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Impact of an artificial digestion procedure on aluminum-containing nanomaterials

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nanoparticles, aluminum, artificial digestion, gastrointestinal tract, agglomeration, ion release, *de novo* emerging of particles

Abstract

Aluminum has gathered toxicological attention due to human exposure and its suspected hazardous potential. Nanoparticles from food supplements or food contact materials may reach the human gastrointestinal tract. Here, we investigated the physico-chemical fate of aluminum-containing nanoparticles and aluminum ions during *in vitro*-digestion throughout the main stages of the human gastrointestinal tract.

Small-angle X-ray scattering (SAXS), transmission electron microscopy (TEM), ion beam microscopy (IBM), secondary ion beam mass spectrometry (TOF-SIMS), and inductively coupled plasma mass spectrometry (ICP-MS) in the single-particle mode were employed to characterize two aluminum-containing nanomaterials with different particle core materials (Al^0 , $\gamma\text{Al}_2\text{O}_3$) and soluble AlCl_3 .

Particle size and shape remained unchanged in saliva, whereas strong agglomeration of both aluminum nanoparticle species was observed at low pH in gastric fluid together with an increased ion release. The levels of free aluminum ions decreased in intestinal fluid and the particles de-agglomerated, thus liberating primary particles again. Dissolution of nanoparticles was limited and substantial changes of their shape and size were not detected. The amounts of particle-associated phosphorus, chlorine, potassium and calcium increased in intestinal fluid, as compared to nanoparticles in standard dispersion. Interestingly, nanoparticles were found in the intestinal fluid after addition of ionic aluminum.

We provide a comprehensive characterization of the fate of aluminum nanoparticles in the simulated gastrointestinal fluids, demonstrating that orally ingested nanoparticles probably reach the intestinal epithelium. The balance between dissolution and *de novo* complex formation should be considered when evaluating nanotoxicological experiments.

Introduction

Aluminum is the most common metal in the biosphere and therefore ubiquitous present in food and consumer products ¹. However, no essential physiological role of aluminum is known, possibly due to its inflexible trivalent oxidation state and its relatively low reactivity ²⁻³. Most aluminum on earth is bound in minerals that are in a chemically inactive state ⁴. In the last two centuries more and more aluminum was transferred into the metallic and in the more reactive ionic form, due to industrial activities and acidification of the environment ⁵⁻⁶. Activation seems to be triggered by acidic pH ⁴. Chronic exposure to aluminum can be harmful for certain groups of people, for example for those with renal dysfunction ⁷. The suspected hazardous potential of aluminum on human health recently led to an increasing attentiveness on this topic, as a correlation between the use of aluminum-containing products and Alzheimer's disease or breast cancer has been proposed ⁸⁻⁹.

Oral ingestion is an important uptake route for aluminum. Exposure might result from natural sources, such as drinking water, but also from food additives, packaging and kitchenware ³. The use of aluminum-containing packaging, consumer products and kitchenware has increased ¹⁰, as well as the use of chemical solvents, leaches and acids. Several metal species are present in a significant amount in food as nano-scaled particles ¹¹⁻¹² and migrate into food from packaging material ¹³⁻¹⁵. Like other orally ingested metals, aluminum nanoparticles overcome the different compartments of the human digestion tract. During this process, the chemical environment changes severely from mouth to stomach and intestine with regard to shifts in pH and the presence of complex mixture of salts, proteins and intestinal bile acids with surface-active properties. These changes may induce nanoparticle modifications including dissolution, agglomeration and deagglomeration and so affect intestinal uptake which differs significantly between dissolved ions and nanoparticles depending on their size, shape and physicochemical properties ¹⁶. These properties include surface coating, protein corona composition, and biological environment ¹⁷. Therefore, it is crucial to characterize nanoparticles under realistic conditions.

To mimic these conditions, different modifications of physiologically buffered fluids have been applied in research, with some of them using buffered solutions with only pH changes ¹⁸, whilst others use more complex systems which include salts, digestion enzymes, proteins or other food components ¹⁹⁻²⁰. Moreover, such models with higher complexity are appropriate to observe changes in the physicochemical characteristics of metallic nanoparticles and also enable detailed studies on the toxicological potential of particles following intestinal digestion ²¹.

The chemical identity of aluminum is an important factor for its toxicological potential. This study focuses on the fate and behavior of different aluminum species during the digestion

process after oral uptake. Therefore, three different aluminum species were used which represent soluble ionic Aluminum (AlCl_3), elementary metallic aluminum (Al^0) and mineral oxidized aluminum (Al_2O_3). These three representative aluminum entities were analyzed separately in a complex artificial digestion system consisting of three steps, namely saliva, gastric juice, and intestinal juice. Differences in ionic content, particle size, shape, element attachment, agglomeration state and stability were investigated using elemental analysis, small angle x-ray scattering (SAXS), transmission electron microscopy (TEM), single particle inductively coupled plasma mass spectrometry (SP-ICP-MS), ion beam microscopy (IBM) and time of flight secondary ion mass spectrometry (ToF-SIMS).

Experimental Section

Chemicals and nanoparticles

Chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany), or Carl Roth (Karlsruhe, Germany) in the highest available purity.

Nanomaterials (Al^0 -core surface-passivated nanoparticles and $\gamma\text{-Al}_2\text{O}_3$ nanoparticles) were supplied by IoLiTec. Al^0 nanoparticles were stored and weighted under an argon atmosphere. Both particles were freshly dispersed at a concentration of 2.56 mg/ml according to the modified NanoGenoTOX protocol (ultrasonication applying an energy of 1176 kJ/ml dispersion using an acoustic power of 7.35 W), stabilized by 0.05% BSA/water before use. BSA was supplied by Carl Roth (Albumin Fraction V, $\geq 98\%$) and AlCl_3 was supplied by Sigma Aldrich (Hexahydrate, $\geq 97\%$).

Artificial *in vitro* digestion

Artificial *in vitro* digestion was originally based on DIN ISO 19738 and distinctly modified for scientific investigations on metals, metallic nanoparticles¹⁹⁻²⁴, and other nanoparticles and biopolymers²⁵⁻²⁶. As described in Figure 1, the artificial *in vitro* digestion consists of three steps with the described composition. Before starting the digestion process, nanomaterials were freshly dispersed via ultrasonication in saliva and 0.05% BSA before addition of digestion enzymes. As a control, ionic aluminum (AlCl_3) was used in the same concentration ranges and treated accordingly. Then, 28 mL of synthetic saliva with the corresponding samples were heated to 37 °C in a water bath and stirred for 5 min. Subsequently, a 10 mL sample was taken for further analysis, 42 mL of artificial gastric juice were added to the solution, and the pH value was set to 2 using hydrochloric acid. The solution was stirred for 2 h at 37 °C and the pH value was monitored every 30 min. Prior to the intestinal step, a 10 mL sample was taken for further analysis. Then, 50 mL of artificial intestinal juice were added,

the pH value was set to 7.5 by adding sodium bicarbonate powder to the reaction solution, and the solution was stirred for 2 more hours. Subsequently, intestinal samples were taken for further analysis.

The activity of the digestion enzymes was verified prior to every set of experiments using distinct control substrates for each step of the digestion process. Amylase activity was confirmed using amylopectin azure, pepsin activity by using an albumin/bromophenol blue complex, tryptic activity by using azocasein, and lipase activity by using 4-methylumbelliferyl oleate as substrates, respectively. All resulting cleavage products were photometrically monitored. In that way we could prove that all enzymes remained functional during the experimental steps.

Transmission Electron Microscopy (TEM)

A drop of each digested or undigested sample was placed on a formvar carbon-coated 300 mesh grid for 20s for adsorption. Excess fluid was wicked off using a filter paper before grids were air-dried. All grids were examined with a JEOL 1400 transmission electron microscope (JEOL, Peabody MA, USA) operated at 120 kV and supplied with a GATAN Orius 1000 camera (GATAN Inc., Pleasanton CA, USA).

Small-angle X-ray scattering (SAXS)

SAXS measurements were conducted in a flow-through capillary with a Kratky-type instrument (SAXSess from Anton Paar AG, Graz, Austria) at 21 ± 1 °C. The SAXSess has a low sample-detector-distance of 0.309 m which is appropriate for the investigation of dispersions with low scattering intensities. The experiments were performed with 120 measurement cycles (each averaged over 10 s). The measurements were background-corrected with the respective mixture of aqueous BSA solution or digestive juices without addition of aluminum species. Deconvolution (slit length desmearing) of the SAXS curves was performed with the SAXS-Quant software (Anton Paar AG). Samples analyzed with SAXS were used as prepared. Curve fitting was performed with the software McSAS (Monte Carlo method, version 1.0.1). This procedure was described before ²⁷.

Single-particle inductively coupled plasma mass spectrometry (SP-ICP-MS)

For single particle analysis of the nanoparticle solutions a quadrupole ICP-MS (Thermo Scientific iCAP Q, Thermo Fisher Scientific GmbH, Dreieich, Germany) with a PFA ST Nebulizer, a quartz cyclonic spray chamber and a 2.5 mm quartz O-ring-free injector (all from

ESI Elemental Service & Instruments GmbH, Mainz, Germany) was used. Using the time-resolved analysis mode for data acquisition intensities as a function of time (counts per dwell-time interval) were collected. The acquisition time for each run was set to 60 s with a dwell time (or data acquisition rate) of 3 ms. The gas flow for the plasma, the nebulizer and the auxiliary (all Ar) were set to 13 L/min, 0.89 L/min and 0.7 L/min. The flow rate of the sample was 0.34 mL/min. Data were exported to a spreadsheet developed by RIKILT (Imperial Quality Control of Agricultural and Horticultural Products for further processing, University of Wageningen, Netherlands). For data processing an established procedure according to Pace *et al.*²⁸ was followed. Determination of nebulizer efficiency was performed according to the described method with reference nanoparticles of known particle size. 60 nm gold reference nanoparticles from the U.S. National Institute of Standards and Technology (NIST, RM 8013) were used as reference nanoparticles.

Ion beam microscopy (IBM)

IBM experiments were performed at LIPSION© nanoprobe. The singletron™ particle accelerator was used to apply a 2.25 MeV proton beam according to a previously described protocol²⁹. To avoid interactions between the ion beam and air molecules a vacuum with a pressure of 5×10^{-5} and 10^{-7} Torr was applied. By focusing the beam, a spatial resolution of around 1 μm was reached. For element analysis we used the X-ray fluorescence technique μ proton-induced X-ray emission (μPIXE) and μ Rutherford Backscattering spectroscopy (μRBS). Detection of μPIXE signals was done by a High Purity Germanium crystal detector (Canberra, Meriden, CT, US). A 60 μm polyethylene layer was used to cover the detector for backscattered protons. A Canberra PIPS-detector was used to detect the μRBS signal. For element analysis the standard dispersion as well as gastric fluid was investigated. The concentration of the nanoparticles in the digestion fluid was set to 1000 μg aluminum/ml. Samples were prepared for measurements by centrifugation at 8,000 $\times g$ for 10 min. Afterwards the supernatant was removed and replaced by mpH_2O followed by vortexing of the sample. This procedure was repeated three times. Finally a small drop of the dispersion was placed on polypropylene foil and the liquid was vaporized.

Time of flight secondary ion mass spectrometry (ToF-SIMS)

10 μL of digested samples were dropped on gold wafers and air-dried. Ion images and spectra were acquired as described before³⁰ using a ToF-SIMS V instrument (ION-TOF GmbH, Münster, Germany) with a 30 keV nano-bismuth primary ion beam source ($[\text{Bi}]x^{(y+)-}$ cluster ion source with a BiMn emitter). The ion currents were 0.5 pA at 5 kHz using a Faraday cup. A pulse of 0.7 ns from the bunching system resulted in a mass resolution that

usually exceeded 9000 (full width at half-maximum) at $m/z < 500$ in positive ion mode. The primary ion dose was controlled below 10^{12} ions cm^{-2} to ensure static SIMS conditions. Charge compensation on the sample was obtained by a pulsed electron flood gun with 20 eV electrons.

The primary ion gun scanned a field of view of $80 \mu\text{m} \times 80 \mu\text{m}$ applying a 512×512 pixel measurement raster. Once the primary ion gun was aligned, a ToF-SIMS mass spectrum was generated by summing the detected secondary ion intensities and plotting them against the mass channels. The data were evaluated using the Surface Lab software (ION-TOF GmbH, Münster, Germany).

Ion release measurements

Ion release of nanoparticles in stock dispersions and digestion fluids was determined by ultracentrifugation ($100,000 \times g$ for 1h at 4°C) followed by acidic hydrolysis of the supernatant (69% HNO_3 , 180°C for 20 min in an MLS-ETHOS Microwave system) and element analysis was conducted using a quadrupole ICP-MS (Thermo Scientific iCAP Q, Thermo Fisher Scientific GmbH) comparable to previous studies³¹. LOD and LOQ for Al were determined as 0.6 respectively 1.8 ppB. Results are given as percentile of the initially used aluminum amount.

Results and Discussion

Aluminum is one of the most abundant metals on earth, occurs in our food and is therefore also taken up orally. The chemical identity of aluminum is an important factor for its bioavailability and toxicological potential^{3, 32}. Therefore, this study focused on the behavior of different aluminum species during the digestion process after oral uptake. Three different aluminum species were used, which represent elementary metallic aluminum, mineral oxidized aluminum, and completely dissolved ionic aluminum. These three representative substances vary strongly in their physicochemical properties including reactivity, solubility and bioavailability^{2, 33} and therefore were analyzed stepwise throughout the digestion procedure (before digestion, in saliva, stomach fluid, and intestinal fluid). Each step is characterized by typical compositions of buffer, salts, protein components and pH values. Differences in ionic content, particle size, shape, element attachment, agglomeration and stability were analyzed using complementary techniques: Element analysis, SAXS, TEM, SP-ICP-MS, ToF-SIMS and IBM.

Shape of the nanoparticles

TEM analysis of undigested nanoparticles in Figure 2 shows polydisperse spherical particles with diameters between 10 and 100 nm with prominent finger-like grow outs for Al^0 and needle-like nanoparticles with about 5 x 30 nm size in loosely packed agglomerates for Al_2O_3 , whereas no particles were detectable in AlCl_3 solution. During the digestion process both nanoparticle species appeared to agglomerate and to be surrounded by organic material, while their size range has not substantially changed. However, it has to be kept in mind that preparation for TEM analysis may cause agglomeration due to the necessary drying step. In the intestinal fluids, deagglomeration was observed. For aluminum ions, nanoparticle-like structures with different densities were observed in intestinal fluid that were not detectable in undigested samples, saliva, or gastric fluid.

Size distribution and agglomeration of the nanoparticles

SAXS results are shown in Figure 3 and Supplementary Figure 1. Both nanoparticle species displayed different agglomeration characteristics in undigested dispersion. Elementary Al^0 nanoparticles display a broad size distribution with primary particle radii ≥ 8 nm. In contrast Al_2O_3 nanoparticles show a more narrow size distribution of primary particles with core radii between 5 and 10 nm (Figure 3B,C). Populations with higher radii resulted from agglomerates and aggregates of these primary particles, as proven by TEM (Figure 2) and represented either aluminum nanoparticles or Al_2O_3 -nanoparticles depending on the nanoparticle species used, as proven by ToF-SIMS (see Figure 6). Aluminum ions formed no detectable particles in undigested stocks. In comparison to the undigested dispersions, the SAXS core radii for both aluminum species are not notably changing by the transfer in saliva (Figure 3E,F). Also, no nanoparticles were detected for ionic aluminum samples (Figure 3D). In contrast to the saliva, the next steps of the digestion procedure strongly influence the agglomeration behavior of the nanoparticles. Especially in gastric juice at low pH, SAXS measurements showed an increased mean radius (Supplementary Figure 1). Moreover, this effect was most prominent at the lowest concentration of Al_2O_3 nanoparticles. At the next digestion step, the intestine, the pH value is shifted to 7.5. There, deagglomeration occurs resulting in primary particles in the nano-scaled range. The core radii were now in the range of the original state as found in the saliva. Surprisingly, the ionic aluminum samples also showed detectable nano-scaled particles in the last step of the digestion process (Figure 3J) which could be attributed to aluminum particles using ToF-SIMS analysis (see Figure 6G).

As a second method to determine size distributions, SP-ICP-MS measurements were performed (Figure 4). Both particles species tended to stay unaffected in the saliva while in the stomach only a small fraction of nanoparticles still remained in the nano-scaled range. Especially for Al_2O_3 nanoparticles, the data indicate very high diameters that derive from

agglomeration in every artificial fluid. The lower limit of the SP-ICP-MS, based on the particle mass, is directly dependent on the particle density, which leads to a particle-specific cutoff diameter. In digested Al_2O_3 samples, there were no primary particles visible up to 200 nm,. This may be due to non-spherical particles and the resulting agglomerates, which was confirmed by TEM measurements (Figure 2). As a second reason, this can be resulting from the complex medium that aggravates the mathematical calculation formula. Up to now, based on the used mathematical algorithm for SP-ICP-MS, reliable size determination is limited to spherical entities. Given the analytical background of aluminum as a consequence of the ubiquitous presence of this element, the limit of detection is higher as compared to rare elements e.g. gold. On the other hand, *de novo*-emerged nanoscaled particles formed from AlCl_3 could not be proven by this method. Some signals were present in the time scan which, however, did not lead to calculable size distributions.

Ion release of the nanoparticles

The free ionic fraction of aluminum was separated by centrifugation from the particulate and matrix-bound aluminum fraction and analyzed via ICP-MS after acidic hydrolysis (Figure 5). As shown in Figure 5B,C, both particle species displayed a very low intrinsic ion release below 0.03% in undigested dispersion. Similar values were obtained for the ionic content in saliva (Figure 5E,F). In gastric media (Figure 5H,I), metallic Al^0 nanoparticles released with a value of 3 % slightly more ions while Al_2O_3 nanoparticles appear to be more inert in terms of solubility. In intestinal fluid, free ions disappeared almost completely. Ion controls showed almost 100% free ions in stocks, saliva and gastric fluid (Figure 5A, D, G), while there was a severe decrease of free ions in the intestinal fluid (Figure 5J). This matches well to the TEM data and SAXS spectra showing particle formation from dissolved aluminum (Figure 2, Figure 3).

Element distribution of aluminum - Time of flight secondary ion mass spectrometry (TOF-SIMS)

ToF-SIMS results are shown in Figure 6. No pronounced agglomeration was found in saliva samples (Figure 6B, C), whereas we determined strong agglomeration spots in the stomach fluid, as indicated by colored circles (Figure 6E, F). For Al_2O_3 nanoparticles, these intense spots disappeared in the intestine, while there were strong agglomerated spots still present for the metallic Al^0 nanoparticles (Figure 6H, I). We could not detect aluminum-containing spots in the AlCl_3 samples in saliva and stomach fluid, while there were some measurable accumulation spots of aluminum in the intestine fluid which, however, appeared much weaker than in the nanoparticle samples (Figure 6G).

Elements associated to aluminum

IBM measurements were used for the analysis of elements associated with the aluminum nanoparticles (Figure 7). We investigated undigested nanoparticle samples as well as digested particles and ionic samples in the intestinal fluid after artificial digestion. μ PIXE images visualize the location of Al particles and co-localization P, K and Ca elements with Al (Figure 7). The amount of co-localized elements for particles prepared following the artificial digestion were analyzed. The concentration of sulfur on the NP surface was the same for undigested Al and Al_2O_3 particles. This finding reflects the presence of the same amount of albumin corona build on the surface of particles after standard treatment. After digestion the amount of sulfur decreased by a quotient larger ten for both particles. It could be concluded that the albumin corona was removed to a large extent during digestion process. The reason could be the change of pH value during digestion treatment. This leads to the recharging of albumin having an isoelectrical point at a value of 4.6. Partially removing of the protein corona might be the result of this treatment. De novo emerged particles deriving from AlCl_3 appear to have a higher sulfur amount than both other species. Precipitation with proteins, such as mucin or trypsin from the artificial media, or attachment of sulfide ions might describe this behavior. The digested Al and Al_2O_3 as well as *de novo* Al particles reveal the same level of P and Ca elements. The concentration of these elements increased by a two order of magnitude in case of Al_2O_3 particles. The high amount of these elements for digested particles might result from attachment of calcium and phosphate ions, which build a calcium phosphate layer on particle surface. Chlorine and Potassium increased more in the metallic Al particle samples than in the Al_2O_3 samples. Only a small amount of iron and zinc, deriving from the digestion fluids, is associated to aluminum after the digestion process.

Discussion

Protein composition in artificial saliva is slightly more complex than BSA used in standard dispersion, but does not contain proteins or salts that are expected to provoke the formation of completely new chemical entities or complexes with altered chemical characteristics. As a result, in artificial saliva the properties of the nanomaterials do not differ strongly from those of the undigested samples. In our experiments, artificial saliva did not lead to aggregation or dissolution of neither Al^0 , nor Al_2O_3 nanoparticles. Also the formation of new particle populations resulting from free ions was not observed for all Al species.

Stomach fluid is characterized by higher ionic strength and a more acidic environment with a pH value in the range of 2²². Previous studies detected a general tendency of nanoparticles to aggregate at low pH values due to the electrostatic destabilization, for example silver^{19, 21}

or silica^{25, 34} or show enhanced ion release in the gastric fluid in case of silver^{20-21, 35} and zinc oxide³⁴. In this study, we were able to prove that these effects are also observable for Al-containing nanoparticles. We found agglomeration in TEM, ToF-SIMS and SAXS combined with a disappearance of nano-scaled particles in SP-ICP-MS. We also detected ion release from both particle species in gastric fluid, but to a very low extent, thus excluding predominant dissolution of the particles. We suggest that a small amount of ions goes into solution without remarkably changing the mean radii of the primary particles. As expected, a slightly higher ion release occurs from the metallic than from the mineral form of Al. Free Al ions can bind to or build complexes with proteins and biological compounds contained in foods, as well as with biological structures of the intestinal tissue^{3, 36-37}. Recent studies showed that this is not necessarily connected to an increased toxicity *in vitro*²¹, but that the phenomenon can lead to increased cellular particle uptake depending on the composition of the digestive juices and therein-contained food components²⁰.

The most remarkable physico-chemical changes occurred at the transition from the artificial stomach fluid to the intestinal fluid, which simulates the passage into the duodenum. There, pH increases to 7.5 and bile extract is added. TEM, SAXS, SP-ICP-MS and ToF-SIMS showed a reconstitution of the state and particle size measured before in saliva fluid. This means that all observed Al species, including soluble AlCl₃, reach the intestine partly as nanoparticles. ICP-MS showed a decrease of free ions in digested samples for both primary particle species (metallic and mineral oxidized Al), suggesting the formation of complexes or aggregates. Furthermore, a strong decrease of free ions in the AlCl₃ solution was detected as well concomitantly to the presence of nano-scaled structures in the intestinal fluid. ToF-SIMS and μ PIXE images also showed agglomerates deriving from AlCl₃ samples that were not detected previously, neither in saliva nor in gastric fluid. TEM-pictures show newly emerging nanoscaled, particle-like structures that differ in shape and density from the other applied primary particles. ToF-SIMS proved that these aggregates contain aluminum and IBM revealed the co-localization of aluminum with sulfur elements. The presence of sulfur detected in *de novo* particles supports the assumption about formation of precipitated Al-protein nanocomplexes. Furthermore, calcium phosphate layer was found on particle surface for all three aluminum species. The formation of this layer could impact significantly on the cellular uptake and the bioavailability of nanoparticles.³⁸⁻³⁹

In conclusion we postulate these structures to be metal-organic particle-like complexes that are predominantly in the nano-scaled range. This difference in density and chemical composition, as compared to the Al⁰ and Al₂O₃ particles also used in the study, is the suspected reason why they cannot be detected in SP-ICP-MS.

With the help of SAXS we observed a particle size distribution with a volume-weighted mean radius of 2.9 nm for the particle population formed *de novo*. The issue of *de novo* formation

of particles as part of a transition between particulate and ionic species has, up to now, not been in the primary focus of research. Comparable *de novo* particle formation, as well as nanoparticles overcoming the digestion process, has also been observed for silver⁴⁰⁻⁴¹. Although soluble compounds precipitate or agglomerate due to changing physico-chemical conditions, it is noteworthy that a significant amount of these *de novo*-emerged particles are in the nano-scaled range. Therefore they might have nano-specific characteristics, including enhanced reactivity and altered uptake. Furthermore, when talking about toxicological analyses of nanomaterials one should keep in mind that there is a certain balance between dissolved, agglomerated and non-agglomerated nanoparticulate species deriving from the same origin. These conversion processes occur bi-directionally and differ significantly among the multiple biological environments. Up to now, little is known about the two-directional solubilization behavior of metallic nanoparticles. We observed a severe change of the chemical state of dissolved aluminum between stomach and small intestine. Figure 8 summarizes possible transitions between free ions, nanoparticles and agglomerates during the digestion process. Experimental evidence for the scenario drafted in Figure 8 has also been depicted in this study for aluminum and was observed in previous studies for silver^{20-21, 35}. Even if *de novo* formation of nano-scaled particles from other metals like Silver has not been experimentally shown in intestinal fluid, physicochemical similarities of different metals, as well as shared affinity to biological structures, suggest that *de novo* formation of nanoparticles might also occur from additional metals following oral uptake of dissolved ions.

Altogether, we used a broad spectrum of methods to characterize the behavior of different aluminum species during an *in vitro* digestion process. As no single method is capable of depicting the different modifications, it is necessary to use complementary analytical techniques for a systematic characterization of physicochemical properties of nanomaterials. Such strategy will bring comprehensive knowledge to investigate nanomaterials and their fate in relevant biological media and to link these results with the toxicological potential. For this purpose, it is indispensable to take into account the different transitions leading to a mixture of ionic, particulate and agglomerated species from one pristine material.

Summary and Conclusions

During the digestion process, metallic and oxidized aluminum particles undergo physico-chemical conversions depending on their biological environment. While staying almost unaffected in saliva, they preferably agglomerate in gastric juice and, also slightly release ions into the fluid. After transition into intestine the agglomerates tend to de-agglomerate into primary particles again, whereas free ions form solid complexes with biological compounds. These complexes can be nano-scaled and differ significantly from primary particles in their size, shape and density. Particles and dissolved ions can be transformed into each other and

their surface element composition can change. A broad spectrum of methods is required to characterize all these parameters adequately. With respect to this knowledge, toxicological investigations of individual nanoparticle species are only purposeful with regard to the respective biological and chemical environment. By demonstrating the mutual conversion of nano-particles and dissolved metal ions, the present data underline that it might not be possible to distinguish between particle- and ion-dependent effects in toxicological studies. Careful physicochemical characterization will be essential for proper interpretation of toxicity data.

Declaration of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1: Scheme of particle dispersion and *in vitro* digestion including the composition of digestion fluids, experimental workflow and nanoparticle concentrations.

Figure 2: Representative TEM pictures of aluminum-containing samples after standard dispersion (A-C), in artificial saliva (D-F), gastric fluid (G-I), and intestinal fluid (J-L). The

sample concentration applied to the grid was 0.8 g/L. AlCl_3 -containing samples showed no measurable particles in stock, saliva and gastric fluid. Representative images are depicted.

Figure 3: Volume-weighted size distributions derived by SAXS measurements of samples containing Al^0 nanoparticles, Al_2O_3 nanoparticles and AlCl_3 , respectively, after standard dispersion (A-C), in artificial saliva (D-F), gastric fluid (G-I), and intestinal fluid (J-L). The distributions of undigested stock solutions and digested Al^0 nanoparticles, Al_2O_3 nanoparticles, and AlCl_3 in their highest concentration are given (6.67 mg Al/mL at the beginning of digestion process in saliva and 1 mg Al/mL at the end in intestinal fluid). AlCl_3 -containing samples showed no measurable particles in stock solution, saliva and gastric fluid. The right hand y-axes mark the cumulative particle fraction presentation given in blue solid lines. The size distributions are fitted by a lognormal distribution function (red solid lines).

Figure 4: Number-weighted size-distributions of aluminum samples after standard dispersion (A-C), in artificial saliva (D-F), gastric fluid (G-I), and intestinal fluid (J-L). Representative images and size distributions are calculated from 60 s measuring time each run. For intestinal samples, the time scan is shown, too (M-O). The number of measurable particles in AlCl_3 -containing samples was too low to calculate a size distribution.

Figure 5: Determination of free aluminum ions of aluminum-containing samples after standard dispersion (A-C), in artificial saliva (D-F), gastric fluid (G-I), and intestinal fluid (J-L). Quantification of ionic percentile determined by UC followed by nitric acid digestion and ICP-MS is given. Each sample was performed twice and measured twice. Error bars show the standard deviation of the mean values.

Figure 6: Representative ToF-SIMS images of aluminum-containing samples in artificial saliva (A-C), gastric fluid (D-F), and intestinal fluid (G-I). AlCl_3 -containing samples showed no measurable particles in saliva and gastric fluid. Densitograms show local agglomerations of measured Al-species, indicated by colored circles.

Figure 7: Digestion impact on surface modification of nanoparticles. (A - F): Element analysis of Al, of Al_2O_3 and *de novo* emerged particles resulted from AlCl_3 . Samples were prepared following standard dispersion (A - C) as well as in intestinal fluid after having performed the

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full artificial digestion protocol (D - F). Each sample was measured at least 3 times on different positions. Error bars represent the standard deviation of the mean values. (G - I): μ PIXE images of element distributions in Al species after artificial digestion process. All images displaying an area of 25 x 25 μm^2 . The color code is as follow: minimal concentration is displayed black, while maximum is shown as white.

Figure 8: Suggested scheme of aluminum particle dissolution and agglomeration during the artificial digestion process. Samples stay unaffected in artificial saliva but agglomerate in the stomach fluid. At the same time, ions are released from particles but incomplete dissolution occurs. In intestinal fluid, agglomerates tend to de-agglomerate into primary particles and free ions form nano-scaled particulate structures, too.

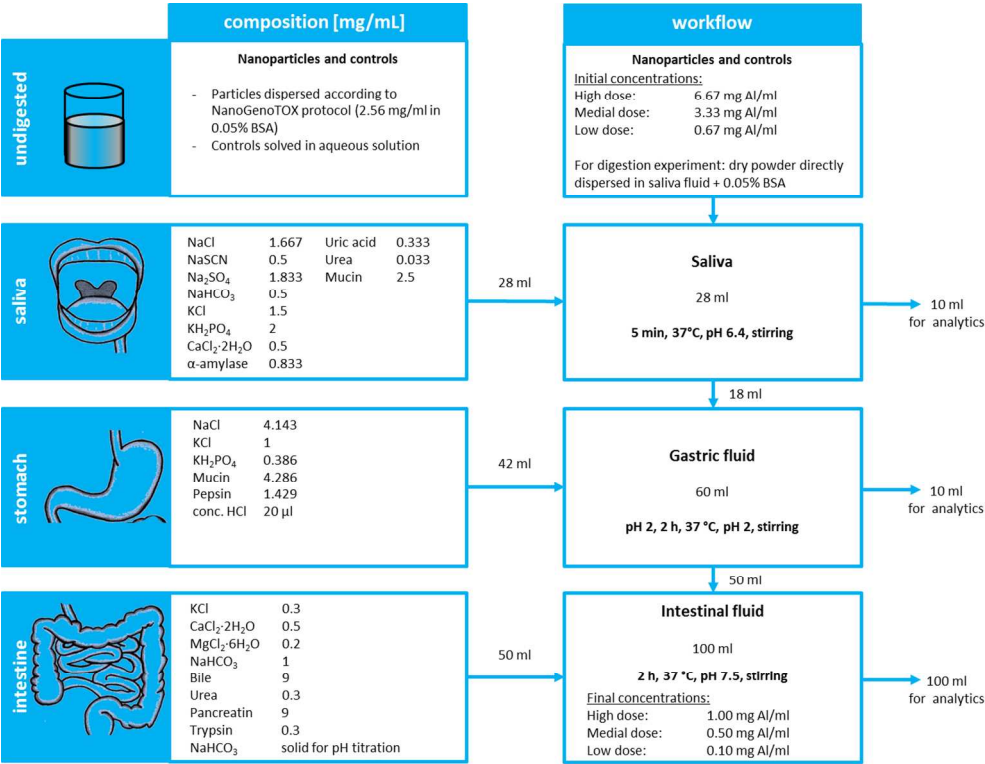


Figure 1: Scheme of particle dispersion and in vitro digestion including the composition of digestion fluids, experimental workflow and nanoparticle concentrations.

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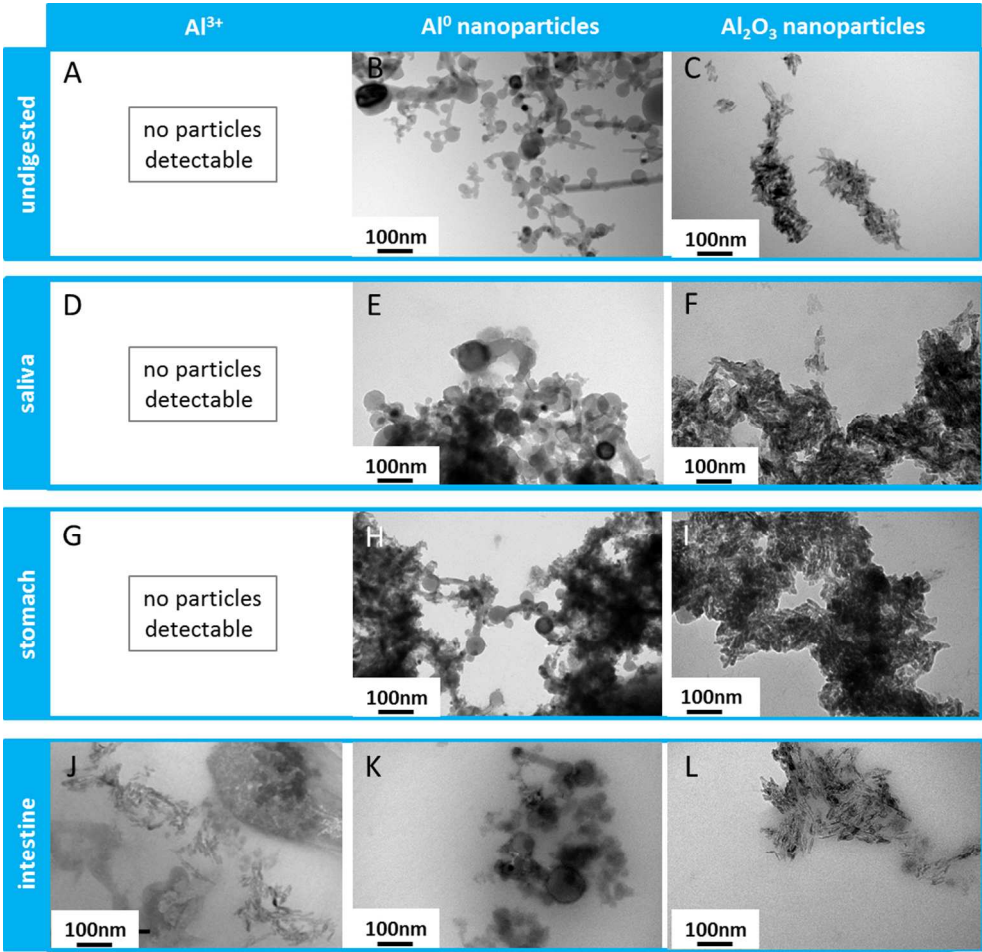


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305x292mm (96 x 96 DPI)

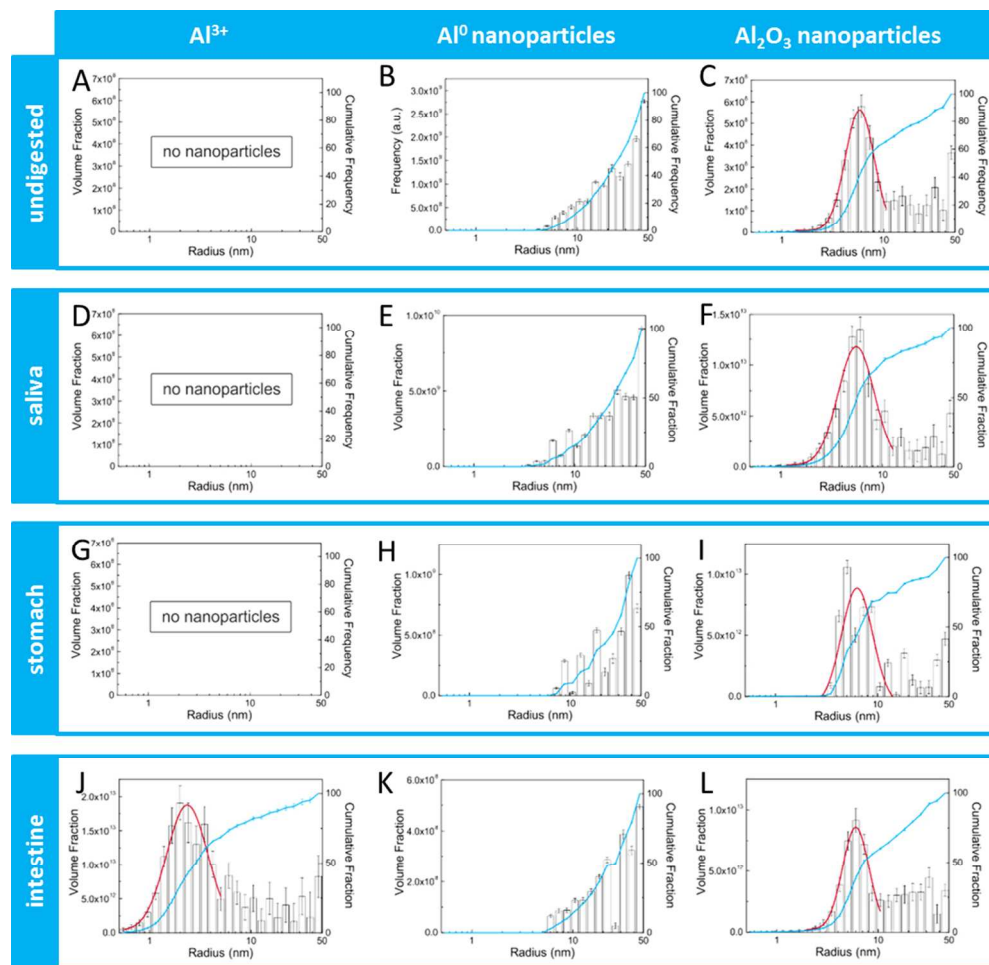


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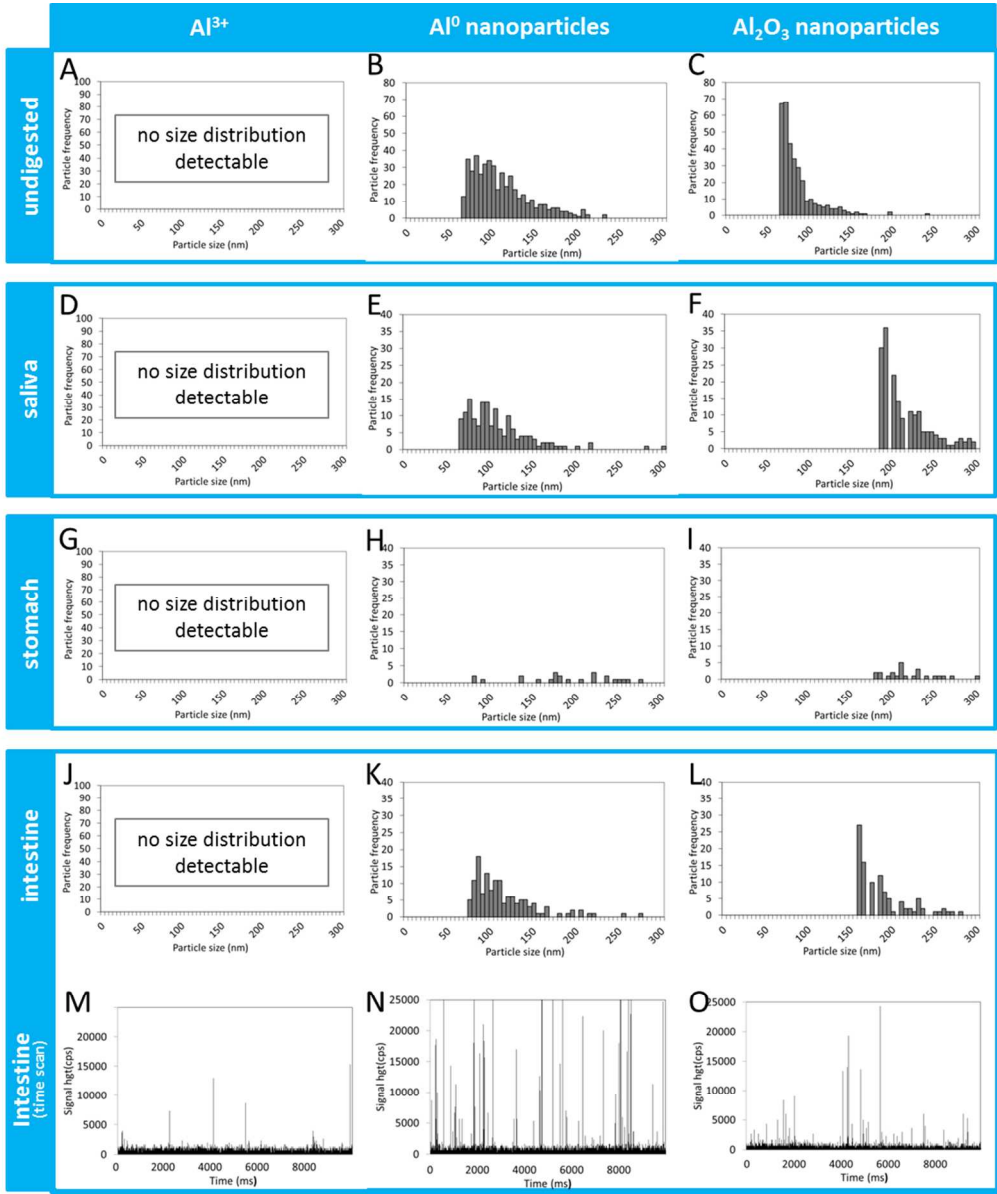


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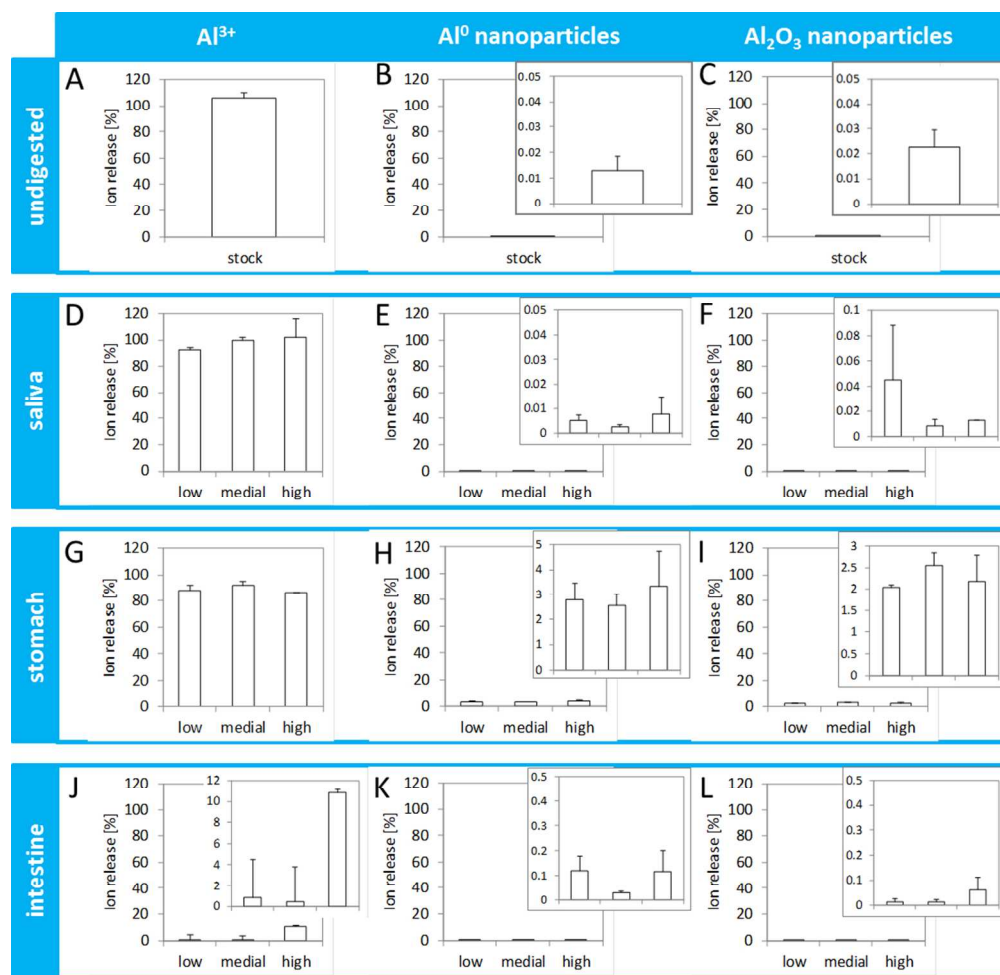


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299x290mm (96 x 96 DPI)

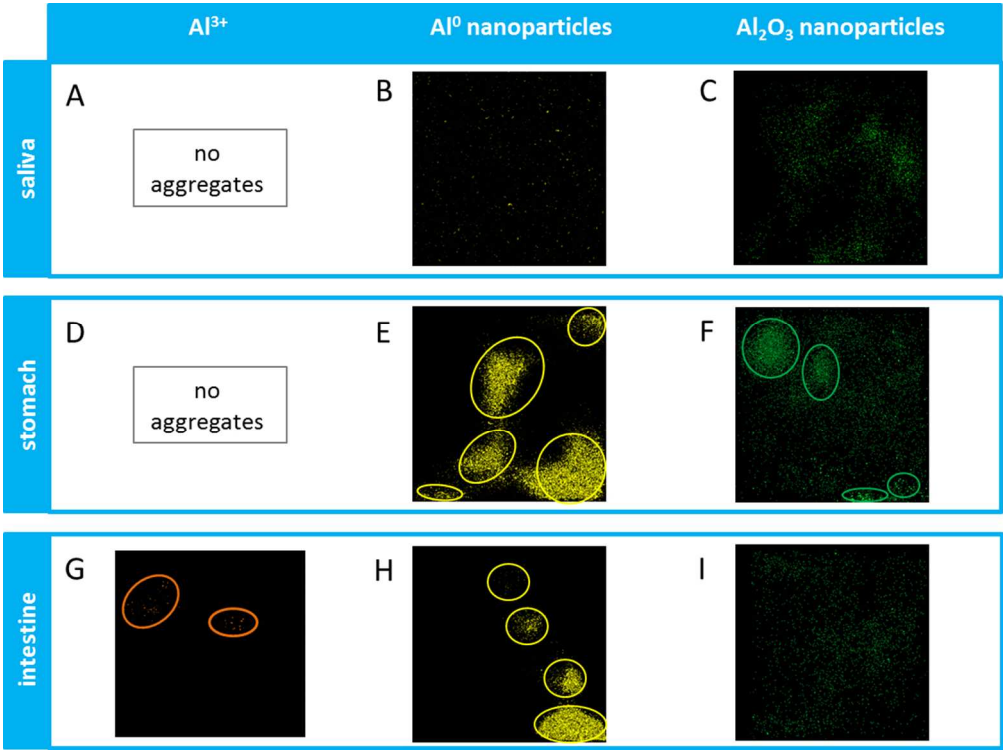


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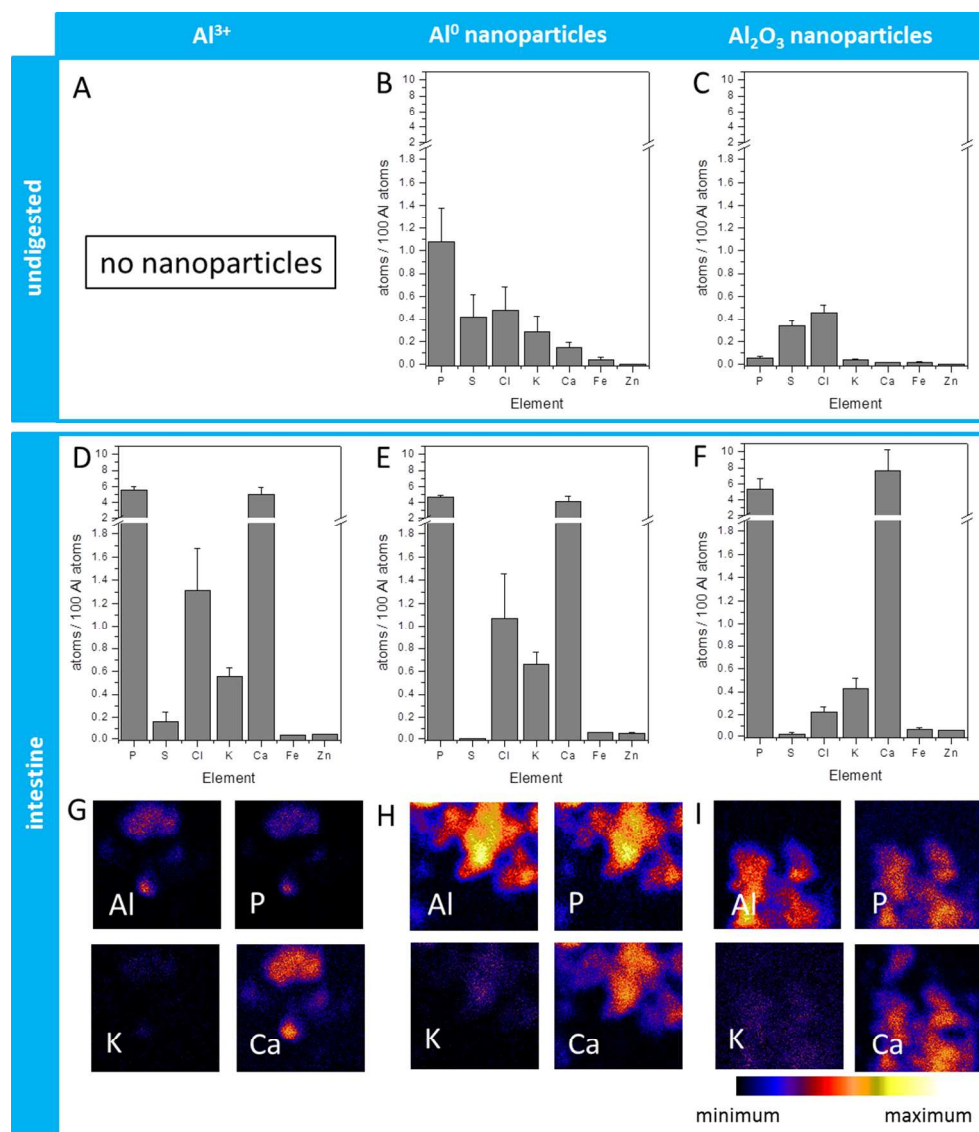


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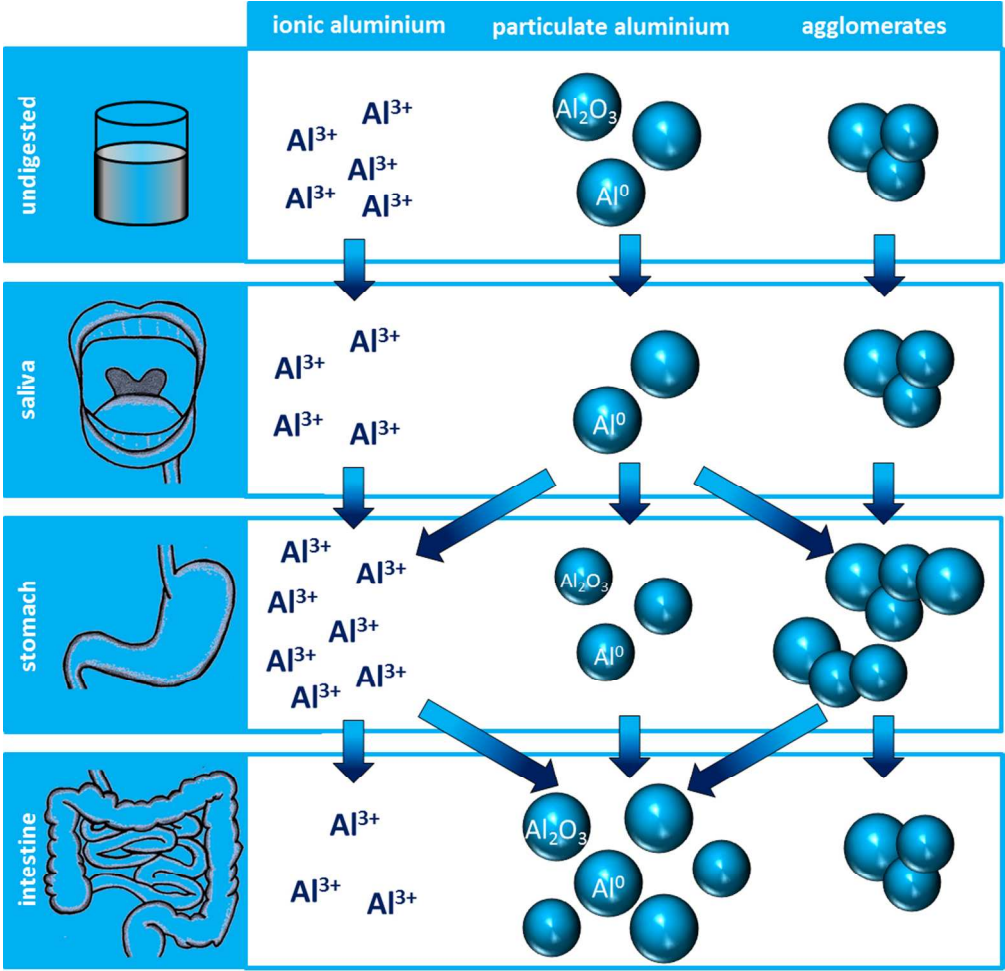


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