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An evaluation of colloidal and crystalline properties of $CaCO_3$ nanoparticles for biological applications

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ABSTRACT

Biodegradable calcium carbonate carriers are a promising and safe nanoparticle platform which might enable various applications as an engineered nanomaterial in health care, food and cosmetics. However, engineered nanoparticles can exhibit new forms of toxicity that must be carefully evaluated before being widely adopted in consumer products or novel drug delivery systems. To this end, we studied four common calcium carbonate particle systems (calcite nanoparticles, amorphous sub-micrometer and vaterite sub-micrometer and micrometer particles) and compared their behavior in biological medium and in cell culture experiments. The thermodynamically stable calcite phase is shown to maintain its morphological features as no phase transformation occurs. Size- and time-dependent phase transformation of the less stable vaterite particles are observed within 96 h in cell medium. The protein serum albumin can be an effective inhibitor of phase-transition and it is shown to improve colloidal stability. The impact of the biological environment goes beyond protein-corona formation, as we observed rapid dissolution of amorphous particles in high ionic strength cell medium, but not in Millipore water. Cellular responses of human osteoblasts against $CaCO_3$ particles indicate that increased intracellular calcium ions improve viability and that particle internalization is not size-dependent. Useful insights for designing $CaCO_3$-based delivery systems are provided and also corroborate to the idea that intrinsic material properties as well as environmental conditions are of relevance for the successful implementation of dispersed $CaCO_3$ particles in drug delivery systems and in other applications.

1. Introduction

The use of engineered nanoparticles (ENPs) in consumer products is becoming increasingly prevalent [1]. Clothing [2], cosmetic [1] and food industries [3] are just a few examples of today's use of ENPs. The particles are mainly applied as pigments and fillers, for the occlusion and delivery of active ingredients and as antimicrobial agents. Another field in which the use of nanoparticles holds great potential is nanomedicine [4,5], especially considering tumor targeting, diagnostics, imaging and treatment [6]. With the increasing use of ENPs, the necessity for nanosafety assessments is well recognized [2], as evidenced by the strong increase in the publications numbers within the past two decades [7].

As a consequence of various strategies to trick the immune system, like change of particle aspect ratio or functionalization with stealth polymers and self-peptides, these colloidal systems are able to avoid clearance [8,6,9]. Accordingly, these materials can exhibit new forms of toxicity, especially as a result of long-term accumulation in the body [10]. To avoid such undesirable effects, biodegradable and biocompatible materials are a promising option. Food and Drug Administration (FDA)-approved polymers like chitosan and poly(lactate) (PLGA) received special attention as they are well tolerated by the body [9]. Another alternative is the use of endogenous materials, like calcium phosphate, calcium carbonate and phosphosilicates [11]. Calcium carbonate ($CaCO_3$) holds great potential, as it is highly pH-sensitive and dissociates into calcium and carbonate ions, which already are ubiquitous in the body.

$CaCO_3$ exists in six different crystal morphologies: the naturally occurring calcite, aragonite, vaterite and amorphous calcium carbonate (ACC) phase and the highly unstable ikaite and monohydrocalcite. Only calcite is thermodynamically stable, while aragonite and vaterite are slightly unstable and tend to transform into calcite in the presence of water/humidity [12–14]. The same is true for amorphous calcium carbonate which in nature often occurs as a precursor to the more stable polymorphs [15]. All $CaCO_3$ polymorphs have a certain solubility in water which is determined by the solubility product and scales with the thermodynamic stability of each crystal phase [16]. This instability is more pronounced at the nanoscale, at which solubility and reactivity increases due to an enhanced surface to volume ratio [17]. Additionally, the dissolution rate is known to be highly dependent on the intrinsic

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nature of the particles (composition and crystal phase) [15] as well as on the environmental conditions (pH, presence of proteins and calcium-chelating moieties, ionic strength) [18–20]. Moreover, the dissolution of nanoparticles in biological medium can impact the biological systems interacting with the material, potentially changing the toxicity behavior from nano to ion-related [7]. Given that, to properly design $CaCO_3$-based drug delivery systems (DDS) or similar nanostructures with dispersed $CaCO_3$ particles considering particle solubility in the respective media is critical. Furthermore, colloidal stability and consequently biological availability of $CaCO_3$ particles could be influenced by their crystal phase and morphology as a consequence of different surface properties of the nanomaterials including their interactions with media biomolecules.

As a result of the detailed and inspired studies of $CaCO_3$ biomineralization, a vast number of publications report the design of $CaCO_3$ particles for drug delivery. However, just for a fraction of these publications in vitro or in vivo studies were performed [21–65]. The latter studies evaluated different aspects of the $CaCO_3$-cellular interactions: intracellular behavior [36,65], pH modulation [37], bubble generation [34,35,53], bone remodeling [24], photothermal therapy [22,28,43,49], drug/protein delivery [21,23,25–27,29–33,38,41,42,46–48,50–52,60–62], gene transfection [44,45,54–59], cellular uptake [63], toxicity [40,65] and bioavailability studies [39,64,65]. Among these publications, crystalline particles like calcite, vaterite and aragonite [38] phases have been utilized. These particle-systems varied in size from bulk material, to micrometer (1–1.5 μm), sub-micrometer (150–800 nm) and nanometer (30–100 nm) particles. In the case of amorphous carriers, the range of particle size was narrower, from 100 to 230 nm [44–49]. In some cases, the interplay of both amorphous and crystalline $CaCO_3$ phases was also reported [24,41–43]. In others, the crystallinity of the particles was not described [50–65]. Moreover, the aforementioned systems were tested with different cell culture media, incubation periods and cell lines. While most studies report viable nanoparticle systems in their own context, comparing the results is a challenging task. The lack of standardization when assessing biological responses [66] along with the incomplete information on the colloidal characterization makes it difficult to generalize the results, especially considering the real-live performance of particle-based drug delivery systems.

With the above discussion in mind, we designed a study that would allow us to compare the biological behavior of different $CaCO_3$ systems with varied crystallinity, solubility and colloidal properties. To this end, we analyzed the material properties of four different types of $CaCO_3$ particles commonly described in the literature as potential drug delivery systems and investigated their biological behavior against human osteoblasts in the Dulbecco's Modified Eagle's Medium (DMEM). Here, we focused on the changes in material properties in the cell medium and on the impact of the particles on the cells. For our study, we chose calcite, ACC particles and vaterite particles in the range of 90 nm to 1 μm which we consider representative in regards to polymorphism and size of those described in the literature as potential drug delivery systems.

2. Materials

Calcium chloride ($CaCl_2$ dihydrate, purity > 96%), sodium carbonate (Na_2CO_3, purity > 99.5%), poly(acrylic acid) sodium salt (PAA, M_w 8000 g/mol, 45 wt% in water), bovine serum albumin (BSA, lyophilized powder), glycerol (Gly, purity > 99.5%), sodium hydrogencarbonate ($NaHCO_3$, purity > 99.5%), ethylene glycol (EG, purity ≥ 99%) were purchased from Sigma-Aldrich and used without further purification. The experiments were performed using double deionized water with a conductivity of 0.04 mS cm^{-1} from Synergy (Millipore, Darmstadt, Germany).

Cell culture tests were carried out on human osteoblast cells (HOB, lot no. 232R020412 obtained from Provitro - Germany). Dulbecco's Vogt modified Eagle's minimal essential medium (DMEM, high glucose), antibiotic-antimycotic (AB/AM), Alexa Fluor 488 phalloidin (AF488) were obtained from Invitrogen (Germany). Fetal calf serum (FCS), phosphate buffered saline (PBS), trypsin-ethylenediaminetetraacetic acid (0.25% trypsin, 0.02% EDTA), Triton X-100, 4′,6-diamidino-2-phenylindole (DAPI, 0.5 μg/mL), paraformaldehyde (PFA, 95.0–100.5%), osmium tetroxide solution (OsO_4, 4% in water), glutaraldehyde solution (Grade 1, 25% in water) and an epoxy embedding medium kit (Epon 812 substitute) were purchased from Sigma-Aldrich (Germany). The water-soluble tetrazolium salt (WST-1) cell toxicity assay (Roche Diagnostics GmbH, Germany) and the lactate dehydrogenase (LDH) Pierce assay (Thermo Scientific, Germany) were directly purchased from the suppliers.

2.1. Synthesis of crystalline and amorphous nanoparticles

Calcite nanoparticles, purchased from PlasmaChem GmbH (Berlin, Germany) are produced by a sol-gel process and used as purchased. Amorphous particles were prepared as previously described [67]. In short, 10 mL aqueous solution with a final concentration of 1.9 g/L of PAA and 12 mM of $CaCl_2$ was prepared. To mineralize the particles, an aqueous solution of Na_2CO_3 was added to the reach the final concentration of 12 mM. The suspension was centrifuged at 5000 rpm for 10 min, the supernatant discarded and particles dried in an oven at 70 °C for 1 h.

Vaterite particles were prepared in a similar way as previously reported [37,68–70]. Micrometer particles were synthesized in a water:EG solution (1:6, v/v). First, 207 μL of 2 M $CaCl_2$ aqueous solution was first added to 4.15 mL of EG solution, stirred for 5 min and followed by 415 μL of 1 M $NaHCO_3$ aqueous solution and 227 μL of water. The final concentration of carbonate was kept equimolar at 83 mM. The solution turned turbid after 5 min, indicating the formation of particles. After 1 h stirring at 500 rpm, 30 mL of 100% ethanol was added to the suspension. Particles were collected by centrifugation at 5000 rpm for 10 min. This washing step was repeated twice and the collected particles were dried at 70 °C for 2 h. Sub-micrometer particles were prepared in a similar fashion but with a few modifications. The 1:6 water:EG solution was substituted by water:EG:Gly 1:1.25:3.7 in volume. Both $CaCl_2$ and $NaHCO_3$ aqueous solutions were added to a 4.15 mL solution containing 1.04 mL of EG and 3.11 mL of Gly. The final concentration of calcium and carbonate was kept equimolar at 83 mM. The solution remained transparent for about 1 h. The dispersion turned turbid after this period. Particles were collected by centrifugation after 2 h 30min, followed by washing the particles twice in 100% ethanol and drying at 70 °C for 2 h. For experiments without BSA-stabilization, the particles were dispersed in the respective medium in an ultrasound bath for 10 min.

2.2. Particle-stabilization by BSA

Dried particles were dispersed in 0.2% BSA in Millipore water to prepare 1.2 g/L particle concentration. The suspension was ultrasonicated for 15 min to ensure that no aggregates remained in solution and to fully coat the particles. Afterwards, it was centrifuged for 10 min at 5000 rpm and dried at 70 °C for 1 h.

2.3. Calcium-ion release from nanoparticles

The calcium content in the supernatant was quantified using the o-cres-olphthalein complexone colorimetric kit (Fluitest CA-CPC). Briefly, an amount of dried particles were added to the respective medium to prepare a particle concentration of 1.2 g/L. The suspension was serially diluted in the same medium to prepare 0.6, 0.3, 0.15 and 0.075 g/L. The suspensions were centrifuged for 10 min at 5000 rpm. 10 μL of the supernatant was added to a 96-well plate containing 50 μL of reaction mixture 1 (reagent obtained in the kit). 50 μL of reaction mixture 2 was added and the well plate was shaken for 30 s to allow homogeneous mixing. The plate was incubated for 10 min at room temperature (RT) and the absorbance was measured at 570 nm with plate reader.