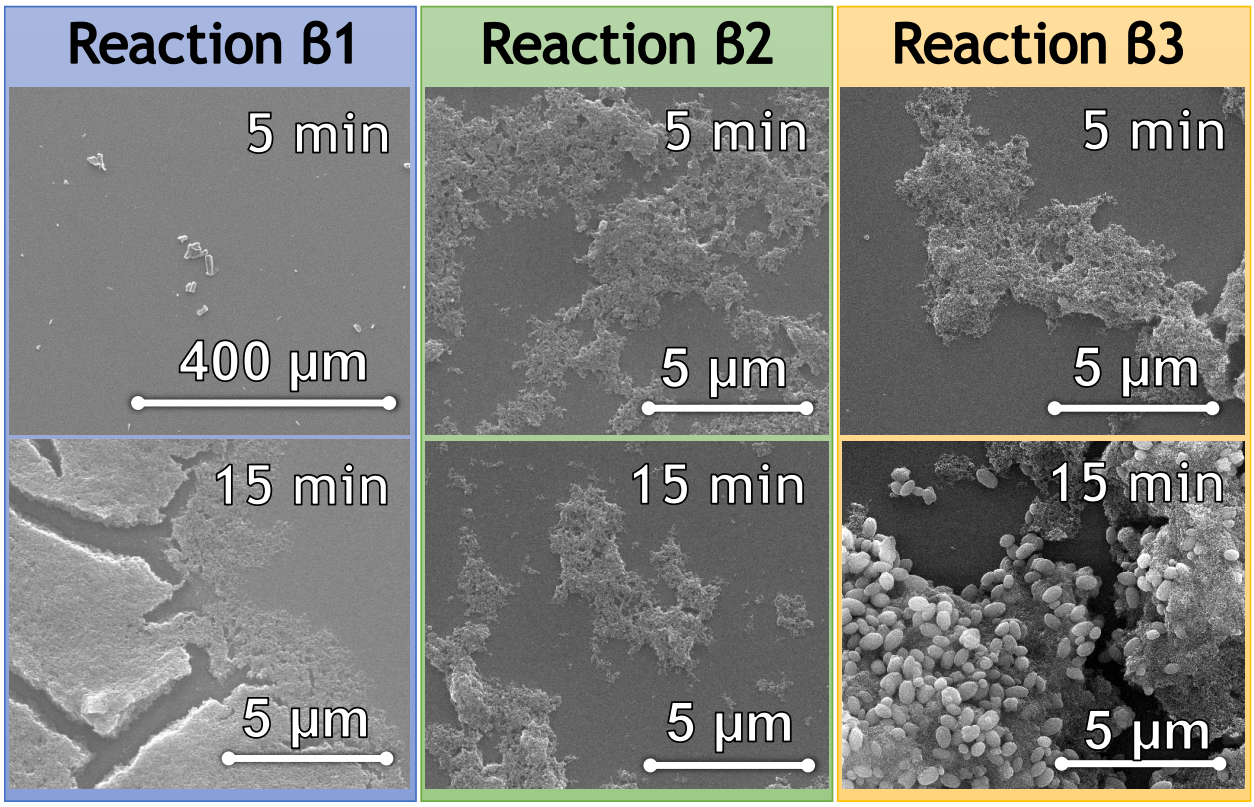

Figure S2. Study of $CaCO_3$ particles formation kinetics using $CaCl_2$ and Na_2CO_3: *SEM images of the products of the **reaction β1** (5 min, 10 min and 15 min), the **reaction β2** (5 min, 10 min and 15 min), the **reaction β3** (5 min, 10 min and 15 min).*

2.2 Reactions (γ1, γ2, γ3, γ4, γ5, γ6, γ7) of $CaCl_2$ with $NaHCO_3$

To slow down the rate of the $CaCO_3$ particles growth, $NaHCO_3$ was applied as a carbonate source. The co-precipitation reaction was performed as described above, $CaCl_2$ was mixed with $NaHCO_3$ under magnetic stirring at 400 rpm for 24 h. After certain periods of time (5, 10, 15, 20, 25, 30, 45, 60, 90, 1440 min) reactions were stopped and formed $CaCO_3$ particles were observed by SEM. The reactions conditions, incl. concentration of used salts, salts ratio, reaction time, was varied as enlisted in the **Table S1**.

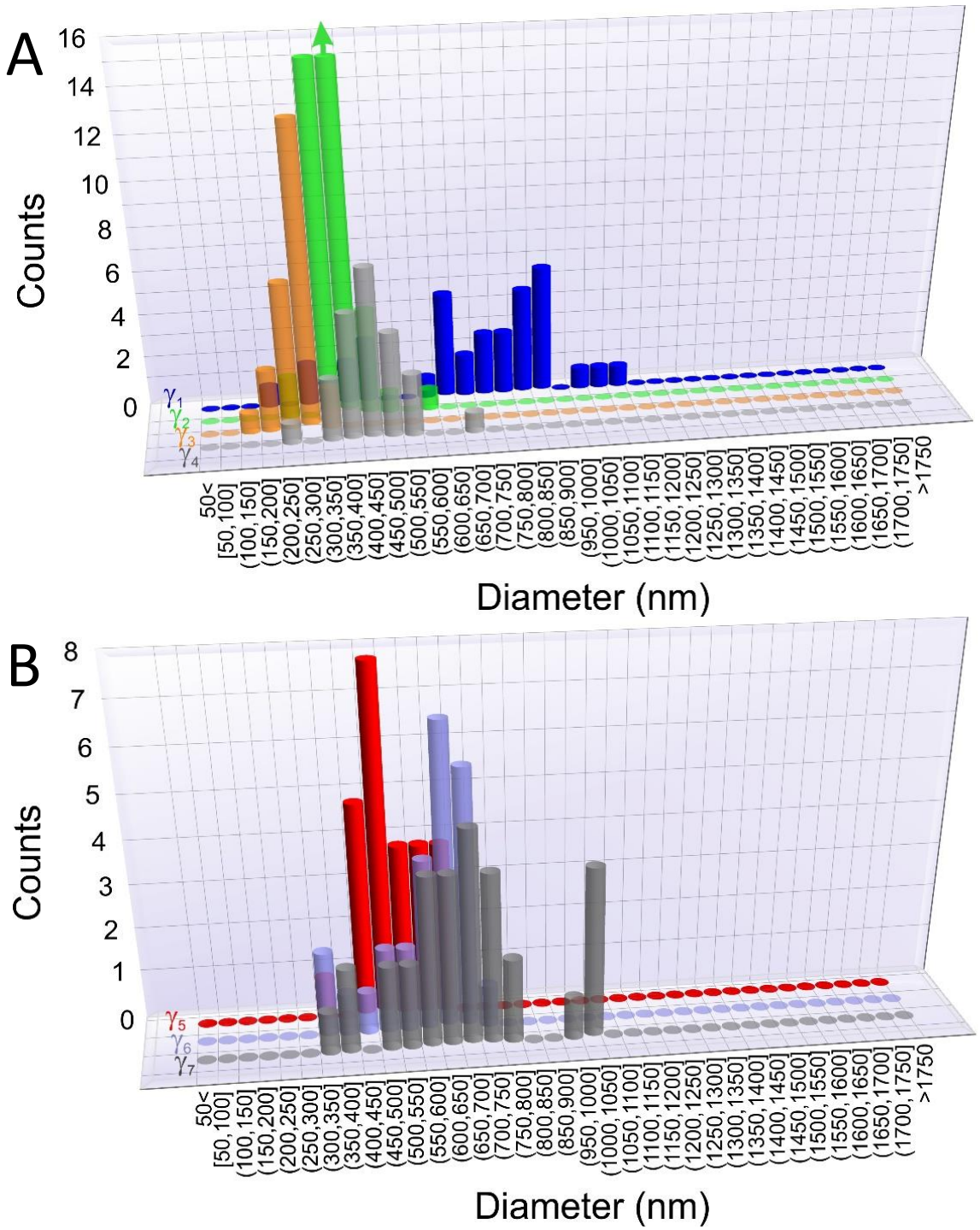

Figure S3. Size distributions *of $CaCO_3$ particles obtained from the reactions* ***A.*** *γ1, γ2, γ3, γ4 and* ***B.*** *γ5, γ6, γ7.*

***Table S1**. Reaction conditions to obtain differently shaped $CaCO_3$ particles.*

Tag	**$CaCl_2$ [M]**	**Na_2CO_3 [M]**	**$NaHCO_3$ [M]**	**Organic Additives [mg/mL]**
α1	0.0005	0.0005	–	–
α2	0.005	0.005	–	–
α3	0.05	0.05	–	–
β1	0.025	0.005	–	–
β2	0.005	0.005	–	–
β3	0.005	0.025	–	–
γ1	0.025	–	0.005	–
γ 2	0.005	–	0.005	–
γ3	0.005	–	0.025	–
γ 4	0.005	–	0.05	–
γ 5	0.005	–	0.075	–
γ 6	0.01	–	0.05	–
γ 7	0.015	–	0.075	–
δ1	0.025	0.005	–	0.5 (PSS)
δ2	0.025	0.005	–	1 (PSS)
δ 3	0.025	0.005	–	2 (PSS)
δ 4	0.005	0.025	–	1 (PSS)
δ 5	0.025	0.005	–	1 (SV)
δ 6	0.025	0.005	–	0.5 (DS)

To study the $CaCO_3$ particles formation over time, the wider ranges 20:1 and 1:20 ratio Ca^{+2}/HCO_3^{-1} were used.

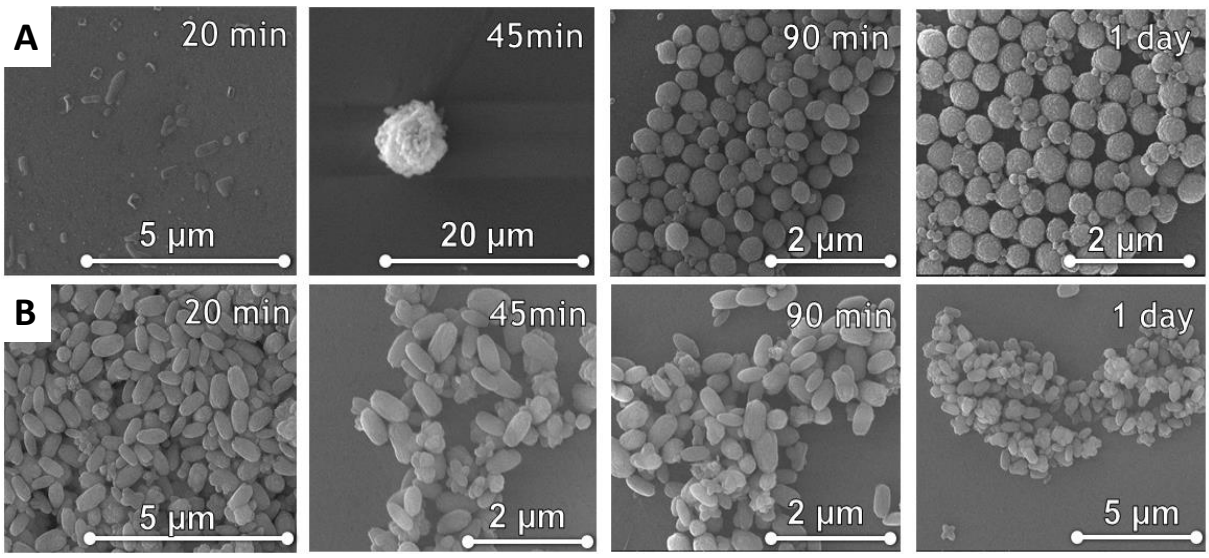

Figure S4. SEM images *of $CaCO_3$ particles formed at different Ca^{+2}/HCO_3^{-1} ratios =* ***A.*** *20:1 and* ***B.*** *1:20.*

Reactions (δ1, δ2, δ3, δ4, δ5, δ6) of $CaCl_2$ with Na_2CO_3 using organic additives

To reveal the influence of organic additives on the formation of $CaCO_3$ particles, PSS, SV and DS were employed for further reactions. Salt solutions $CaCl_2$ and Na_2CO_3 were mixed together under magnetic stirring at 400 rpm for 1440 min. The reaction conditions are enlisted in the **Table S1**.

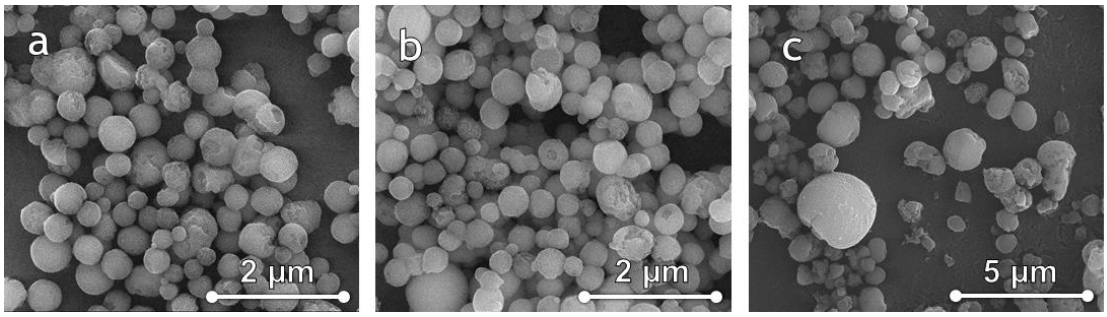

Figure S5. Reaction δ6 *with increased concentrations of DS. Time of the reaction was 1 day,* ***A.*** *c(DS)=0.5mg/mL.* ***B.*** *c(DS)=1mg/mL DS,* ***C.*** *c(DS)=2mg/mL.*

It should be noted that $CaCO_3$ particles are stable in ethanol-based solvents, while unstable in aqueous solutions (**Figure S6**): the storage of freshly formed particle in ethanol makes them stable for a long period of time. However, transferring them from ethanol into water, will induce rapid growth of $CaCO_3$ particles up to several micrometers.

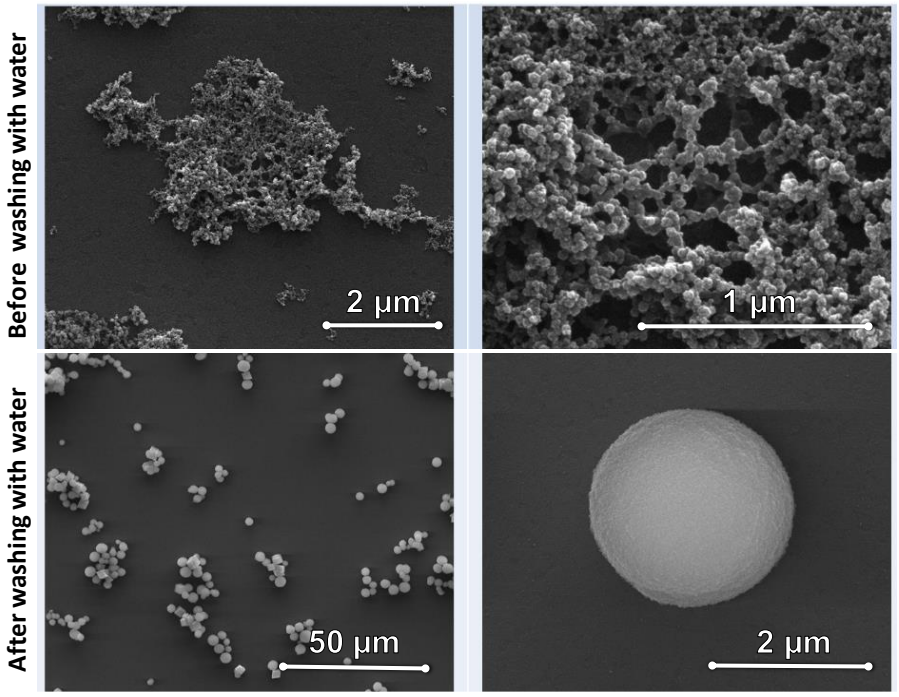

Figure S6: Re-crystallization of calcium carbonate particles. *SEM images of amorphous $CaCO_3$ particles before washing with water (upper row) and after washing with and dispersion in water (bottom row).*

3. X-ray diffraction (XRD)

To measure XRD spectra of obtained particles, 5 µL drop of $CaCO_3$ particles stock solution was placed onto the glass SiO_2 substrate. The XRD spectra of synthesized particles were obtained with a Bruker's D8 Discover Diffractometer (setup: q:q geometry with the source was copper anode and the detector a LYNXEYE XE linear detector). The raw data was smoothed followed by baseline subtraction, which allowed to obtain the content of vaterite and calcite fractions in the reaction products. The following relations were used for the ratio assessment:

$$\boldsymbol{X_v = \frac{7.691 \cdot I_{110v}}{I_{104c} + 7.691 \cdot I_{110v}} \quad X_C = 1 - X_v}$$

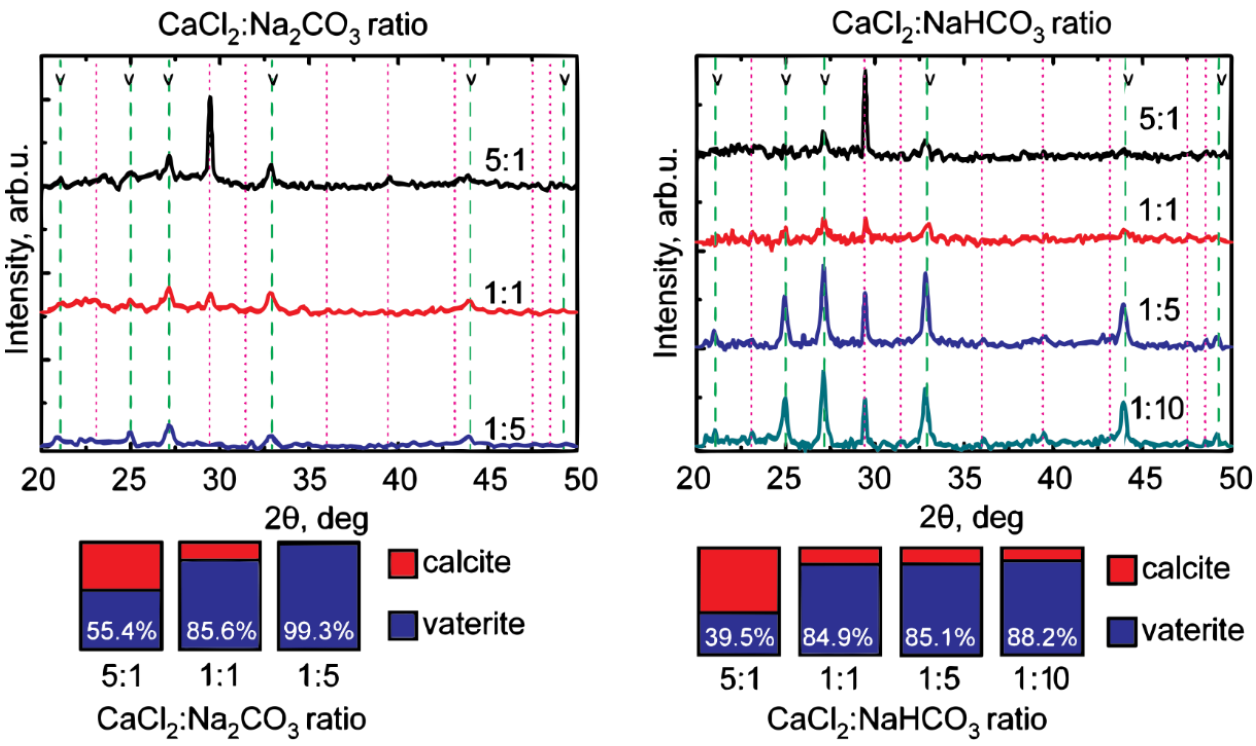

Figure S7: XRD analysis *of $CaCO_3$ samples obtained by mixing of (left) $CaCl_2$ and Na_2CO_3 at different ratios and (right) and $CaCl_2$ and $NaHCO_3$ at different ratios.*

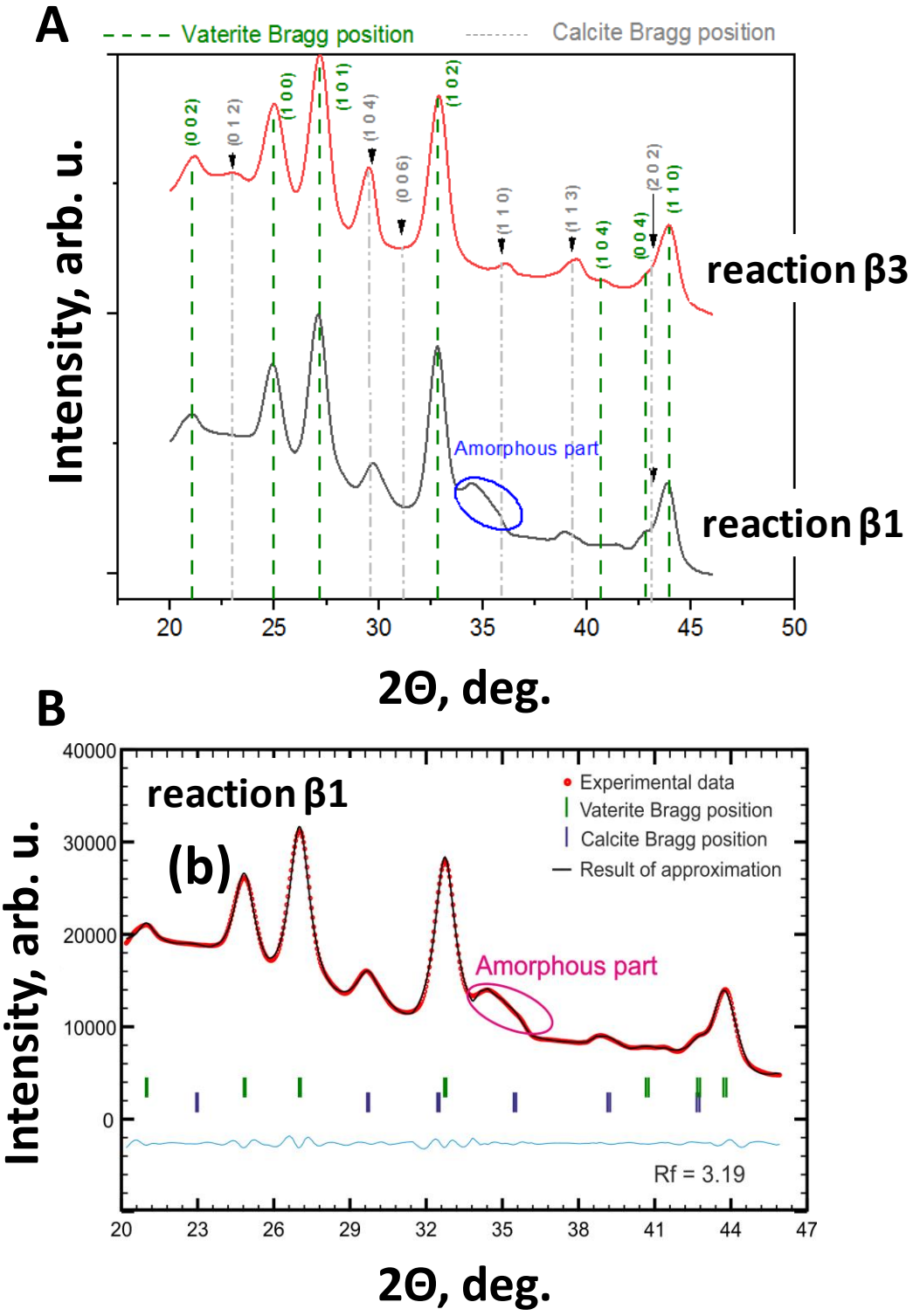

Figure S8: XRD analysis A. *XRD Θ-2Θ scans of $CaCO_3$ samples obtained in the* ***reactions β1 and β3***. ***B.*** *Example of Rietveld fit approximation of the XRD Θ-2Θ scan from $CaCO_3$ particles obtained in the* ***reaction β1****, where blue bars are calcite Bragg positions, green bars are vaterite Bragg positions, red circles are experimental data and black line is result of approximation.*

Table S2: XRD** data of $CaCO_3$ particles obtained in the **reactions δ1-δ5.

Sample	**Fraction Vaterite, %**	**Calcite Fraction, %**	**<D_v> vaterite, nm**	**<D_v> calcite, nm**	**Volume lattice vaterite,** Å^3	**Volume lattice calcite,** Å^3
reaction δ1	72.44	27.56	8.5 ± 1	5.7 ± 3	124.994	367.873
reaction δ2	58.25	41.75	15±3	14±4	126.539	373.491
reaction δ3	100	-	13.8±4	-	125.942	-
reaction δ4	100	-	15.8±3.9	-	125.054	-
reaction δ5	94.6	5.4	18.1±4.6	8.9±4	125.483	371.074

4. Scanning Electron Microscope (SEM)

To perform SEM measurements of $CaCO_3$ particles, the dispersed in ethanol particles were dropped (5μL) onto indium tin oxide (ITO) glass or n-doped silicon substrate and let dry for a few minutes. Dried particles were coated by sputtering with several nanometers of gold-palladium film. Quanta 200 FEG Environmental Scanning Electron Microscope (SEM) was used to obtain SEM images of synthesized particles.

5. Confocal Laser Scanning Microscopy (CLSM)

The fluorescence profile assay of $CaCO_3$ particles was performed with CLSM (Zeiss LSM 710 equipped with a helium-neon laser 543 nm). Images were obtained using Objective Plan-Apochromat 63x/1.40 Oil DIC objective, and the pinhole was set to 1 airy unit.

6. Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) images were collected with a Jeol JEM 1011 (Jeol, Japan) electron microscope operating at an acceleration voltage of 100 kV, and recorded with a 11 Mp fiber optical charge-coupled device (CCD) camera (GatanOrius SC-1000). For the sample preparation, 3 μL of the diluted sample was dropped onto a carbon-coated copper grid, and the solvent was removed by evaporation at room temperature.

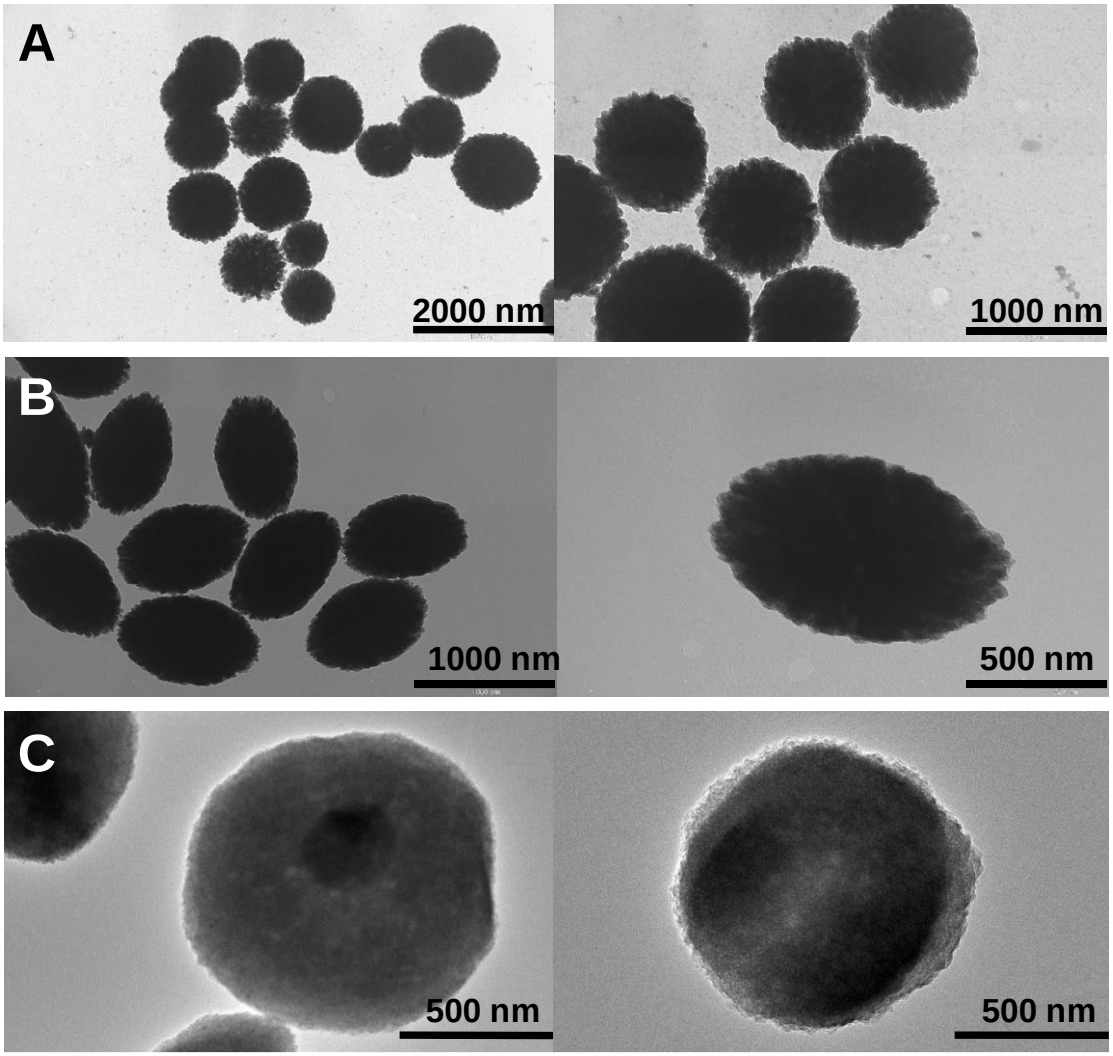

Figure S9: TEM images *of A. Spheroids, B. Ellipsoids, C. Toroids.*

7. Thermogravimetric analysis (TGA)

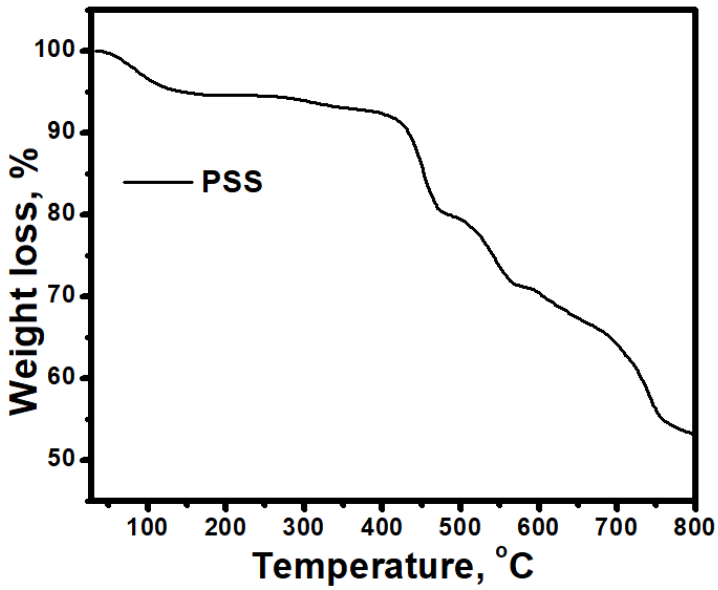

Figure S10: Thermogravimetric analysis*. TGA curve of PSS.*

8. Adsorption capacity of differently shaped $CaCO_3$ particles

The magnitude of TRITC adsorption on the surface of differently shaped (spheres, ellipses, toroids) $CaCO_3$ particles was determined as the difference between its initial and equilibrium concentrations in a water solution (pH 7.4) after contact with a sorbent from the formula:

$$Q_e = \frac{(C_o - C_{ads})V}{m}$$

where Q is the magnitude of TRITC adsorption, C_0 and C_{ads} are the initial and equilibrium concentrations of TRITC in a water solution, C is the volume of the solution, and m is the mass of the sorbent sample. The 100 µL of differently shaped particles were incubated TRITC at concentrations 0.5 mg/mL, 0.25 mg/mL, 0.12 mg/mL, 0.6 mg/mL and 0.3 mg/mL for 2 h. Afterwards, all samples were centrifuged at 10 000 rpm for 3 min, an supernatant was measured with multiplate reader (CLARIOstar®, BMG LABTECH, 488/532). The concentration of adsorbed TRITC was calculated using calibration curve (**Figure S11**). To determine mass (m) of 100 µL $CaCO_3$ particles, they were dispersed in ethanol, dried and weighted. Finally, the adsorption capacity was plotted against concentration of sorbent.

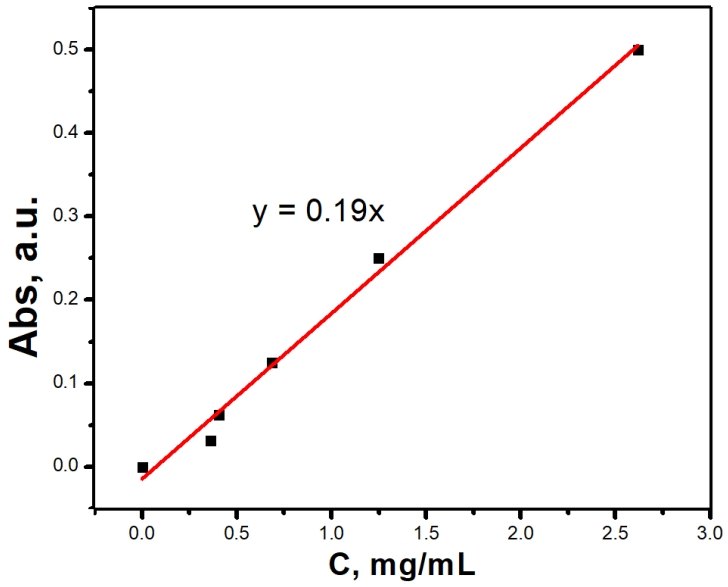

Figure S11. Calibration curve. *Absorbance of TRITC at 525 nm measured at known concentrations.*

Release studies

To study the release of TRITC from the differently shaped CaCO3 particles, 10^7 particles were shaken in water (1, 2, 4, 6, 8, 20, 24 h). Afterwards, particles were spun down at 10 000 rpm for 3 min and the absorbance spectra of supernatants were measured with multiplate reader (CLARIOstar®, BMG LABTECH). The percentage of released TRITC was then plotted versus the time of incubation.

9. Cell culture

The C6 glioma cell line was kindly provided by Dr G. Yusubalieva from the Federal Research Clinical Center of the FMBA of Russia. The C6 glioma cell line was cultured in DMEM supplemented with 10% in vol. FBS and 1% in vol. P/S and cultured at the standard conditions (37˚C, 5% CO_2).

10. Toxicity studies

To study toxicity of differently shaped $CaCO_3$ particles, LIVE/DEAD assay was used. For this, C6 glioma cells were seeded into 96-well plate at amount 10 000 cells/ well. At the same day, $CaCO_3$ particles were added to the cells at different concentrations (maximum concentration 100 particles/cell). Next day, C6 glioma cells were stained with 0.2 μM Calcein AM (live staining) and 3 μM Propidium Iodide (dead staining) for 30 min at 37˚C and 5% CO_2. Afterwards, cells were observed under confocal microscope (Carl Zeiss LSM 710). To visualize live cells (cells stained with Calcein AM), argon laser emitting at 488 nm was used. To visualize dead cells (cell nuclei stained with Propidium iodide), helium neon laser emitting at 543 nm was used. The confocal pinhole was set to 1 airy unit and images were taken with an Objective EC Plan-Neofluar 40x/1.30 Oil DIC. Images were then analyzed with FIJI open source image analysis software. Finally, cell viability was plotted versus added amount of differently shaped $CaCO_3$ particles.

11. Uptake studies

To study uptake efficiency, 150 000 C6 glioma cells were seeded onto 3.5 cm confocal culture dishes (V=1 mL). At the same day, differently shaped $CaCO_3$ particles labeled with TRITC were added to the cells at amount 10 particles/cell. Next day, the cytoskeleton and cell nuclei were stained with plalloidin conjugated with AlexaFluor 488 (AF 488) and Propidium Iodide (PI). For this, cells were washed 2 times with PBS and permeabilized with PBS supplemented with 10% vol. formalin for 5 min. Afterwards, cells were washed again with PBS and PBS containing 1 μM phalloidin-AF488 and 0.1 mg/mL PI was added to the cells. C6 glioma cells were leaved for 2 h in incubator at 37˚C and 5% CO_2. Confocal microscopy was used to evaluate $CaCO_3$ particles

uptake. For this, cells were scanned using Z-stack option. At least 50 cells per experiment was analyzed. Taking into account that the cytoskeleton was fluorescently stained with phalloidin (green), nuclei with PI (blue) and particles with TRITC (red), the red fluorescence signal surrounded with the green fluoresce was an indication for intracellular particle localization. $CaCO_3$ particles adherent to the outer cell plasma membrane could be clearly distinguished from the internalized. Based on the confocal microscopy images, the histograms showing the number of internalized particles per cell and the corresponding cumulative distribution functions were plotted.

12. Statistical data

The particle size and geometry were obtained with SEM (FEI Quanta) and further analyzed using *Scandium software*. For all synthesis protocols average sizes of particles, particles elipticity were measured. Elliptical 3D symmetry was assumed to be identical in two minor axes of the ellipsoid. Collected data were further proceeded with MATLAB software. The measurements were repeated in triplicated. Errors were calculated as standard deviation.