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Research paper

Detection and dissolution of needle-like hydroxyapatite nanomaterials in infant formula

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ABSTRACT

The unknowns surrounding presence, composition and transformations during the use phase of engineered nanoparticles (ENPs) in consumer products raises potential human and environmental health concerns and public discourse. This research developed evidence and confirmatory analytical methods to determine the presence and composition of ENPs in a consumer product with a complex organic matrix (six different infant formula samples). Nano-scale crystalline needle-shaped hydroxyapatite (HA; appx. 25 nm × 150 nm) primary particles, present as aggregates (0.3–2 μm), were detected in half the samples. This is the first report of these ENPs in infant formula. Dissolution experiments with needle-shaped HA were conducted to assess potential transformations of nano-HA particles. Rapid dissolution of needle-shaped HA occurred only under lower pH conditions present in simulated biological fluids (acidic gastric fluids), but not in simulated drinking water (near-neutral pH). Other non-nanosized HA minerals exhibited less dissolution under the same low pH conditions. This work demonstrates the occurrence of engineered nanomaterials in the food supply of a sensitive population (infants) and the need to consider transformations in nanomaterials that occur during use, which result in different exposures between pristine/as-produced ENPs and nanomaterials after passing through the human gut.

1. Introduction

Many minerals exist in both natural and engineered nanoparticle (ENP) forms. While the occurrence of naturally occurring nanoparticles (e.g., hematite, hydroxyapatite) is well recognized in natural systems, the environmental behavior of ENPs raises new regulatory and health concerns. These concerns primarily stem from existing knowledge gaps in understanding the ENP risks, which could be summarized in two categories: (1) discovering where ENPs are used in commerce and hence might enter the environment, and (2) elucidating ENP transformations from pristine materials, to synthesis in the lab or factory, and through use and end-of-life phases. We and others have previously shown that silicon- and titanium-oxide ENPs exist in foods, are ingested by humans, and pass through wastewater treatment plants, which results in their release to surface waters and terrestrial systems where sewage solids are land applied (Kiser et al., 2010; Kiser et al., 2009; Keller and Lazareva, 2014; Mueller and Nowack, 2008; Piccinno et al., 2012; Gottschalk et al., 2013; Reed et al., 2012; Robichaud et al., 2009). These two ENPs undergo little dissolution (i.e., transformation) during this process, which differs from antimicrobials like silver, copper, or zinc nanomaterials (Kaegi et al., 2013; Thalmann et al., 2014; Conway et al., 2015; Hong et al., 2015).

Calcium phosphate minerals are an example of solids present in nature and used in environmental remediation/treatment processes (Lenton et al., 2015; Miretzky and Fernandez-Cirelli, 2008; Piccoli and Candela, 1994; Vance et al., 2015; de- Bashan and Bashan, 2004; Wiesner et al., 2011) or human nutritional supplements. Intentional formation of calcium phosphate is used to immobilize heavy metals in soil (Boisson et al., 1999; Fuller, 2002), remove fluoride from water to protect public health, (Fan et al., 2003) or remove phosphate from wastewaters to limit the eutrophication potential of wastewater discharges (de- Bashan and Bashan, 2004). Calcium phosphate, also referred as tricalcium phosphate (TCP), is used as a leavening agent in foods, a polishing material in toothpaste, an antioxidant activity promoter and texture stabilizer in canned vegetables, a firming agent or to avoid formation of clumps in food. Hydroxyapatite (HA; $Ca_5(PO_4)_3$ or $Ca_5(PO_4)_3(OH)$) is a common form of calcium phosphate. Many people take calcium supplements, including calcium carbonate, calcium citrate and hydroxyapatite forms, but the literature is mixed on which form leads to greater bioavailable calcium for health bone development (Straub, 2007; Ruegsegger et al., 1995). In other applications, nanoforms of calcium minerals have raised concern. For example, the European Union Scientific Committee on Consumer Safety 2015 opinion on nano-HA states that the safety of its use in oral and cosmetic products

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cannot be currently decided due to limitations in available data, including the exact size, shape and crystallinity of the nano-HA, but that the available information indicates nano-HA in needle form is potentially toxic when used in dermally-applied cosmetic products (SCCS, 2015).

Calcium is an essential element for all biological organisms, and is widely used in human food supplements. For example, infant formula is intended to be the sole nutrition source for infants for the first 12 months. Although regulations (e.g. 21 CFR 107.100 in the USA) stipulate the elements required in the infant formula, they lack guidance on the type or size of the compounds used to provide the nutrients. Regulations refer to HA as generally regarded as safe (GRAS); however, new bottom up manufacturing processes that create nanomaterials compared to top down processes create new concerns if the GRAS status applies. Given potential toxicity concerns raised in the EU on nano-needle-shaped hydroxyapatite in products intended for human use, the need for infants to have calcium and other elements (P, Fe) in their diets, and potential transformations for HA under different pH conditions, we undertook a study to separate and identify HA and other nanomaterials in powdered infant formulas. This challenging work with infant formulas that contain salts, sparingly soluble minerals, fats and other components is a precursor to understanding the occurrence and role of nano-scale HA minerals in complex environmental matrices (soil, biota, and water).

To identify initially unknown nanomaterials in infant formula, samples were separated by centrifugation after dispersing powders in water and then analyzed by transmission electron microscopy (TEM) with energy dispersive X-ray spectroscopy (EDS) and X-ray diffraction (XRD). Findings from these samples were compared against reference calcium phosphate materials. We focused on HA because it was found in three out of six samples, although it has not yet been widely considered by the health and safety exposure community as a risk in the food supply system. Within a complex food matrix, HA nanoparticles are difficult to be detected using conventional analytical paradigms. A secondary focus was the dissolution of HA in synthetic biological fluids to explore potential transformation in human body of these nano- and micron-sized minerals. Because the intended function of calcium phosphate in infant formula is to promote nutrient uptake, we used aqueous matrices representing simple drinking water and simulated gastric fluids. Understanding nanomaterial transformations during their intended use emerges as a critical discussion and conclusion point around the benefits of using nanotechnology (e.g., rapid dissolution of HA to deliver calcium and phosphate ions).

2. Materials and methods

2.1. Chemicals

Six infant formulas from different companies (Gerber, Similac, Enfamil, and Well Beginnings) were purchased in the United States and identified, for confidentiality, as S1–S6. Samples S1–S5 were dry powders, and S6 was a liquid concentrate. Dry powders and a liquid concentrate were chosen to compare suspected different additives used for each product. Three reference powder samples of food-grade calcium phosphate, labeled as hydroxyapatite, were procured from three different vendors. Samples R1 (American Elemental) and R2 (Hebei Shunye Import and Export Limited Company) were labeled as 99% pure and containing needle-like nano-HA. Sample R3 (NOW Foods) was an HA supplement provided in a gelatin pill capsule; only the contents of an opened capsule were used in analysis and dissolution tests.

2.2. Electron microscopy analysis

Infant formula (0.15 g) samples S1–6 and HA reference samples R1–3 were suspended in 40 mL ultrapure water (18.2 MΩ cm, Nanopure Infinity, Barnstead) and sonicated (80 W/L, Branson Ultrasonic Bath, Emerson) for 30 min to disperse particles. This mass to liquid ratio was used to parallel work other food samples analyzed by our group (Yang et al., 2014a; Yang et al., 2016a). Additional electron microscopy experiments were conducted at solid to liquid ratios based upon recommended sample preparation on the infant formula packaging, and showed no dependence of outcomes on solid to liquid ratios. Other detailed control and validation experiments are summarized in Table SI.4 and described in the Results section.

Step-by-step description of sample preparation of electron microscopy samples are summarized in Figs. SI.2 through SI.5. Briefly, samples in 50 mL vials were centrifuged at $F = 14.000$ G for 15 min. The organics-rich supernatant was poured off, leaving a pellet of particulate matter at the bottom of the centrifuge tube. The pellet was re-suspended in 20 mL ultrapure water and inverted by hand for 30 s, then 50 μL volumes were pipetted onto a copper/lacey carbon transmission electron microscopy (TEM) grid and allowed to air-dry overnight. Microscopy was performed on a Philips CM200 HR-TEM with energy dispersive X-ray spectroscopy (EDS). To confirm HA was not an artifact from sample preparation, a pure powder reference sample of HA was procured, deposited on a SEM stub (Fig. SI.3) and directly analyzed as a powder by scanning electron microscopy (SEM; FEG XL30 ESEM with EDS system) with energy dispersive spectroscopy. Mean particle diameter, particle size distributions, and cumulative distribution below 100 nm were determined by manually measuring the particles sizes of 250 particles from the images using ImageJ software and conducting statistical analysis.

2.3. Sample preparation for confirmation and quantification of hydroxyapatite

Fig. SI.6 provides a step-by-step description of sample preparation. To determine the relative amount of hydroxyapatite nanoparticles in infant formula, 10 g of each formula sample (six in total) was weighed into 50 mL centrifuge tubes with 40 mL of ultrapure water (18.2 MΩ cm, Nanopure Infinity, Barnstead). The mixed samples were then centrifuged for 20 min at $F = 14.000$ G to separate lighter components. The pellet collected at the bottom of centrifuges was washed three additional times with UP water. The washed pellet was freeze-dried under vacuum for 48 h (FreeZone Freeze Dry System, Labconco), weighed, and compared with the weight of starting material to calculate the relative concentration of collected minerals. The mineral phases of pellets and reference powders were prepared (Fig. SI.7) and analyzed using powder X-ray diffraction (pXRD) using a Siemens D5000 diffractometer with a monochromated Cu–Kα radiation at 40 kV and 30 mA. Each sample was scanned at 2θ values from 10° to 70° to collect diffractograms, which were compared with the diffraction patterns of standard materials in ICDD database.

2.4. Dissolution experiments using hydroxyapatite in aqueous media

Ultrapure water and simulated biological fluids were used to examine the dissolution potential of the two reference HA and calcium bioavailability after ingestion. A detailed procedure is outlined in Fig. SI.1. A Fed-State Gastric Fluid (Fed-SGF, pH 5.0) and a Fasted-State Gastric Fluid (Fast-SGF, pH ~ 1.6) were prepared following recipes reported previously (Marques et al., 2011) and detailed in Table SI.1. For HA dissolution, 40 mL of the media was placed in 50 mL plastic centrifuge vials followed by the addition of 8 mg of reference HA to achieve a final concentration of 200 mg/L. The HA concentration was chosen to represent the serving size of HA per serving of infant formula. Immediately after mixing HA with simulated media, the suspensions were placed on a rotational shaker (45 rpm). The fed-state gastric fluid and fasted-state gastric fluid were rotated for 2 h to mimic the average contact time of food in the human stomach (Marques et al., 2011). Within 5 min of the completion of mixing, 15 mL of each suspension was filtered through 30 kDa centrifugal ultrafilters (NMWL = 30 K Da, ultracel