



Scientific Committee on Consumer Safety

SCCS

OPINION ON

Hydroxyapatite (nano)

The SCCS adopted this opinion by written procedure

on 16 October 2015

Revision of 16 March 2016

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.

They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of independent experts.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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http://ec.europa.eu/health/scientific_committees/consumer_safety/index_en.htm

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This opinion has been subject to a commenting period of eight weeks after its initial publication. Comments received during this time have been considered by the SCCS and discussed in the subsequent plenary meeting. Where appropriate, the text of the relevant sections of the opinion has been modified or explanations have been added. In the cases where the SCCS after consideration and discussion of the comments, has decided to maintain its initial views, the opinion (or the section concerned) has remained unchanged. Revised opinions carry the date of revision.

Keywords: SCCS, scientific opinion, hydroxyapatite, nano, Regulation 1223/2009, CAS 1306-06-5, EC 215-145-7

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1. BACKGROUND

Article 2(1)(k) of Regulation (EC) No 1223/2009 establishes that "nanomaterial" means an insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm.

That definition covers only materials in the nano-scale that are intentionally made, and are insoluble/partially-soluble or biopersistent (e.g. metals, metal oxides, carbon materials, etc), and it does not cover those that are soluble or degradable/non-persistent in biological systems (e.g. liposomes, emulsions, etc). Article 16 of the Cosmetics Regulation requires any cosmetic product containing nanomaterials to be notified to the Commission six months prior to being placed on the market, and Article 19 requires nano ingredients to be labelled (name of the ingredient, followed by 'nano' in brackets). If there are concerns over the safety of a nanomaterial, the Commission shall refer it to the Scientific Committee on Consumer Safety (SCCS) for a full risk assessment.

The Commission received 35 notifications of cosmetic products containing Hydroxyapatite (CAS No 1306-06-5) in nano form. This ingredient is reported in the CosIng database without any reference to the nano form with the function of abrasive, bulking and emulsion stabilising, but it is not regulated in Cosmetic Regulation (EC) No 1223/2009. According to the applicants, the ingredient is used in nano uncoated form both in leave-on and rinse-off oral cosmetics products including toothpastes, tooth whiteners and mouth washes with maximum reported concentration limit of 10% and specifications.

The Commission has concerns on the use of Hydroxyapatite in nano form because of the potential for nanoparticles of Hydroxyapatite to be absorbed and enter into the cells. Therefore, we would like to request the SCCS a safety assessment of the nano form of Hydroxyapatite covered in the notifications listed in the annex to this mandate, in the above-mentioned categories of products, taking into account the reasonably foreseeable exposure conditions.

2. TERMS OF REFERENCE

1. In view of above, and taken into account the scientific data provided, the SCCS is requested to give its opinion on the safety of the nanomaterial Hydroxyapatite when used in oral cosmetics products including toothpastes, tooth whiteners and mouth washes with a maximum concentration limit of 10%, taking into account the reasonably foreseeable exposure conditions.

2. SCCS is requested to address any further scientific concerns with regard to the use of Hydroxyapatite in nano form in cosmetic products.

3. OPINION

At the Applicant's request, the names of companies and materials have been coded in this opinion alphabetically (company names) and numerically (material names).

3.1 Chemical and Physical Specifications

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

Hydroxyapatite

3.1.1.2 Chemical names

Hydroxyapatite
Calcium Phosphatetribasic
Calcium Hydroxyphosphate
Hydroxylapatite

3.1.1.3 Trade names and abbreviations

Confidential

3.1.1.4 CAS / EC number

CAS: 1306-06-5
EC: 215-145-7

3.1.1.5 Structural formula

N.A.

3.1.1.6 Empirical formula

$\text{Ca}_5 (\text{PO}_4)_3 (\text{OH})$

3.1.2 Physical form

Materials added to products are listed either as powder (to be used in toothpastes) or suspension (mouthwash).

Suspension is described as an odourless white paste (Material codes 1000440; 1000320; 1001387; 1001388; 1001240; 1001241; 1001727); and all other materials as powder.

The material is described to be composed of rod/thumb shaped particles that are in the form of dispersed free particles and agglomerates (Company C).

Submission of company B refers to hydroxyapatite of any shape including nanofibres of 2-4 nm width and 20 – 50 nm in length.

All materials are stated to be in uncoated form.

SCCS comment:

Hydroxyapatite is a naturally occurring mineral. Hydroxyapatite has a hexagonal crystal structure comprising different crystalline phases. The OH⁻ ions in hydroxyapatite can be replaced by different counter anions to form other members of the apatite group. Therefore

hydroxyapatite can bear a variable degree of other anions, most commonly carbonate, phosphate and fluoride, which leads to modified atomic composition and also properties (e.g. see www.astm.org/Standards/F1185.htm).

There are several established pathways to produce hydroxyapatite, among them wet chemical synthesis and precipitation, biomimetic preparation and electrodeposition. A number of different pathways also exist for preparing nanoforms of hydroxyapatite. The processing of natural hydroxyapatite sources like bovine bone to extract hydroxyapatite is also used. There is a huge body of literature on hydroxyapatite, its manufacturing and biocompatibility due to use as an implant material and also on the generation of different nanoforms.

According to the information provided by the applicants, the nano-hydroxyapatite in the raw material is fully synthetic, made from inorganic reactants using food-grade quality calcium and phosphorous salts, i.e. it is not produced from bone material.'

3.1.3 Molecular weight

Molecular weight: 502.31 g/mol

3.1.4 Purity, composition and substance codes

Company A and C (material 1): Hydroxyapatite as the main component; Potassium chloride (KCl) as preservative and water as excipient.

Company B (material 2): "Artificially synthesized, amorphous, nano-disperse calcium hydroxyapatite in powder form or aqueous suspension does not contain reaction by-products." Calcium content (by weight): $\geq 39.0\%$; phosphorus content (by weight): $\geq 18.0\%$.

3.1.5 Impurities / accompanying contaminants

Company A and C (material 1): Total heavy metals < 20 ppm.

Company B (material 2): Moisture content of the powder (by weight): $\leq 3.0\%$; content of heavy metals (by weight): $\leq 0.002\%$.

3.1.6 Solubility

Insoluble in water (aqueous solubility < 0.01 mg/l).

3.1.7 Partition coefficient (Log P_{ow})

Log P_{ow}: /

3.1.8 Additional physical and chemical specifications

Specific surface area:

Company A and C, material 1: $80 - 100$ m²/g (according to BET nitrogen adsorption)

Company B, material 2: BET specific surface area SSA: 147 m²/g

Volume specific surface area VSSA: 440 m²/cm³.

Zeta potential:

Company A and C, material 1: + 30 ± 1 mV
 Company B, material 2: not measurable.

| | |
|-------------------|---|
| Density: | 1.1 – 1.2 g/cm ³ (Company A and C, material 1) |
| Viscosity: | 1.0 ± 0.3 Pa.s (assessed with a Physica UDS 200, oscillatory shear testing at 30°C, deformation 100% at 10Hz) (Company A and C, material 1) |
| pKa: | / |
| Refractive index: | / |
| pH limits: | 10.0 ± 0.5 (measured directly on the product) (Company A and C, material 1) |
| Porosity: | hydroxyapatite nanoparticles with mesopores (Company A and C, material 1) |
| UV_Vis spectrum: | / |
| Melting point: | / |
| Boiling point: | / |
| Flash point: | / |
| Vapour pressure: | / |

3.1.9 Homogeneity and Stability

Material 1:

Hydroxyapatite is a chemically stable compound; therefore its degradation is not expected under normal conditions of storage, avoiding freezing, and keeping the product in the original container at room temperature, in a clean, dry place.

To insure homogeneity of the material that contains the hydroxyapatite (nano) – (material 1, company A and C) - it should be stirred before every use. It is a thixotropic material, which means that it is very viscous under normal conditions, but it becomes less viscous over time when shaken, agitated, or otherwise stressed. However, this is a reversible microstructural change of the material. The shelf-life of this material is 18 months.

Material 2 (company B):

Calcium hydroxyapatite is chemically and thermally stable. Shelf-life of suspension: 2 months; shelf-life of powder: 2 years.

3.1.10 Particle Size Distribution and Crystalline Shape

Shape:

The reported particle shapes of the materials are either hexagonal plate, nanofibre, or amorphous, with some materials reported to be composed of nano-rod or nano-thumb shaped particles.

The hydroxyapatite nanoparticles are reported to be aggregated/agglomerated to larger clusters.

Needle-like hydroxyapatite particles have also been reported in scientific literature.

Company B (material 2) mentions: 'isometric aggregates consisting of particles of any shape, sized 5-10 nm, and fibres formed from the same particles.'

Particle size/particle size distribution

Company B (material 2)

According to **TEM**: nanofibre and plate form;

Nanofibres: 2 – 4 nm thickness; 20 – 50 nm length

Plates: mean size 15 nm

Formation of aggregates in size range from 50 nm to 1.4 µm

According to **DLS**: size distribution 50 nm to 1.4 µm; mean size: 1 µm

Company A (material 1):

Form of nanoparticles: nanorod or nanothumb

Malvern Mastersizer: no agglomerates with particle size < 100 nm; mean particle size of agglomerates: 1.213 µm

TEM: shows nanorod shaped entities with size below 100 nm; structures larger than 100 nm can be observed due to agglomeration of particles.

Company C

Mean particle size of individualised particles measured by TEM in terms of particle number is reported as 64 nm.

Primary particle size measured using Malvern Mastersizer 2000 is reported as 20 nm (lowest cut-off level); 60 to 400 nm (volume weighted median); and 30 nm to 80 nm (number weighted median). Mean particle size of the agglomerates (in terms of particle number) is reported as 1.213 µm.

General comments on physicochemical characterisation

The characterised materials do not necessarily correspond to those materials which have been used for toxicity testing.

One submission refers to hydroxyapatite of any shape including nanofibres of 2-4 nm width and 20 – 50 nm in length.

3.2 Function and uses

Nano-hydroxyapatite is used as a drug delivery material and as a bone defect filling material. Oral care products containing nanoparticulate hydroxyapatite are intended to be used for teeth remineralisation and apparently are already on the market. Products listed in the dossiers are either in the form of paste or suspension. All products listed in the dossiers are intended for oral application. All products are listed as rinse off - except 3 mouthwash products that are listed by Company A as leave on. The material is intended for use in toothpaste, tooth whitener and mouthwash products. Based on the explanation provided by one Applicant (Company C) the concentration of hydroxyapatite of 3-15% refers to the concentration in the raw material 1, which contains 15.5% nano-hydroxyapatite. Therefore, the 3-15% of the raw material 1 corresponds to 0.465 – 2.325% nano-hydroxyapatite in the final product. The submission also describes the use of 90% of raw material 1 which corresponds to 13.95% of nano-hydroxyapatite (w/w) in the final product.

All materials are described as having no surface reactivity or photoreactivity.

SCCS comment:

Two applicants (company A and C) have used the same ingredient. One of the applicants (company A) later clarified that the minimum concentration used in final products can be as low as 0.08% of the raw material (corresponding to 0.0125% nano-hydroxyapatite). The second Applicant (company C) clarified that although 90% raw material corresponds to 13.95% nano-hydroxyapatite (w/w), for this type of application, the expected maximum raw material concentration is 20%, which corresponds to 3.1% of nano-hydroxyapatite in the final product.

The applicants should provide further information on the level of any significant impurities other than heavy metals, and provide an upper limit of total impurities in the raw material.

3.3 Toxicological Evaluation

3.3.1 Acute toxicity

3.3.1.1 Acute oral toxicity

A material named "Hydroxyapatite 5%, aqueous solution, 20 nm" was intra-gastrically administered in a dose range between 1,600 and 36,000 mg/kg bw to white rats of both genders (strain unclear) and the animals were observed for 14 days. As described in the study report, death of the animals occurred from 10,000 mg/kg bw in both sexes as a result of cardiac and respiratory arrest. Important findings on animals surviving 14 d: lung vessels were moderately congested, "alveoli filled with air"; signs of "fatty degeneration of the liver". The LD₅₀ was 20,800 mg/kg bw in both sexes.

Ref.: Nechiporenko, S.P. and Stepanov, S.V. (2011)

SCCS comment:

The study did not adhere to any OECD or EU test guideline and it is not clear whether it was performed according to GLP principles. The test material was insufficiently characterised and it is not clear whether the doses used correspond to test material as 5% aqueous solution or to the active ingredient. It is also not clear whether the tested material belongs to the materials covered by the submission. The study is therefore of little value to this assessment.

3.3.1.2 Acute dermal toxicity

/

3.3.1.3 Acute inhalation toxicity

A material named "Hydroxyapatite 5%, aqueous solution, 20 nm" was investigated for acute inhalation toxicity in white mice. Signs of irritation of the upper respiratory tract were observed in this study.

Ref.: Nechiporenko, S.P. and Stepanov, S.V. (2011)

SCCS comment:

Due to poor reporting and insufficient characterisation of test material, no conclusions can be drawn from that study.

3.3.1.4 Acute intravenous toxicity

| | |
|---------------------|---|
| Guideline: | ISO 10993-11 |
| Species/strain/sex: | mouse, Hla [®] : (ICR) CVF [®] , male |
| Group size: | 5 |
| Test substance: | Nano-Hydroxyapatite paste (a white viscous paste composed of nanoparticles of hydroxyapatite and water; 38% hydroxyapatite) |
| Batch: | 1309-12 |
| Purity: | > 95% |
| Vehicle: | 0.9% Sodium Chloride in water |
| Dose levels: | 10 g paste/kg bw |
| Administration: | intravenous |
| GLP: | Yes |
| Study period: | September – October 2012 |

A single dose of test material extracted with 0.9% sodium chloride was intravenously administered (no further information) at a dose of 50 ml/kg bw to five male animals, five further animals received vehicle control. Animals were observed for clinical reactions

immediately after and 4, 24, 48 and 72 hr after injection. Animals were weighed daily for three days after dosing. There was no mortality during the study and all animals (control and treated) appeared normal during the course of the study. Treatment did not adversely affect the animals' body weight.

Ref.: NAMSA (2012a)

SCCS comment:

No material characterisation is given in the study report. However the applicant confirmed that this study had been performed with material 1 of the submission. The study did not correspond to study types usually used to investigate acute toxicity of cosmetic ingredients and the observation period after treatment was much shorter than usually applied in acute toxicity studies according to the OECD test guidelines. However, the study demonstrated that a single intravenously administered dose of 10 g/kg bw nano-hydroxyapatite paste (corresponding to 3.8 g active ingredient) apparently did not adversely affect mice within an observation period of 3 days. Apparently, this is the "acute systemic toxicity study" as described in Ding *et al.*, 2012, where further study details are missing.

Information from open literature

In a publication from the open literature (article in Chinese, only some parts of the abstract in English), it was concluded that the "medium" (wording from the abstract) lethal dose of nano-hydroxyapatite is 200 mg/kg bw. In this study, some clinical-chemical parameters were also investigated. It could be demonstrated that some enzymes dramatically increased 30 min after injection, reaching peaks at 2 hr after injection and returning to normal levels 24 hr after injection. BUN and ALP peaked 24 hr after injection and returned to normal levels afterwards. As the study is in Chinese, no further detail on study conditions, material characterisation or explanation of abbreviations could be obtained.

Ref.: Liu, L.P. *et al.* (2005)

In a further publication from the open literature, 6 male Sprague-Dawley rats received single injections of 50 mg/kg bw nano-hydroxyapatite (needle-like; long diameter: 80 nm, short diameter: 20 nm; hydrodynamic diameter in physiological saline: 245.1 nm (i.e. agglomeration in physiological saline), Zeta potential in physiological saline: - 16.3 mV; purity: \geq 99.0%) into the tail vein; 6 control rats received vehicle alone (physiological saline). 48 hr later, blood samples were collected for haematological and clinical-biochemical investigations and the left lateral lobe of the liver was removed for histopathological analysis and determination of oxidative biomarkers. Compared to control livers which had normal histology, the liver tissues from the rats exposed to nano-hydroxyapatite showed inflammatory cell infiltration at the portal areas of the liver. Haematological analysis demonstrated increased white blood cells, elevated levels of the inflammatory cytokine TNF- α , and increased alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bile acid (TBA), cholesterol, uric acid, lactate dehydrogenase (LDH) and low density lipoprotein (LDL) compared to controls. In the livers, increased levels of H₂O₂ and MDA (malondialdehyde) and decreased levels of glutathione ($p < 0.05$) were observed.

Ref.: Chen, Q. *et al.* (2014).

SCCS comment:

The study demonstrates that needle-shaped nano-hydroxyapatite can lead to biochemical changes and indications of oxidative damage after i.v. administration in rats within 48 hr.

3.3.1.5. Acute intraperitoneal toxicity

| | |
|---------------------|---|
| Guideline: | ISO 10993-11 |
| Species/strain/sex: | mouse, Hla [®] : (ICR) CVF [®] , male |
| Group size: | 5 |
| Test substance: | Nano-Hydroxyapatite paste (a white viscous paste composed of nanoparticles of hydroxyapatite and water; strength: 38% hydroxyapatite) |
| Batch: | 1309-12 |
| Purity: | > 95% |
| Vehicle: | Sesame Oil |
| Dose levels: | 10 g/kg bw |
| Administration: | intraperitoneal |
| GLP: | Yes |
| Study period: | September – October 2012 |

Test article in sesame oil and sesame oil alone were “extracted” by continuous agitating for 24 hr at 70°C. The extracts were not centrifuged, filtered or otherwise altered prior to dosing (meaning that the test materials were in a suspension form).

A single dose of test material extracted with sesame oil was intraperitoneally administered (no further information) at 50 ml/kg to five male animals, 5 further animals received vehicle control. Animals were observed for clinical reaction immediately after and 4, 24, 48 and 72 hr after injection. Animals were weighed daily for three days after dosing. There was no mortality during the study and all animals (control and treated) appeared normal during the course of the study. Treatment did not adversely affect body weight in the animals.

Ref.: NAMSA (2012a)

SCCS comment:

No material characterisation is given in the study report. However the applicant confirmed that this study had been performed with material 1 of the submission. The study did not correspond to the study types usually used to investigate acute toxicity of cosmetic ingredients and the observation period after treatment was much shorter than usually applied in acute toxicity studies according to the OECD test guidelines. However, the study demonstrated that a single intraperitoneally administered dose of 10 g/kg bw nano-hydroxyapatite paste (corresponding to 3.8 g active ingredient) apparently did not adversely affect mice within an observation period of 3 days.

SCCS conclusion on acute toxicity

The acute toxicity of nano-hydroxyapatite was investigated through the oral, inhalation, intraperitoneal and intravenous routes. No study was performed in accordance with any OECD or EU Test guidelines and apart from one intravenous study published in the open literature (Chen *et al.*, 2014), proper material characterisation was not given. Therefore, no conclusions on acute toxicity can be drawn from these studies. The study from the open literature used needle-shaped nano-hydroxyapatite and indicated that needle-shaped crystals of hydroxyapatite can be a concern in relation to acute toxicity.

3.3.2 Irritation and corrosivity

3.3.2.1 Skin irritation

| | |
|---------------------|---|
| Guideline: | ISO 10993-10 |
| Species/strain/sex: | Rabbit, White New Zealand, male |
| Group size: | 3 |
| Test substance: | Nano-Hydroxyapatite paste (a white viscous paste composed of nanoparticles of hydroxyapatite and water; 38% hydroxyapatite) |

Batch: 1309-12
Purity: > 95%
Vehicle: 0.9% sodium chloride or sesame oil
Dose level: approximately 40 mg/kg bw
Dose volume: 0.2 ml
Observation: up to 72 hr after administration
GLP: Yes
Study period: October 2012

Test article in the respective vehicles, and vehicles alone, were "extracted" by continuous agitating for 24 hr at 70°C. The extracts were not centrifuged, filtered or otherwise altered prior to dosing (meaning that test materials were in a suspension form).

Groups of 3 animals received intracutaneous injections of substance dissolved in the respective vehicle at five sites of the clipped right back side of the animals; respective vehicle controls were intracutaneously injected at five sites of the clipped left back side of the animals. The treatment sites were observed immediately after injections; animals were then returned to cages and observations for edema and erythema (on a score from 0 to 4) were made 24, 48 and 72 hr after injection. Erythema and edema for test article and vehicles at each scoring interval were calculated by adding erythema and edema scores. The differences between mean overall scores of treated versus vehicle scores were used to assess local dermal irritation.

Results:

Two animals treated with the sesame oil vehicle had reduced faeces at the 24 hr examination, all other animals appeared normal. The score difference (treated versus control sites) in animals treated with sodium chloride vehicle was zero, whereas score difference (treated versus control sites) in animals treated with sesame oil vehicle was 0.2. A substance is considered to meet the requirements of this test when the difference between mean score of treated versus control is < 1). As differences were 0 and 0.2., the material tested met the requirements of the test.

Ref.: NAMSA (2012b)

SCCS comment:

No material characterisation is given in the study report. However the applicant confirmed that this study had been performed with material 1 of the submission. The study did not correspond to study types usually used to investigate skin irritation. However, it demonstrates that under the conditions used, intracutaneous injection of 40 mg/kg nano-hydroxyapatite paste (corresponding to 15.2 mg a.i.) does not cause erythema and oedema.

In another study, a material termed as "Hydroxyapatite 5%, aqueous solution, 20 nm" was investigated for skin effects in rats. Tails of white rats (no information on strain and sex) were placed 2/3 in vials (no information on duration or the concentration of test material). Tails were examined for erythema and edema 1 hr and 16 hr after treatment. No changes of tail morphology, epidermis and skin appendages were observed.

Ref.: Nechiporenko, S.P. and Stepanov, S.V. (2011)

SCCS comment:

The study did not adhere to an OECD or EU test guideline and it is not clear whether it was performed according to GLP principles. No conclusion can be drawn from this study.

For skin irritation in humans see section 3.3.11.

3.3.2.2 Mucous membrane irritation / Eye irritation

The eye irritation potential of material 1 was investigated in the Hen`s Egg Chorioallantoic Test. Three fertilised hen`s eggs were incubated with each substance respectively (material 1 at a concentration of 25%, diluted in water, and 2 positive controls (1% SDS and 0.1M NaOH) and 1 negative control (0.9% NaCl)) for nine days, and on the 10th day, the eggs were opened and the CAM (chorioallantoic membrane) exposed. 0.3 g of the test substance was applied to the surface of the CAM and after a 20-second exposure period, the CAM was rinsed with 5 ml of sterile Milli-Q water. Morphological analysis observing hyperaemia (increased blood flow), haemorrhage (blood from a ruptured vessel) and coagulation (presence of blood clots) was performed 0.5, 2, and 5 minutes after rinsing-off the test substance. Observations were subjectively assessed and scored according to published literature. Photographs were integrated in the report. According to the HET-CAM, 25% of the material was considered weakly or slightly irritant with a score of 2.8 on the CAM, based on hyperaemia at the 2 min score.

Ref.: Inovapotek (2013a)

SCCS comment:

The HET-CAM test does not belong to the internationally accepted tests for eye irritation testing. The study report contains no information on GLP-compliance. Reporting on findings on test material, positive and negative controls is considered insufficient by the SCCS as e.g. no information on scores obtained from positive or negative controls is given. Also, there is no information on positive or negative scores obtained from historical controls. Based on the findings of the study, mucous membrane irritation of material 1 of the submission cannot be excluded.

A material termed as "Hydroxyapatite 5%, aqueous solution, 20 nm" was investigated for effects on mucous membrane of the eye. 2 drops of substance were instilled into the conjunctival sac of a rabbit (apparently one animal, no further information) which was observed for 1 month. From the study it was concluded that the test material causes slight and reversible irritation.

Ref.: Nechiporenko, S.P. and Stepanov, S.V. (2011)

SCCS comment:

The study did not adhere to an OECD or EU test guideline and it is not clear whether it was performed according to GLP principles. The test material is insufficiently characterised and it is not clear whether the tested material belongs to the materials covered by the submission. No conclusion can be drawn from this study.

SCCS conclusion on irritation and corrosivity

No guideline compliant skin or mucous membrane irritation test was provided by the applicants. The studies/study descriptions partly lacked proper material characterisation. A skin- and eye irritating potential cannot be excluded based on the studies provided.

3.3.3 Skin sensitisation

In a study from the open literature, needle-shaped nano-hydroxyapatite particles synthesised by a wet chemical method yielding particles of a size below 50 nm and a Zeta potential of -13.4 mV in buffer (the authors state that the material was unstable in water due to a Zeta potential below 3.19 mV) were investigated for sensitising effects in Albino Guinea pigs (Hartley) (sex not given). 10 test animals and 5 control animals were used. A paste of 80 mg nano-hydroxyapatite in physiological saline was applied topically to the clipped back, covered and kept for 6 hr. The procedure was repeated three times a week for three weeks. Two weeks after the last induction application, a challenge application was

made comparable to the induction applications. Following removal of the patch, the sensitisation potential was evaluated at 24, 48 and 72 hr after patch removal. No indications for skin sensitisation were observed in the study.

In the same animals, blood, livers and brains were taken at termination and haematological and biochemical parameters were determined in blood. Parameters indicative of oxidative stress (total protein (TP), lipid peroxidation (malondialdehyde (MDA) formation), glutathione reductase (GR), reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and 8-hydroxyl-2-deoxyguanosine (8-OHdG)) were investigated in livers and brains.

Results

Haematology: whereas white blood cells were comparable between treated and untreated animals, there was a statistically significant increase in red blood cells, haemoglobin and haematocrit and a statistically significant decrease in platelet counts in treated animals.

With respect to biochemical parameters, there were slight decreases in serum glutamic oxaloacetic transaminase, glucose and urea and statistically significant increases in serum glutamic pyruvate transaminase and alkaline phosphatase in treated animals compared to controls, however, the study authors considered these latter two variations as being in the normal range. Among the markers of oxidative stress investigated, there were no significant changes between treated and control animals. The findings might be indicative of systemic effects after dermal administration of nano-hydroxyapatite.

Ref.: Geetha, C.S. *et al.* (2013)

SCCS comment:

It is not clear whether the study was performed according to an OECD/EU guideline and according to GLP principles. According to OECD test guideline 406, the concentration to be used for induction should be the highest to cause mild irritation, whereas for challenge the highest non-irritation dose should be used. It is not clear whether such considerations were made. The study is of limited value for assessment of the sensitising potential of nano-hydroxyapatite. However, the findings might be indicative of systemic effects after dermal administration of needle-shaped nano-hydroxyapatite.

Another study used a material termed as "Hydroxyapatite 5%, aqueous solution, 20 nm" for skin sensitisation in guinea pigs (no information on sex, strain and number of animals used). The test material was injected once at a dose of 20 mg/kg bw (unclear whether this refers to test material or active ingredient) into the outer skin surface of the ear. 10 days later, a solution of the test material was applied to the skin (2 x 2 cm) of the shaved back (100 mg/cm²). It is unclear whether this refers to test material or active ingredient. Control animals received distilled water. Blood was taken 3 hrs after skin application and analysed for WBC, eosinophils, lymphocytes, monocytes and RSL (no explanation is given for the abbreviations "RSL" and "lysis" used in the report). No signs of irritation were observed in treated skin, and there was no difference in the blood parameters investigated between control and treated animals.

Ref.: Nechiporenko, S.P. and Stepanov, S.V. (2011)

SCCS comment:

The study did not adhere to an OECD or EU test guideline and it is not clear whether it was performed according to GLP principles. The test material is insufficiently characterised and it is not clear whether the tested material belongs to the materials covered by the submission. No conclusion can be drawn from this study.

SCCS conclusion on skin sensitization

No guideline-compliant study for skin sensitisation was provided. In a study from the open literature using needle-shaped nano-hydroxyapatite, there were no indications of skin sensitisation. An *in vivo* study performed in guinea pigs cannot be used to assess skin sensitisation due to poor study description and material characterisation.

No conclusion on skin sensitisation can be drawn from the available information.

3.3.4 Dermal / percutaneous absorption

According to information from the applicant(s), a dermal absorption study is underway. However, as submissions are intended for oral use, a dermal absorption study is not necessary for this type of use.

3.3.5 Repeated dose toxicity**3.3.5.1 Repeated Dose (short-term) toxicity**

7-day study, intravenous administration

Two types of hydroxyapatite nanomaterials were used (number 1#: rod-like; 10 – 20 nm in diameter; 30 – 50 nm in length; number 4#: rod-like, 20 – 40 nm in diameter; 70 – 90 nm in length). SD rats (no further explanation of abbreviation, most probably Sprague-Dawley rats) from Shanghai XIPUER-BK Lab Animal Co. Ltd. were used. Nanomaterials were sonicated for 30 min at 300W/50 kHz and suspended at a concentration of 1 mg/ml in normal saline solution containing 10% foetal calf serum. Groups of 5 male and 5 female animals received one of the two types of nanomaterial at 10 mg/kg bw or vehicle control once daily for 7 days by the i.v. route. On day 8, blood was taken for determination of blood cell count with differential and clinical-chemical analysis. Animals were killed; a variety of organs was taken and prepared for histopathological analysis. Haematology revealed no statistically significant differences between control and treated animals. Clinical-chemical analysis revealed statistically significant differences from control of glucose and total bilirubin for #1 material and statistically significant differences from control of glucose, ALT, UA and Na ($p < 0.05$) (no explanations given for abbreviations. UA most probably means uric acid). Histopathological evaluation revealed "pseudotubercles in lung, performing intracavity embolism, inflammatory cell infiltrate and hyperplasy of stroma cell." 1# nanomaterials (i.e. the smaller ones) resulted in a "vacuolar degeneration of nephric tubule epithelium in the kidney." The authors of the publication conclude that a long-term study of systemic toxicity is needed and that the kidney may be the main organ of discharge of nanoparticles. The authors also state that the smaller particles were more toxic than the larger particles and explained this by the larger surface area of the smaller particles.

Ref.: Ding, T. *et al.* (2012)

SCCS comment:

Only the information as presented in the publication was available and there is no information on GLP- or guideline adherence. From the design of the study (only one dose tested, only 7 d administration period, type of administration) the study cannot be taken to determine a point of departure for toxicological risk assessment. However, it can be deduced from the study that smaller particles of nano-hydroxyapatite might be of higher

toxicological potency when systemically available. Furthermore, kidneys and lungs might be considered as target tissues for the systemically available nano-hydroxyapatite.

Oral 3-week study

Synthesised, needle-shaped hydroxyapatite nanoparticles (diameter: 3 – 7 nm; length: 27 – 46 nm; no further specification) were used for the study. No information on GLP is available. The study design did not correspond to an OECD or EU guideline for repeat-dose testing. Groups (n=7) of female Sprague-Dawley rats were either kept untreated (control) or received daily oral treatments (no further specification) with nano-hydroxyapatite at 150 or 300 mg/kg bw/d for 3 weeks. A further group was once injected subcutaneously with 600 mg/kg bw nano-hydroxyapatite and left for 5 weeks. Body weight was recorded weekly. At the end of the experimental period, blood samples were taken for determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), uric acid and urea, animals were killed and liver and kidney samples were taken for histopathology.

No statistically significant effects on body weight were observed. With respect to liver function tests (ALP, ALT and AST), statistically significant increases compared to controls were observed in all treatment groups for ALT and AST. For ALP, statistically significant increases compared to control were only observed in the oral high dose group. There was no change in kidney function parameters investigated apart from a statistically significant decrease of urea in the s.c. group. Kidneys and livers of all animals showed normal structure.

Ref.: Hafez, A.I. *et al.* (2012)

SCCS comment:

Based on changes in liver function enzymes after oral administration of needle-shaped nano-hydroxyapatite particles of the above-mentioned size, it can be deduced that the material investigated will lead to systemic effects related to hepatotoxicity. No histopathological changes were detected in the treated animals' livers or kidneys. However, no other tissue was investigated in this study. Due to the poor description, the poor material characterisation given in the publication and the design of the study, it is considered inappropriate for setting a point of departure for toxicological risk assessment.

Oral 20 day study

A material termed as "Hydroxyapatite 5%, aqueous solution, 20 nm" was intra-gastrically administered for 20 days at a dose of 2,000 mg/kg bw to 3 white rats (strain and sex unclear). Animals were sacrificed one day after the last administration and a variety of parameters were investigated (e.g. weights of certain organs, urinary protein, clinical-chemical parameters). Internal organs were examined macroscopically and lungs, myocardium, livers, kidneys and gastric mucosa were examined histopathologically in treated animals. Amongst the parameters investigated, there was no apparent difference between treated and control animals. In tissues examined histopathologically, there were no abnormalities.

Ref.: Nechiporenko, S.P. and Stepanov, S.V. (2011)

SCCS comment:

The study did not adhere to an OECD or EU test guideline and it is not clear whether it was performed according to GLP principles. The test material is insufficiently characterised and it is not clear whether the doses used correspond to test material as 5% aqueous solution or to the active ingredient. It is also not clear whether the tested material belongs to the materials covered by the submission. No conclusion can be drawn from this study.

Repeated (4 week) intraperitoneal (i.p.) study

In a study from the open literature, a group of male Sprague-Dawley rats (8 per dose group) received a freshly prepared and sonicated saline solution of nano-hydroxyapatite (rod-shaped material with a mean size of 28.47 ± 0.4 nm (20 – 30 nm in length and 5 nm in width) at a concentration of 8.3 mg/ml, dose applied not mentioned), three times per week for three weeks. Further groups of 8 animals received either saline alone, chitosan or a composite of nano-hydroxyapatite and chitosan. At the end of the 4th week, animals were killed and blood was taken for analysis of biochemical markers. Livers and kidneys were taken for histopathological investigation.

After treatment with nano-hydroxyapatite, a statistically significant increase in sodium and a statistically significant decrease in potassium ($p < 0.01$) were observed. All other parameters investigated exhibited no significant change compared to control. Histopathology revealed apoptotic cells in the kidneys and livers of animals treated with nano-hydroxyapatite.

Ref.: Wang, L. *et al.* (2012)

SCCS comment:

The study was not performed according to a guideline and GLP adherence is unclear. The exact dose administered is not clear. The study, however, demonstrates that systemically available nano-hydroxyapatite can induce apoptosis in the liver and kidney of rats. No other organs were investigated. The finding of apoptosis confirms the findings of a panel of *in vitro* studies, where nano-hydroxyapatite induced apoptosis in a variety of cells. However, the study cannot be used as a point of departure for risk assessment. The nanomaterials used in the publication were only poorly described. With respect to size and shape, they might be of relevance for the materials considered for this opinion; however, Zeta potential of the materials tested in the study is not comparable with that of the materials provided in the submissions by applicants (Company A and C, material 1).

Dermal 28-day study

In a study from the open literature, needle-shaped nano-hydroxyapatite particles synthesised by a wet chemical method yielding particles of a size below 50 nm and a Zeta potential of -13.4 mV in buffer (the material was stated to be unstable in water due to a Zeta potential below 3.19 mV) were investigated in a dermal 28-day repeat-dose study. Groups of Wistar rats (5/sex/dose) received a paste of nano-hydroxyapatite in water at 0, 25, 50 and 100 mg/kg bw/d up to 6 hr/day for 7d/week for 28 days. At the end of the exposure period, blood was taken for determination of haematological and biochemical parameters. Gross necropsy was performed in all animals, and major organs (heart, liver, lungs, spleen and skin) were processed for histopathology. Liver homogenate was prepared and analysed for total protein, lipid peroxidation, reduced glutathione and antioxidant enzymes (glutathione reductase, glutathione peroxidase and superoxide dismutase).

There was no effect on food intake, body weight or behaviour. Apart from a statistically significant reduction in haemoglobin, platelets and mean corpuscular volume (MCV) at the highest dose, haematological investigations revealed no other statistically significant differences between control and treated animals. With respect to biochemical parameters there were single incidences of differences between treated and control animals (for chlorides at 50 mg/kg bw/d and alkaline phosphatase at 200 mg/kg bw/d). Gross pathology of major organs revealed no abnormalities. Upon histopathology, a mild infiltration of mononuclear inflammatory cells was observed in the dermis of treated animals. In lungs, seven cases of bronchial associated lymphoid tissue proliferation were observed.

Investigation of liver homogenates revealed changes in lipid peroxidation and reduced glutathione when compared to controls; however, there was no dose-dependency. All antioxidant enzymes investigated were lower compared to control with a dose-dependency for glutathione reductase. Further, superoxide dismutase was decreased in homogenates from treated animals. However, all changes in enzymes investigated did not reach statistical significance. The study authors conclude that the nano-material synthesised did not cause any treatment-related adverse effects after 28 d repeated dermal administration to the skin of Wistar rats.

Ref.: Valappil, M.P. *et al.* (2014)

SCCS comment:

No information on guideline or GLP adherence is available. It remains to be clarified whether some of the effects observed (e.g. increased alkaline phosphatase, reduced glutathione reductase) might be an indication of systemic effects. Also the lung effects observed after histopathological examination deserve clarification. As it is unclear whether the effects observed could be substance-related or not and as the submissions are intended for oral administration, the study is not adequate for setting a point of departure for risk assessment. From the shape and the Zeta potential, the material used is different from the materials under consideration for the SCCS opinion.

3.3.5.2 Sub-chronic (90 days) toxicity (oral)

No data available

3.3.5.3 Chronic (> 12 months) toxicity

No data available

SCCS conclusion on repeat-dose toxicity

No guideline-compliant repeat-dose toxicity study was provided by the applicants or retrieved by literature search. However, some studies with repeated administration of nano-hydroxyapatite have been performed for up to 28 days by the intravenous, intraperitoneal, oral and dermal route. The studies mostly lack proper material characterisation and only a limited amount of parameters and tissues usually investigated in guideline-compliant repeat-dose studies have been addressed in the available studies/study descriptions and only a few doses (in part only single doses) were tested.

The intravenous and intraperitoneal routes are considered as being of minor relevance for the risk assessment of cosmetics. A 7-day repeated dose intravenous study using two types of rod-shaped nano-hydroxyapatite indicated that kidneys and lungs might be the target tissues for systemically available nano-hydroxyapatite. Apoptosis was observed in kidney and liver cells from rats treated i.p. for 4 weeks with rod-shaped nano-hydroxyapatite, although the informative value of this study is limited due to the administration route, insufficient material characterisation and the use of one dose only.

An oral 3-week study pointed to systemic effects of needle-like nano-hydroxyapatite due to increases in liver function enzymes; however, the questionable relevance of the material used and the limited amount of parameters investigated render the study inappropriate for setting a point of departure for toxicological risk assessment. A further oral study is also inappropriate due to major deficits (only one dose investigated, poor reporting, no material characterisation provided). In a dermal 28-day repeat dose toxicity study using needle-shaped nano-hydroxyapatite, there were some indications of systemic effects. However, again due to limited reporting, the questionable relevance of the material used for the

materials under consideration and the route of application different from that of the intended use, the study is not adequate for setting a point of departure for risk assessment. In summary, there is no study available which would allow establishing a point of departure for risk assessment. However, the available studies indicate that needle-shaped nano-hydroxyapatite might be of concern in relation to systemic availability and possible toxic effects.

3.3.6 Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Ames test, described in open literature

Two types of Hydroxyapatite nanomaterials were used (number 1#: rod-like; 10 – 20 nm in diameter; 30 – 50 nm in length; number 4#: rod-like, 20 – 40 nm in diameter; 70 – 90 nm in length). Extracts of nanoparticles were prepared at 0.1 g/ml and 0.1 ml of test article extract was used in the Ames test with *S. typhimurium* strains TA97, TA98, TA 100 and TA 102. S9 mix (no further details) was used as metabolic system. Based on the outcome of the test and comparison with concomitantly tested positive and negative controls (no further information provided) it was concluded that the two nanomaterials investigated were negative in the Ames test.

Ref.: Ding, T. *et al.* (2012)

SCCS comment:

Based on the poor reporting, no conclusions can be drawn from this study; in addition, the SCCS notes that, although the bacterial reverse mutation test is a reliable genotoxicity screen test for soluble chemicals, it does not appear to be suitable for nano-materials. This may in part be related to the size of bacteria, presence of the bacterial cell wall, and the limited or total lack of uptake of nanoparticles by bacteria (Doak *et al.*, 2012; Magdolenova *et al.*, 2014). Consequently, the results obtained with the gene mutation test in bacteria have no value for the assessment of the genotoxic potential of the material(s) tested.

Mouse lymphoma assay

Two types of hydroxyapatite nanomaterials were used (number 1#: rod-like; 10 – 20 nm in diameter; 30 – 50 nm in length; number 4#: rod-like, 20 – 40 nm in diameter; 70 – 90 nm in length). Extracts of nanoparticles were prepared at 0.1 g/ml and 4.5 ml of test article extract was used in the Mouse Lymphoma assay performed. As stated by the authors, the tests were carried out according to the OECD TG 476. MMS (abbreviation not explained in the publication) at 10 µg/ml in treatment medium was used as positive control without S9 and CP (abbreviation not explained in the publication) at 3 µg/ml in treatment medium was used as positive control with S9 mix. Incubation time was 3 hr with S9 and 24 hr without S9. No further information on the source of S9 was given.

Based on the outcome of the study, the authors concluded that the extracts of the two nanomaterials investigated did not cause a positive response with or without S9.

Ref.: Ding, T. *et al.* (2012)

SCCS comment:

Based on the poor description, no conclusions can be drawn from this study.

Turkez *et al.* (2014) investigated sister chromatid exchanges, micronucleus formation, chromosome aberration and 8-oxo-2-deoxyguanosine formation by needle-shaped nano-hydroxyapatite with average particle diameters of 10 – 50 nm in cultured peripheral blood lymphocytes from 6 human donors. In comparison to untreated cultures, dose-dependent increases - in part of statistical significance - of sister chromatid exchanges, micronuclei, chromosome aberration rates and 8-oxo-2-deoxyguanosine levels were observed in treated cultures.

Ref.: Turkez, H. *et al.* (2014)

SCCS comment:

The study used poorly characterised needle-like nano-hydroxyapatite, with limitations in both study design and description, so it is not of value for the SCCS opinion. However, despite these limitations it documents potential genotoxic effects of needle-shaped nano-hydroxyapatite.

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

No data available

SCCS conclusions on Mutagenicity / Genotoxicity

Information on three genotoxicity studies (described above) performed with nano-hydroxyapatite was available from the open literature. It is not clear whether the studies were performed in accordance with the respective OECD- or EU test guidelines. A gene mutation test performed in bacteria cannot be used for the assessment of genotoxicity of nano-hydroxyapatite as this type of study is inappropriate for nanomaterials. No positive response was observed in a mouse lymphoma assay performed with two types of rod-shaped nano-hydroxyapatite. In a study using needle-shaped nano-hydroxyapatite, dose-dependent increases in sister chromatid exchanges, micronuclei, chromosome aberration rates and 8-oxo-2-deoxyguanosine levels were observed, pointing to genotoxic potential of needle-shaped nano-hydroxyapatite. Due to poor material description and limitations in study design used in the mouse lymphoma assay and in the latter study, the relevance of the findings for the materials of the submission remains unclear. No conclusion could thus be drawn on the genotoxicity/mutagenicity of nano-hydroxyapatite, although the available information from Turkez *et al.* (2014) indicated that needle-shaped nano-hydroxyapatite might be of concern in relation to genotoxicity.

3.3.7 Carcinogenicity

No data available

SCCS conclusion on carcinogenicity

Due to the absence of data, no conclusions can be drawn on the carcinogenicity of nano-hydroxyapatite.

3.3.8 Reproductive toxicity

3.3.8.1 Two generation reproduction toxicity

/

3.3.8.2 Other data on fertility and reproduction toxicity

No data available

3.3.8.3 Developmental Toxicity

No data available

SCCS conclusion on reproductive toxicity

Due to the absence of data, no conclusions can be drawn on the reproductive toxicity of nano-hydroxyapatite.

3.3.9 Toxicokinetics**3.3.9.1 Toxicokinetics in laboratory animals****Absorption**

A low oral bioavailability can be anticipated due to high molecular weight and low water solubility at least for the non-micronised and non-nano-sized forms of hydroxyapatite. However, as described in Scheel and Hermann (2011), as the pH goes down in the acidic range (starting from pH values of appr. 5), hydroxyapatite becomes increasingly soluble, thus explaining biodegradation by macrophages in the lysosomes.

From the oral 3-week repeat-dose study described in section 3.3.5.1, it can be deduced that needle-shaped hydroxyapatite nanoparticles (diameter: 3 – 7 nm; length: 27 – 46 nm) may exert systemic effects, as statistically significant changes were observed in the liver markers ALT, AST and ALP after oral administration of this material. No conclusion on the oral absorption is possible based on the available information.

In order to assess possible translocation into cells or cell layers, penetration through a mucosa-like human corneal epithelial (HCE) tissue model (SkinEthic®) was investigated. Following pre-incubation of HCE tissues models in SkinEthic HCE culture medium overnight, models were treated with 50 µl of test samples (0.5% and 1% of each a hydroxyapatite-protein composite (non nano-material), plate-like nano-hydroxyapatite (dimension in nm: 3 x 20 x 45) and needle-like nano-hydroxyapatite (dimensions in nm: 3 x 20 x 100) for 3 min by simulating slight mechanic stress (tooth brushing) by placing the cultures on a shaker and rinsing two times afterwards with 0.5 ml phosphate-buffered saline. Afterwards, HCE models were investigated by Cryo-TEM analysis in the different compartments of the tissue. No indication of penetration of the composite into tissue and cells could be detected in the TEM images, and only rarely there was an indication for penetration for the needle-shaped material.

Ref.: Scheel, J. and Hermann, M. (2011)

SCCS comment:

Despite the insufficient description and the screening nature, the experiment demonstrates that needle-shaped nano-hydroxyapatite may penetrate into mucosa-like human corneal epithelial cells.

Distribution

From the 7-day intravenous study described in section 3.3.5.1, it can be deduced that systemically available nano-hydroxyapatite particles might be distributed into lungs and kidneys as histopathological changes were observed in these two organs.

Ref.: Ding, T. *et al.* (2012)

From a further intravenous study it can be inferred that nano-hydroxyapatite particles are transported to the liver and cause inflammatory cell infiltration in the portal areas and oxidative damage.

Ref.: Chen, Q. *et al.* (2014)

Metabolism and excretion

Nano-hydroxyapatite particles are assumed to be converted into Ca^{2+} and PO_4^{3-} and excreted afterwards.

Ref.: Hafez, A.I. *et al.* (2012)

3.3.9.2 Toxicokinetics in humans

No information provided

SCCS conclusion on Toxicokinetics

The available information points to the fact that some forms of nano-hydroxyapatite might exert systemic effects after administration by the dermal and oral route. Further, nano-hydroxyapatite might be taken up by mucosal-like corneal epithelial cells. These indications mainly stem from studies performed with needle-shaped nano-hydroxyapatite. Apart from one study demonstrating uptake in human buccal cells of spherically shaped nano-hydroxyapatite (see section 3.3.12.2), no information is available on the systemic effects or systemic uptake (absorption) of nano-hydroxyapatite in non-needle-like shape.

Based on the available information, systemic effects or systemic uptake of orally administered nano-hydroxyapatite cannot be excluded. This is supported by the observed cellular uptake in *in vitro* studies performed with nano-hydroxyapatite. From the limited information available it can be supposed, that if systemically available, nano-hydroxyapatite will be distributed to liver, kidneys and lungs.

Nano-hydroxyapatite particles have been assumed to be converted into Ca^{2+} and PO_4^{3-} and excreted afterwards.

3.3.10 Photo-induced toxicity

3.3.10.1 Phototoxicity / photo-irritation and photosensitisation

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3.3.10.2 Photomutagenicity / photoclastogenicity

/

3.3.11 Human data

An acute cutaneous irritation test (patch test) was performed in order to evaluate the cutaneous compatibility of material 1 (Company C) after a single application to the skin. The test was performed with 10 Caucasian healthy volunteers of both genders, aged between 18 and 65 years old selected according to inclusion/exclusion criteria as laid down in the report after informed consent. The test consisted of a single application of material 1 (Company C)

at 25% (aqueous solution with adjusted pH of 5.2) to the volunteers' backs with an occlusive patch (Finn chambers on Scanpor) for 48 hours. Purified water served as negative control, SDS (1% in aqueous solution) served as positive control. The skin compatibility was assessed visually for erythema and oedema 30 minutes after patch removal and scored on a four-point scale. The score obtained from negative controls was deduced from the scoring of the test material. Erythema score 1 was reported in 2 volunteers after deduction of negative control score (no further information). Dryness of skin was reported for 5/10 volunteers. The study authors concluded that material 1 (Company B) 25% adjusted to pH 5.2 is non-irritating.

Ref.: Inovapotek (2013b)

SCCS comment:

A skin irritating potential of material 1 (Company B) 25% adjusted to pH 5.2 cannot be excluded.

3.3.12 Special investigations

3.3.12.1 Cytotoxicity studies

| | |
|-------------------|---|
| Guideline: | ISO 10993-5 |
| Test system: | L-929 mouse fibroblast cells |
| Replicates: | 3 |
| Test substance: | Nano-Hydroxyapatite paste (a white viscous paste composed of nanoparticles of hydroxyapatite and water; strength: 38% hydroxyapatite) |
| Batch: | 1309-12 |
| Purity: | > 95% |
| Concentrations: | |
| Vehicle: | 1X DMEM |
| Treatment: | 10 g paste/kg bw |
| Treatment period: | |
| GLP: | Yes |
| Study period: | September - October 2012 |

Test substance, vehicle control, positive control (powder free latex gloves (30.8 cm² in 10 ml 1X DMEM) and negative control (high density polyethylene (60 cm² in 10 ml 1X DMEM)) were "extracted" in vehicle by agitating for 24 hr at 37°C. Extracts were not centrifuged, filtered or otherwise altered prior to dosing (i.e. the test material was in a suspension form). Triplicate, subconfluent monolayers of L-929 mouse fibroblast cells cultivated in 10 cm² dishes were treated with 2.0 ml of either test substance extract (0.2 g substance/ml, corresponding to 0.076 g a.i./ml) or vehicle control or extracts of positive and negative controls. Cell wells were then incubated at 37°C in 5% CO₂ for 48 hr. Following incubation, cells were examined microscopically to evaluate cellular characteristics and percent lysis. The test article showed no evidence of causing cell lysis or toxicity, no pH shift was observed. In extracts from positive control on the other hand, rounding of cells, 100% of cells without intracytoplasmic granules and 100% lysis was observed.

Ref.: NAMSA (2012c)

SCCS comment:

No material characterisation is given in the study report. However the applicant confirmed that this study had been performed with material 1 of the submission. There were no indications of cytotoxicity of material 1 under the test conditions applied.

A description of a cytotoxicity assay with material 1 (Company C) in osteoblasts conducted in a university laboratory was provided by one of the applicants.

The following hydroxyapatite formulations were used:

- hydroxyapatite spray dried powder with 5.0 µm (no further information)
- nano-hydroxyapatite paste at 30%

The formulations were added to cells diluted in culture medium at concentrations of 50, 100, 500, 1000 and 5000 µg/mL and cells were then incubated for 1, 3 and 7 days. Control cell cultures were also used, in the absence of the test formulations. The cell viability was assessed by the MTT assay, the cell's cytoskeleton and nucleus were stained and observed by confocal microscopy. At each time point cell viability was not affected when incubated with hydroxyapatite spray dried powder at concentrations up to 100 µg/mL and the morphology of cells incubated at this concentration was similar to controls used. For higher concentrations, cell viability decreased in a dose-dependent manner. At a concentration of 5000 µg/mL the cell viability was impaired and the "cytoplasmic volume" of the cells decreased. When the nano-hydroxyapatite paste at 30% was used, no significant alterations on cell viability were observed up to a concentration of 1000 µg/mL and no morphologic differences were observed up to this concentration. For both products at concentrations of 5000 µg/mL, the cytoplasmic volume of cells decreased.

Ref.: submission dossier Company C

SCCS comment:

The study report was not provided for evaluation, the summary given by the applicant is considered insufficient. From the summary it can be deduced that concentrations above 100 and 1000 µg/ml of hydroxyapatite spray dried powder and nano-hydroxyapatite paste, respectively, are toxic to osteoblasts.

Fan, *et al.* (2011) investigated the cytotoxicity of nano-hydroxyapatite (particle size about 67.8 ±22.5 nm; colloidal size about 93 nm, SSA about 32.2 m²/g, Zeta potential -10 ±1.8 mV (the medium in which Zeta potential was determined was not stated) in human bronchial epithelial BEAS-2B cells. Cytotoxicity was assessed by quantifying double-stranded DNA by the Pico Green assay and by the WST-8 assay. Cells were grown in the presence of 30, 100 and 300 µg/ml nano-hydroxyapatite and readouts were made by fluorescence (Pico Green) or photometry (WST-8 assay). Although cell viability decreased dose-dependently by both assays, there was no statistical significance of the findings.

Ref.: Fan, Q. *et al.* (2011)

SCCS comment:

The study points to only mild cytotoxic effects of the material used. The Zeta potential of the test materials is different from the materials of the submission.

The same study investigated the influence of a 1% solution of nano-hydroxyapatite on a commercially available clinical pulmonary surfactant. A time-dependent deterioration of the surfactant was observed attributed to protein adsorption on the nanomaterials. When combining these results with the cytotoxicity experiments performed in this same study, the authors conclude that determination of cytotoxicity alone might not be sufficient for concluding on toxicological effects in lung tissue.

Two independent measurements of cytotoxic effects of hydroxyapatite nanoparticles (no further information given in the report) by the Neutral red uptake assay were performed in

BALB/c 3T3 cells. Sodium Lauryl Sulfate (SLS) was used as positive control. As a result, only raw data from microplate readings were given and no conclusions were drawn from these readings by the study authors.

Ref.: Feofanov, A.V. (2015)

SCCS comment:

The study report is of no value as no characterisation was provided for the material investigated.

3.3.12.2. Further *in vitro* investigations

The *in vitro* studies available in the published scientific literature are summarized in a Table (Table 2) annexed to this opinion. However, only the relevant *in vitro* studies from the large number of available publications are described below.

In a study from the open literature, the subcellular distribution and apoptotic profile of nano-hydroxyapatite with a view to the use of the material in oral care products was investigated in commercially available human buccal epithelial cells TR146. Commercially available nano-hydroxyapatite of spherical shape, with a size of 51.1 ± 12.1 nm and a Zeta-potential of -5.41 ± 0.59 mV in DMEM/F12 biological medium, was used. For determination of cellular uptake, fluorescein isothiocyanate (FITC)-labelled nano-hydroxyapatite was prepared from the commercially obtained material (no further characterisation given). For determination of cellular localisation, cells were cultivated for 12 hr with 125 and 1250 μ M of FITC-labelled nano-hydroxyapatite, cytosolic and membrane fractions were prepared and fluorescence measurements performed. Furthermore, cells treated for 12 hr with 1250 μ M FITC-labelled nano-hydroxyapatite were analysed microscopically. Intracellular reactive oxygen species was investigated by the 2',7'-dichlorofluorescein diacetate assay (DFCH-DA assay) after cultivating cells for 24 hr in the presence of 0, 62.5, 125, 250, 500, and 1250 μ M nano-hydroxyapatite. To detect mitochondrial specific superoxide, cells were subsequently counterstained with MitoSox Red and analysed by cytometer. Inflammatory response was investigated by IL-6 determination via PCR, whereas NF- κ B was investigated by reporter gene assay after 6 hr treatment of cells with 0, 62.5, 125, 250, 500 and 1250 μ M nano-hydroxyapatite. Apoptosis and signalling pathways were investigated by a commercially available Annexin V and dead cell assay kit and by immunoblotting.

Results:

At 125 μ M of FITC-labelled nano-hydroxyapatite the distribution between cytoplasmic and membrane fractions was almost equal, whereas a significant retention in the membrane fraction was observed at 1250 μ M. Cross-sectional confocal acquisition of live cells incubated with FITC-labelled nano-hydroxyapatite revealed a higher amount of nanomaterial located nearer to the apical (top) end of the cells. A significant, concentration-dependent increase in ROS formation was observed reaching a 40% increase at 1250 μ M. Counterstaining with MitoSox suggested that nano-hydroxyapatite stimulates mitochondrial superoxide production in TR146 cells. In the concentration range investigated, nano-hydroxyapatite significantly increased IL-6 (up to 4-fold) expression and NF- κ B transcriptional activity compared to untreated controls. With respect to apoptosis, cell retained high viability (>90%) and percentage of necrotic cells did not change in the concentration range investigated. Cells treated with nano-hydroxyapatite concentrations at 250 μ M and higher displayed only a modest increase in early apoptotic cells by approximately 4%, but a significant increase in percentage of late apoptotic cells by almost 2 fold compared to the untreated control.

Ref.: Tay, C. *et al.* (2014b)

SCCS comment:

The study demonstrates that FITC-labelled nano-hydroxyapatite can be taken up by human oral epithelial cells and preferentially accumulates near the apical cell membrane. In the cells treated with non-FITC-labelled nano-hydroxyapatite, oxidative stress along with increased expression of inflammatory genes and apoptosis can be induced. The Zeta potential of the material investigated differs from that reported for material 1 of the submissions.

Albrecht *et al.* (2009) investigated the biocompatibility of five hydroxyapatite materials of different size and morphology as described in Table 1, i.e., nano/needle-shaped (HA-NN), nano/rod-like (HA-NR), nano/plate-like (HA-NP), fine/dull needle-shaped (HA-FN), and a hydroxyapatite–protein-composite (HPC). Materials were investigated in rat NR8383 cells and primary alveolar macrophages from female Wistar rats at concentrations up to 3000 µg/ml.

Table 1: Material characterisation for hydroxyapatite forms investigated in Albrecht *et al.*, 2009.

| Material | Morphology | Average particle size [nm] | Specific surface area [m ² /g] |
|----------------|--|----------------------------|---|
| HPC (non-nano) | irregular shaped | 1200 x 2100 | 67 |
| HA-NP | mainly nano-sized plates | 3 x 20 x 45 | 154 |
| HA-NR | mainly nano-sized rods | 5 x 90 | 166 |
| HA-NN | needles | 3 x 20 x 100 | 106 |
| HA-FN | intermediate morphology between rods and needles | 95 x 740 | 27 |

Lipopolysaccharide (LPS) and DQ12 quartz served as positive controls. In the water-soluble tetrazolium salt 1 (WST-1) and lactate dehydrogenase (LDH) assays with NR8383 cells, no cytotoxicity was observed for HPC and the pure hydroxyapatite samples up to 3000 µg/ml, while HA-FN showed a significant effect at the highest dose in the LDH assay. In primary cells, no cytotoxicity based on LDH was observed with all samples up to 300 µg/ml. ROS generation measured by electron paramagnetic resonance (EPR) technique was significantly enhanced with HA-NN and HPC in NR8383 cells. No effect was detected in primary cells, which are considered more relevant to physiological conditions. All hydroxyapatites elicited TNF-α release from the NR8383 cells, but with significantly lower potency than DQ12 quartz and LPS.

Ref.: Albrecht, C. *et al.* (2009)

SCCS comment:

The original study report for this publication was not available for evaluation. The study demonstrates that nano-hydroxyapatite needles enhanced ROS formation in macrophage cell lines but not in primary cells. Furthermore, based on LDH, the highest dose of

hydroxyapatite nanoneedles was cytotoxic in cell lines. The Zeta potentials are not given in the publication. Therefore it remains unclear whether the results of this study are relevant to the materials under evaluation and can be considered for the SCCS opinion.

The same hydroxyapatite materials as used in Albrecht *et al.* (2009) were investigated in RAW264.7 macrophages, a murine macrophage cell line, for cytotoxicity (XTT release), inflammatory mediators (TNF- α -release) and nitrogen oxide production. RAW 264.7 macrophages were incubated with:

- Nano-hydroxyapatite, rod-like (HA-NR),
- Nano-hydroxyapatite, plate-like (HA-NP) (glycerol/water dispersion with 5.3% HA-NP),
- Nano-hydroxyapatite, needle-shaped (HA-NN)
- Fine hydroxyapatite, blunt-ended needles (HA-FN) and
- Hydroxyapatite–protein-composite (irregularly shaped - HPC) (glycerol/water dispersion with 9.5% HPC).

The used concentrations ranged from 50 to 5000 $\mu\text{g/ml}$. Cells were analysed for viability, cytokine production and induction of nitrogen oxide after 18 and 42 hr with DQ12 quartz and lipopolysaccharide (LPS) as positive controls.

Results Cytotoxicity:

Viability of RAW 264.7 macrophages was impaired above concentrations of 500 $\mu\text{g/ml}$ by the test samples at both time points.

Results TNF- α -release:

- HA-NR: significant induction after 18 hr at a concentration of 100 $\mu\text{g/ml}$ (4.8 times higher than the induction by DQ12 at 75 $\mu\text{g/ml}$, and about 28% of the TNF- α release induced by 10 $\mu\text{g/ml}$ LPS). At 500 $\mu\text{g/ml}$, the signal induced by HA-NR was significantly reduced compared to 100 $\mu\text{g/ml}$, and further decreased towards the cytotoxic range. After 42 hr, a slight TNF- α secretion was found at the lowest concentration.
- HA-NP: highest TNF- α release after 18 hr at a concentration of 500 $\mu\text{g/ml}$ (slightly but significantly lower than for DQ12 quartz at 75 $\mu\text{g/ml}$). After 42 hr, detectable induction through HA-NP could only be found at 1000 $\mu\text{g/ml}$.
- HA-NN: dose-dependent increase at both time points, peak-levels reached at 1000 $\mu\text{g/ml}$.

Results nitrogen oxide formation:

Nitrogen oxide release in RAW 264.7 macrophages after 18 hr was within the range between 0.4 and 8.4 nmol nitrogen oxide/ml for HA-NR, HA-NP and HA-NN, and was thus an order of magnitude below the LPS-induced effects. Incubation of macrophages with HA-NR and HA-NN for 42 hr resulted in a slight increase in nitrogen oxide release relative to the other test substances, in particular at 1000 and 5000 $\mu\text{g/ml}$.

Ref.: Scheel, J. *et al.* (2009)

SCCS comment:

The original study report from this publication was not available for evaluation. The study demonstrates that the nanomaterials investigated have an effect on cell viability, TNF- α release and nitrogen oxide production (indicating macrophage activation). As Zeta potentials are not given in the publication, it remains unclear whether the results of this study are relevant to the materials under evaluation and can be considered for the SCCS opinion.

Four different nano-hydroxyapatite materials with different nanocrystal morphologies (short rod-like (SSA 68.45 m²/g), long rod-like (specific surface area (SSA) 45.00 m²/g), spherical (SSA 122.48 m²/g) or needle-shaped (SSA 148.14 m²/g) crystals), and different sizes (10–20, 10–30 or 20–40 nm) were investigated in primary cultured rat osteoblasts with respect to growth, inhibition and apoptosis. The osteoblasts were treated with the four types of nano-hydroxyapatite at various concentrations (20, 40, 60, 80 or 100mg/L). The cell growth rate was detected using the MTT assay; apoptotic alterations and the level of reactive oxygen species in osteoblasts were measured using flow cytometry; the amounts of apoptotic p53 and cytochrome c proteins were measured using Western Blotting. All four types of nano-hydroxyapatite inhibited the growth of osteoblasts in a dose-dependent manner. These nano-hydroxyapatite materials significantly induced apoptosis in osteoblasts. Nano-hydroxyapatite materials with smaller specific surface areas induced lower apoptosis rates. Reactive oxygen species production was significantly ($p < 0.01$) increased compared to negative control after treatment with all the nano-hydroxyapatite materials investigated. The needle-shaped and the short rod-like particles induced greater cellular injury than the spherical and long rod-like particles, respectively. The increased apoptosis rates were accompanied by increased p53 and cytochrome c expression. These findings indicate that nano-hydroxyapatite materials inhibit the proliferation of osteoblasts and induce their apoptosis *in vitro*.

Ref.: Xu, Z. (2012)

SCCS comment:

This study from the open literature points to cytotoxic effects of different forms of nano-hydroxyapatite to osteoblasts. However, Zeta potentials of the tested materials are not given in the publication and it remains unclear whether the results of this study are relevant to the materials under consideration.

Liu *et al.* (2010) explored the effect of nano-hydroxyapatite (rod-like crystals, smallest parts 20 – 30 nm; less than 10% > 100 nm) on steroid hormone production and apoptosis in human ovarian granulosa cells. Uptake of nanoparticles in human ovarian granulosa cells was evaluated by transmission electron microscopy (TEM). The cell cycle was assessed with propidium iodide-stained cells (distribution of cells in G0/G1, S, and G2/M phases) by flow cytometry. The pattern of cell death (necrosis and apoptosis) was analysed by flow cytometry with annexin V-FITC/PI staining. The expression of mRNAs encoding cholesterol side chain cleavage enzyme (P450scc), P450 aromatase (P450arom) and steroidogenic acute regulatory protein (StAR) were determined by RT-PCR. Progesterone and estradiol levels were measured by radioimmunoassay.

TEM results confirmed uptake of nano-hydroxyapatite into granulosa cells and distribution into membrane-bound compartments, including lysosomes, mitochondria and intracellular vesicles. The increased percentage of cells in S phase when cultured with nanoparticles indicated that there was an arrest at the checkpoint from phase S-to-G2/M (from $6.28 \pm 1.55\%$ to $11.18 \pm 1.73\%$, $p < 0.05$). The increased ratio of S/(G2/M) implied the inhibition of DNA synthesis and/or impairment in the transition from the S phase. The apoptosis rate of normal granulosa cells was $7.83 \pm 2.67\%$ and increased to $16.53 \pm 5.56\%$ ($p < 0.05$) after the cells had been treated with 100 μM nano-hydroxyapatite for 48 hours. Treatment of cells with nano-hydroxyapatite at concentrations between 10-100 μM did not significantly change the progesterone or estradiol levels in culture medium, or the expression levels of mRNAs encoding P450scc, P450arom and StAR after 48 hr and 72 hr treatments.

Ref.: Liu, X. *et al.* (2010)

SCCS comment:

This study from the open literature indicates uptake, apoptotic effects and effects on cell cycle of nano-hydroxyapatite in human ovarian granulosa cells, but steroid hormone production was unaffected. As Zeta potentials are not given in the publication, it remains

unclear whether the results of this study are relevant to the materials under evaluation and can be considered for the SCCS opinion.

Summary on *in vitro* studies (described in the above text and annexed in table 2 at the end of the opinion):

From the literature search performed on behalf of the Commission, a considerable number of *in vitro* studies on nano-hydroxyapatite was identified and reviewed by the SCCS. A variety of different cell types were investigated in these studies. As material characterisation in many of these studies was insufficient, it is not clear whether and to what extent the results from these studies are of relevance to the materials under consideration and, therefore, for this SCCS opinion. However, the results of the *in vitro* studies indicate a few important aspects relating to the safety assessment of hydroxyapatite materials which are summarised as follows:

Cellular uptake

Nano-hydroxyapatite materials are readily taken up by exposed cells. Some *in vitro* studies have shown preferential localisation close to the membrane, which might be accompanied by formation of a special cellular structure called SCC (surface-connected compartment, considered as a structure, where nano-hydroxyapatite might be sequestered). Other studies demonstrate the occurrence on nano-hydroxyapatite in the cytoplasm and also in the nucleus.

Studies also point to the fact that cellular uptake is dependent on physicochemical parameters (e.g. shape, size, crystallinity) but also that (small) agglomerates of hydroxyapatite nanoparticles might be taken up by cells.

Studies using red blood cells have demonstrated that external adherence of nano-hydroxyapatite to erythrocytes (which might be of relevance to the nano-hydroxyapatite material available in blood) might also have an impact on these cells (e.g. on sedimentation, aggregation).

Cellular changes

Studies have demonstrated morphological changes of cells after treatment with nano-hydroxyapatite; contrasting results were reported for cytotoxicity. Whereas the majority of studies have reported cytotoxic effects when treating cells with nano-hydroxyapatite, some studies report proliferative effects or absence of effects. Cytotoxicity seems to be dependent on the cell type used and physicochemical parameters (e.g. shape, size, crystallinity) of the nano-hydroxyapatite material. It is also hypothesised that increased amounts of intracellular calcium are responsible for cytotoxic effects.

Studies in osteoblasts point to an influence of nano-hydroxyapatite on increasing cell proliferation. Some studies indicate an influence of nano-hydroxyapatite on the cell cycle.

Downstream effects:

Studies point to the induction of apoptosis as well as necrosis after treatment with nano-hydroxyapatite. Studies further demonstrate the induction of oxidative stress and inflammatory changes in cells after treatment with nano-hydroxyapatite.

Thus, if systemically available, nano-hydroxyapatite might affect cells when it adheres to the plasma membrane from outside (e.g. of blood cells) or when it reaches the intracellular compartment.

3.3.13 Safety evaluation (including calculation of the MoS)

There is no data available which would allow setting a point of departure for risk assessment. However, there are indications that nano-hydroxyapatite might exert systemic effects after oral administration either via oral mucosa or by swallowing.

CALCULATION OF THE MARGIN OF SAFETY

Not possible

3.3.14 Discussion

Only a limited amount of data in line with section 1.3. of SCCS 1484/12 was provided. Almost all studies provided were not compliant with relevant test guidelines in terms of study design and some of them did not use the materials of the submission (material 1 and 2).

In some of the available studies/ study descriptions, material characterisation was poor or absent, and it is not clear whether and to what extent the investigated materials corresponded to the materials of the submission.

No study has been identified that would allow the identification of a point of departure for risk assessment. On the other hand, there are indications from studies published in the open literature for hydroxyapatite materials that are different from the materials 1 and 2 showing that they might be taken up locally (e.g. into buccal cells) and that nano-hydroxyapatite might exert systemic effects after oral administration. As no information on long-term exposure is available, it is not possible to draw any conclusion whether repeated, long-term systemic exposure to nano-hydroxyapatite (which can be anticipated from the product type) would manifest in adverse effects as indicated in the scientific literature (e.g. expressed in Fox *et al.*, 2012).

Also if locally taken up into cells, there are clear indications that this might be a concern as many nano-hydroxyapatite materials have been shown to be easily taken up into cells where they have been shown to exert a variety of effects (cytotoxicity, induction of oxidative stress, apoptosis, inflammatory responses).

In summary, based on the information available, SCCS considers needle-like nano-hydroxyapatite as a concern in relation to potential toxic effects. It is of note that material 2 of the submission is stated to cover nano-hydroxyapatite of any shape and that it definitely contains nanofibres of 2-4 nm width and 20 – 50 nm in length which can be interpreted as nanoneedles and which are very similar in shape to nano-needle-shaped material as described in the literature. However, whether other shapes of nano-hydroxyapatite including material 1 of the submission might be of concern or not for the consumer when used up to a concentration of 10% in oral cosmetic products cannot be decided on the basis of the data available.

Physicochemical properties

Hydroxyapatite is a naturally occurring, water-insoluble mineral of a molecular weight of 502.31 g/mol. Hydroxyapatite is of hexagonal crystal structure comprising different crystal phases. The OH⁻ ions in hydroxyapatite can be replaced by different counter anions to form other members of the apatite group. Nano-hydroxyapatite materials added to oral cosmetic products are listed either as powder or suspension. Two different materials (material 1 and 2) are covered by the submission.

Material 1 is characterised by a specific surface area of 80 – 100 m²/g and a Zeta potential of + 30 ± 1 mV. The material is reported to be in the form of nanorod or nanothumb shape forming agglomerates with particle size > 100 nm, however according to TEM nanorod shaped entities with size below 100 nm are observable.

Primary particle size is reported as 20 nm (lowest cut-off level); 60 to 400 nm (volume weighted median); and 30 nm to 80 nm (number weighted median).

Material 2 is characterised by a specific surface area of 147 m²/g and Zeta potential is stated not to be measurable. The material is reported to consist of 'isometric aggregates consisting of particles of any shape and fibres formed from the same particles.' In theory,

this could also include needle-shaped crystalline form of nano-hydroxyapatite. Size of nanofibres is reported to be 2 – 4 nm thickness and 20 – 50 nm length, whereas a mean size of 15 nm is reported for plates. Material 2 forms aggregates in a size range from 50 nm to 1.4 µm.

Reported shelf-lives of both materials are > 18 months.

These characterised materials however do not necessarily correspond to those materials which have been used for toxicity testing.

Function and uses

Nano-hydroxyapatite is used as a drug delivery material and as a bone defect filling material. Oral care products containing nanoparticulate hydroxyapatite are intended to be used for teeth remineralisation. The material is intended for use in toothpaste, tooth whitener and mouthwash products at a final concentration of up to around 3.1% of nano-hydroxyapatite. Products listed in the dossiers are either in the form of paste or suspension. All products listed in the dossiers are intended for oral application.

Toxicological Evaluation

Acute toxicity

The acute toxicity of nano-hydroxyapatite was investigated by the oral, inhalation and intravenous routes. Neither study was performed in accordance with an OECD or EU Test guideline and apart from one intravenous study published in the open literature (Chen *et al.*, 2014), proper material characterisation was not given. Therefore, no conclusions on acute toxicity could be drawn from these studies. The study from the open literature that used needle-shaped nano-hydroxyapatite indicated that needle-shaped crystals of hydroxyapatite can be a concern in relation to acute toxicity.

Irritation and Corrosivity

No guideline compliant skin or mucous membrane irritation test was provided by the applicants. The studies/study descriptions mainly lacked proper material characterisation. No conclusions could be drawn with respect to skin irritation. There are however indications that nano-hydroxyapatite might be irritating to mucous membranes.

Dermal absorption

Not relevant for this opinion. However, one study using needle-shaped nano-hydroxyapatite indicated systemic effects after dermal administration of nano-hydroxyapatite indicating potential dermal absorption.

Skin Sensitization

No guideline-compliant study for skin sensitisation was provided. In a study from the open literature using needle-shaped nano-hydroxyapatite there were no indications of skin sensitisation. An *in vivo* study performed in guinea pigs could not be used to assess skin sensitisation due to poor study description and material characterisation.

No conclusion on skin sensitisation could be drawn from the available information.

Repeated-dose toxicity

No guideline-compliant repeat-dose toxicity study was provided by the Applicants or retrieved from literature search. However, some studies with repeated administration of nano-hydroxyapatite have been performed for up to 28 days by the intravenous, intraperitoneal, oral and dermal route. These studies mostly lacked proper material characterisation and only addressed a limited amount of parameters and tissues compared to those usually investigated in guideline-compliant repeat-dose studies and only few doses (or only single doses) were tested.

The intravenous and intraperitoneal routes are considered as being of minor relevance for the risk assessment of cosmetics. However, a 7-day repeated dose intravenous study using two types of rod-shaped nano-hydroxyapatite indicated that kidneys and lungs might be target tissues for systemically available nano-hydroxyapatite. Apoptosis was observed in kidney and liver cells from rats treated i.p. for 4 weeks with rod-shaped nano-hydroxyapatite, although the informative value of this study is limited due to the administration route, insufficient material characterisation and the use of one dose only.

A 3-week oral study pointed to systemic effects of needle-like nano-hydroxyapatite due to increases in liver function enzymes; however, the relevance of the material used and the limited amount of parameters investigated render the study inappropriate for setting a point of departure for toxicological risk assessment. A further oral study is also inappropriate due to major deficiencies (only one dose investigated, poor reporting, no material characterisation provided). In a dermal 28-day repeat dose toxicity study using needle-shaped nano-hydroxyapatite there were some indications of systemic effects. However, again due to limited reporting, the questionable relevance of the material used for the materials under consideration, and the route of application being different from that of the intended use, the study is not considered adequate for setting a point of departure for risk assessment.

In summary, there is no study available which would allow establishing a point of departure for risk assessment. However, available studies indicate that needle-shaped nano-hydroxyapatite might be of concern in relation to systemic availability and possible toxic effects.

Mutagenicity

Information on three genotoxicity studies performed with nano-hydroxyapatite was available from the open literature. It is not clear whether the studies were performed in accordance with the respective OECD- or EU test guidelines. A gene mutation test performed in bacteria cannot be used for the assessment of genotoxicity of nano-hydroxyapatite as this type of study is inappropriate for nanomaterials. No positive response was observed in a mouse lymphoma assay performed with two types of rod-shaped nano-hydroxyapatite. In a study using needle-shaped nano-hydroxyapatite, dose-dependent increases in sister chromatid exchanges, micronuclei, chromosome aberration rates and 8-oxo-2-deoxyguanosine levels were observed pointing to genotoxic potential of needle-shaped nano-hydroxyapatite. Due to poor material description and limitations in study design used in the mouse lymphoma assay and in the latter study, the relevance of the findings for the materials of the submission remains unclear. No conclusion could thus be drawn on the genotoxicity/mutagenicity of nano-hydroxyapatite. The available information indicated that needle-shaped nano-hydroxyapatite might be of concern in relation to genotoxicity.

Carcinogenicity

Due to the absence of data, no conclusions could be drawn on the carcinogenicity of nano-hydroxyapatite.

Reproductive toxicity

Due to the absence of data, no conclusions could be drawn on the reproductive toxicity of nano-hydroxyapatite.

Toxicokinetics

The available information points to the fact that nano-hydroxyapatite might exert systemic effects after administration by the dermal and oral route. Further, nano-hydroxyapatite might be taken up by mucosal-like corneal epithelial cells. These indications mainly stem from studies performed with needle-shaped nano-hydroxyapatite. Apart from one study demonstrating uptake in human buccal cells of spherically shaped nano-hydroxyapatite, no information is available on possible systemic uptake (absorption) or systemic effects of nano-hydroxyapatite with non-needle shape.

Based on the available information, systemic effects or systemic uptake of orally administered nano-hydroxyapatite could not be excluded. This is supported by the observed cellular uptake in *in vitro* studies performed with nano-hydroxyapatite. From the limited information available it can be supposed, that if systemically available, nano-hydroxyapatite will be distributed to liver, kidneys and lungs.

Nano-hydroxyapatite particles have been assumed to be converted into Ca^{2+} and PO_4^{3-} and excreted afterwards.

Special investigations (in vitro studies)

Nano-hydroxyapatite materials are readily taken up into exposed cells. Some *in vitro* studies showed preferential localisation close to the membrane, others demonstrate the occurrence on nano-hydroxyapatite in the cytoplasm and also in the nucleus.

Studies also point to the fact that cellular uptake is dependent on physicochemical parameters (e.g. shape, size, crystallinity) but also that (small) agglomerates of hydroxyapatite nanoparticles might be taken up by cells. Studies using red blood cells have demonstrated that external adherence of nano-hydroxyapatite to erythrocytes might also have an impact on these cells.

Studies have demonstrated morphological changes of cells after treatment with nano-hydroxyapatite; contrasting results were reported for cytotoxicity, which seems to be dependent on the cell type used and physicochemical parameters (e.g. shape, size, crystallinity) of the nano-hydroxyapatite material. It has been hypothesised that increased amounts of intracellular calcium are responsible for cytotoxic effects. Some studies have indicated an influence of nano-hydroxyapatite on the cell cycle.

Studies have also pointed to the induction of apoptosis as well as necrosis after treatment with nano-hydroxyapatite. Studies further demonstrated the induction of oxidative stress and inflammatory changes after treatment with nano-hydroxyapatite. Thus, if systemically available, nano-hydroxyapatite might affect cells when it adheres to the plasma membrane (e.g. of blood cells) or when it reaches the intracellular compartment.

4. CONCLUSION

1. In view of above, and taken into account the scientific data provided, the SCCS is requested to give its opinion on the safety of the nanomaterial Hydroxyapatite when used in oral cosmetics products including toothpastes, tooth whiteners and mouth washes with a maximum concentration limit of 10%, taking into account the reasonably foreseeable exposure conditions.

Only a limited amount of data was provided by the Applicants that corresponded to the SCCS Guidance on Safety Assessment of Nanomaterials in Cosmetics (SCCS 1484/12). The provided data were also not in line with the SCCS Memorandum on Relevance, Adequacy and Quality of Data in Safety Dossiers on Nanomaterials (SCCS/1524/13). To facilitate the assessment, the SCCS therefore also considered additional information gathered through a search of the published scientific literature. However, after detailed evaluation, the SCCS has concluded that the evidence, both that provided in the submission and that available in the scientific literature, is insufficient to allow drawing a conclusion on the safety of nano-hydroxyapatite when used in oral cosmetic products. This is because:

- The test materials used in toxicological studies mostly lacked information on characterisation, were poorly described, or were different from those under evaluation (Materials 1 and 2). It is not clear in most cases whether and to what extent the investigated materials correspond to the materials under evaluation.
- Almost all of the toxicological studies provided were not compliant with relevant test guidelines in terms of study design. In most cases, study reports included in the submission provided only a poor description of the studies. The quality of the information from scientific publications could not be assessed because detailed study reports were not available.
- No study, either from those provided by the Applicants or obtained from the scientific literature, could be identified that would allow the identification of a point of departure for use in risk assessment.
- Some studies published in the open literature for hydroxyapatite materials, which are different from the materials under evaluation, point to the possibility that nano-hydroxyapatite might be taken up locally (e.g. into buccal cells), and that it might exert systemic effects after oral exposure. Since no information on long-term exposure is available, it is not possible to draw any conclusion on whether repeated, long-term oral exposure to nano-hydroxyapatite would manifest in adverse effects as indicated in the scientific literature (e.g. expressed in Fox *et al.*, 2012).

Based on the information available, SCCS considers that the safety of nano-hydroxyapatite materials included in the submission to the consumer, when used up to a concentration of 10% in oral cosmetic products, cannot be decided on the basis of the data submitted by the applicants and that retrieved from literature search. Since the available data/ information could not be related to the hydroxyapatite materials under evaluation, the SCCS will need toxicological data specific for the materials included in the submission for safety assessment, unless a close similarity with the materials used in the available studies can be demonstrated to allow data read-across.

Guidance on the types of data important for safety evaluation of nanomaterials in cosmetic products is detailed in the SCCS Nano-Guidance (SCCS/1484/12). Further clarification on certain aspects relating to relevance, adequacy and quality of the data required for safety assessment of nanomaterials is provided in the SCCS Memorandum (SCCS/1524/13).

2. SCCS is requested to address any further scientific concerns with regard to the use of Hydroxyapatite in nano form in cosmetic products.

The available information indicates that nano-hydroxyapatite in needle-shaped form is of concern in relation to potential toxicity. Therefore, needle-shaped nano-hydroxyapatite should not be used in cosmetic products. It is of note that Material 2 of the submission also includes nanofibres of needle-like structure.

5. MINORITY OPINION

6. REFERENCES

Submission files from applicants as well as references listed below were used.

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Annex

Table 2: Overview on *in vitro* studies performed with nano-hydroxyapatite:

| Reference | Endpoint(s) investigated | Cell type / Model description | Hydroxyapatite characterisation | Experimental procedure/Testing conditions | Results and conclusion |
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| Banik, M. and Basu, T. (2014): Calcium phosphate nanoparticles: a study of their synthesis, characterization and mode of interaction with salmon testis DNA. Dalton Trans 34, 3244-3259. | Interaction of nano-HYAP with DNA. | Salmon testis DNA (no further information on source). | Synthesized, citrate-stabled nano-HYAP (co-precipitation), characterized by DLS, XRD, atomic force microscopy (AFM), SEM-EDX (energy-dispersive X-ray); TEM, FT-IR differential thermal and thermo-gravimetric analysis. Shape: round; mean hydrodynamic radius 160 ± 5 nm; calculated size (Debye-Scherrer) 36.4 nm, zeta potential: -23 mV at pH 8, average size (AFM): 45 nm. Authors state that material is stable for several days. | nano-HYAP-DNA interaction assessed by UV-absorption, CD-spectrum, viscometry, DNA melting, gel retardation, FTIR, voltametry, AFM digestion. | The results revealed that calcium phosphate nanoparticles interacted with DNA with $\sim 1 : 3.3$ stoichiometry with a binding constant of the order of 10^4 M^{-1} through groove-interacting mode and a single nanoparticle covered about 6.2 base pairs of the DNA chain. Binding interaction was spontaneous, cooperative, exothermic and enthalpy-driven and some electrostatic nature of the binding was also evident; however, the non-polyelectrolyte contribution was dominant. The binding interaction finally caused an increase in the melting temperature of DNA from 70.8 °C to 75 °C and alteration of its secondary structure from the naturally occurring B-form to C-form. |
| Bauer, I.W., Li, S., Han, Y., Yuan, L. and Yin, M. (2008): Internalization of hydroxyapatite nanoparticles in liver cancer cells. J. Mater. Sci: Mater. Med. 19, 1091 - 1095. | Internalisation of nano-HYAP into cells. | Hepatocellular carcinoma cell lines (not further specified). | Synthesized nano-HYAP characterised by X-ray diffraction, TEM and particle-size distribution. Particle size < 100 nm, agglomeration in cell culture medium; no further characteristics given, however as mentioned in the text, apparently needle-shaped. | Determination of intracellular calcium depletion after 1 hr incubation with nano-HYAP sol at 1.4 mg/ml. | nano-HYAP particle agglomerates are taken up by cells via clathrin-mediated endocytosis (nano-HYAP internalized in intracellular vacuoles (i.e. vesicles of 100 - 800 nm in diameter)); potassium-depleted cells did not internalize nanoparticles. |
| Chen, L., McCrete, J.M., Lee, J. C.-M., and Li, H. (2011): The role of surface charge on the uptake and biocompatibility of hydroxyapatite nanoparticles with osteoblast cells. Nanotechnology 22, 105708. doi:10.1088/0957-4484/22/10/105708. | Cell viability and uptake of untreated and surface-treated nano-HYAP. For surface treatment, three different carboxylic acid compounds with very similar molecular structure except for one functional group (amine group, carboxyl group, or methyl group) were selected and grafted on HYAP nanoparticles to provide different nanoparticles surface charge, while keeping other properties unchanged, in order to systematically investigate the influence of surface charge with minimum interference from other factors. | Commercially available MC3T3-E1 cells (osteoblast cell line). | Untreated and 3 different surface-modified nano-HYAP materials used characterised by FT-IR, Zeta-potential, XRD and TEM; Zeta potentia (in PBS at pH 7.4): untreated nano-HYAP: $-11.7 \pm 0.7 \text{ mV}$; negatively charged nano-HYAP: $-28.3 \pm 3.9 \text{ mV}$; neutral nano-HYAP: $-11.4 \pm 2.5 \text{ mV}$; positively charged nano-HYAP: $48.6 \pm 5.2 \text{ mV}$; Compared to untreated nano-HYAP, no significant difference on size and morphology was observed on the nano-HYAP modified with 12-aminododecanoic acid (positive) and dodecanedioic acid (negative). nano-HYAP size increased from 100nm in length, 20nm in diameter to about 150 nm in length and 50 nm in diameter after the surface modification with dodecanoic acid (neutral). | MTT and LDH assays performed after 4 and 7 days of exposure; concentrations: 0.05, 0.1, 0.5 and 1.0 mg/ml in MTT assay; LDH assay: 1.0 mg/ml. | Positively charged surface modified nano-HYAP is more easily taken up into cells than negatively charged or unmodified nano-HYAP; all nano-HYAP forms investigated could be internalized by the cells. Cytotoxicity assays revealed proliferative effects of nano-HYAP materials investigated. |
| Chen, Q., Xue, Y. and Sun, J. (2014): Hepatotoxicity and liver injury induced by hydroxyapatite nanoparticles. J. Appl. Toxicol. 34, 1256 - 1264. | Cell viability, apoptosis, MAPK signalling pathway, cellular uptake. | Commercially available Buffalo rat liver (BRL) cells. | Commercially available nano-HYAP; needle-like; long diameter: 80 nm, short diameter: 20 nm; hydrodynamic diameter in cell culture medium: 278.2 nm, Zeta potential in cell culture medium: - 12.4 mV; Zeta potential in physiological saline: -16.3 mV; purity: $\geq 99.0 \%$; agglomeration in cell culture medium. | Cellular uptake: by TEM after 1 hr incubation; cell viability: CCK-8 assay and LDH leakage in a concentration range of nano-HYAP of 25 - 800 $\mu\text{g/ml}$, incubation period 24 hr; apoptosis: Annexin V-FITC/propidium iodide detection kit and flow cytometry; analysis of p-ERK, ERK, p-p38, p38, p-JNK and JNK by Western Blotting. | Cellular uptake: aggregates in cytoplasmic vesicles and in cytoplasm. Cell viability: concentration-dependent loss of cell survival from 200 $\mu\text{g/ml}$ and concomitantly increased LDH leakage pointing to damage of cell membrane. Apoptosis: dose-dependent increase of apoptotic and necrotic cells at 400 and 800 $\mu\text{g/ml}$. Dose-dependent increase of p38 phosphorylation at 200 and 800 $\mu\text{g/ml}$, increased phosphorylation of ERK and JNK at 400 and 800 $\mu\text{g/ml}$; no change in total p38, ERK and JNK. |

Revision of opinion on hydroxyapatite (nano)

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| Chen, X., Deng, C., Tang, S. and Zhang, M. (2007): Mitochondria-dependent Apoptosis Induced by Nanoscale Hydroxyapatite in Human Gastric Cancer SGC-7901 Cells. <i>Biol. Pharm. Bull.</i> 30, 128 - 132. | Cell viability, apoptosis. | Commercially available human gastric cancer SGC-7901 cells. | nano-HYAP (purity \geq 99.9 %; $\phi \leq$ 50 nm), no further information given. | Determination of cell viability by MTT assay at 25, 50, 100, 150, 200 μ g/ml for 0, 24, 48, and 72 hr. Apoptosis: Caspase inhibition by flow cytometry, TEM, DNA-fragmentation, Mitochondrial membrane potential; Western Blot analysis for Bcl-2, Bax and Cytochrome c. | Dose- and time-dependent decrease of cell viability. Induction of apoptosis. Activation of Caspase-3 and caspase-9 but not of caspase-8; down-regulation of Bcl2, upregulation of Bax, decrease of mitochondrial membrane potential, release of cytochrome c from mitochondria into cytosol. |
| Chu, S.H., Feng, D.F., Ma, Y.B. and Li, Z.Q. (2012): Hydroxyapatite nanoparticles inhibit the growth of human glioma cells in vitro and in vivo. <i>Int. J. Nanomedicine</i> 7, 3659 - 3666. | Cell viability, apoptosis. | Human glioma 251 and human glioma SHG44 cells. | Unclear, only information on size is given: 50 nm ("very uniform size"). | Cell viability: MTT assay, concentrations: 0, 15, 30, 60, 120, and 240 mg/L, duration: 24, 48 and 72 hr. Apoptosis: TEM, flow cytometry and Western Blotting, concentrations: 0, 120 and 240 mg/l, duration: 48 hr. | Nano-HYAP induced decrease in cell viability in a dose-dependent manner. At 120 and 240 mg/l, apoptotic morphological changes in cells were seen. The expression of c-Met, SATB1, Ki-67, and bcl-2 protein decreased, and the expression of SLC22A18 and caspase-3 protein decreased noticeably. The findings indicate that nano-HYAP has an evident inhibitory action and induces apoptosis of human glioma cells in vitro. |
| Costa-Rodrigues, J., Silva, A., Santos, C., Almeida, M., Costa, M. and Fernandes, M. (2014): Complex effect of hydroxyapatite nanoparticles on the differentiation and functional activity of human pre-osteoclastic cells. <i>J. Biomed. Nanotechnol.</i> 10, 3590-3600. | Osteoclastic differentiation. | Peripheral blood mononuclear cells (PBMC cells) (containing CD14+ osteoclastic precursors) isolated from blood of male healthy donors aged 25-35 years and cultured either unstimulated or under osteoclastogenic-induced conditions. | Synthesized (hydrothermal precipitation method, citric acid assisted), elongated rod-like particles characterized by XRD, TEM, EDX, BET, FT-IR, Zeta sizing. Crystalline phase: hexagonal; specific surface area: 55 m^2/g ; Zeta potential: -60 mV at pH 7.5; average size (length x width): 100 x 25 nm. | PBMC cells were cultivated either unstimulated or under osteoclastogenic conditions. In both conditions, nano-HYAP (1 - 100 μ g/ml) was added at different time-points (day 1, 7 or 14) of a 21 day culture time. Cultures in the absence of nano-HYAP served as negative controls. Osteoclastic cell response was determined on days 7, 14 and 21 by using different markers of osteoclastic cell response (e.g. tartrate resistant acid phosphatase (TRAP), osteoclast-related gene expression). | Results showed that nano-HYAP modulated the differentiation and function of osteoclastic cells in a dose- and time-dependent manner. Effects were dependent on the stage of osteoclastic differentiation. In unstimulated PBMC, nano-HYAP significantly increased osteoclastogenesis, leading to the formation of mature osteoclasts, as evident by the significant increase of TRAP activity, number of TRAP-positive multinucleated cells, osteoclastic gene expression and resorbing ability. In a population of mature osteoclasts (formed in osteoclastogenic-induced PBMC cultures), nano-HYAP caused a dose-dependent decrease on the osteoclastic-related parameters. These results highlight the complex effects of nano-HYAP in osteoclastic differentiation and activity and suggest the possibility of nano-HYAP to modulate/disrupt osteoclastic behavior, with eventual imbalances in the bone metabolism. This should be carefully considered in bone-related and other established and prospective biomedical applications of nano-HYAP. |
| Dey, S., Das, M. and Balla, V.K. (2014): Effect of hydroxyapatite particle size, morphology and crystallinity on proliferation of colon cancer HCT116 cells. <i>Materials Science and Engineering C39</i> , 336 - 339. | Cell growth and cell viability. | Commercially available human colon carcinoma cells HCT116. | 3 different synthesized HYAP materials: microparticles (MP), nanoparticle (NP) and nanorods (NRD), characterized by X-ray diffraction, FTIR and SEM. Crystallite size (nm): MP: 177.2, NP: 11.2, NRD: 22.1 nm; crystallinity: MP: 0.92, NP: 0.12; NRD: 0.43. | Cell viability: MTT assay at HYAP concentration of 0.1 mg/ml, incubation times: 24, 48 and 72 hr. | Cell viability significantly reduced with all HYAP materials used. The lower the particle size, the higher the decrease in viability. Rod-shaped material decreased viability to the greatest extent. In addition to size, also crystallinity has an influence on cell viability. |
| Fu, Qiang, Rahaman, M., Zhou, N., Huang, W., Zhang, L. and Li., H. (2008): In vitro study on different cell response to spherical hydroxyapatite nanoparticles. <i>Journal of Biomaterials Applications</i> 23, 37 - 50. | Cytotoxicity and cell proliferation. | Commercially available mouse fibroblasts (L929) and commercially available osteosarcoma U2-OS cells. | Synthesized material, characterised by scanning electron microscopy (SEM), X-ray diffraction (XRD), FT-IR analysis and BET analysis: particle size range: 20 - 40 nm; specific surface area: 75 m^2/g . Nearly spherical shape. Commercially available HYAP (no further information, apparently non-nano) was used for comparison. | Concentrations of nano-HYAP tested in L929 cells: 62.5, 125, 250 and 500 μ g/ml for 1 - 10 days. | Inhibition of proliferation and induction of necrosis in U2OS cells by nano-HYAP (necrosis discussed as consequence of increased Calcium uptake into cells). In L929 cells, nano-HYAP was less toxic than commercially available HYAP. |

Revision of opinion on hydroxyapatite (nano)

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| Geetha, C.S., Remya, N.S., Leiji, K.B., Syama, S., Reshma, S.C., Sreekanth, P.J., Varna, H.K. and Mohanan, P.V. (2013): Cells-nano interactions and molecular toxicity after delayed hypersensitivity, in Guinea pigs on exposure to hydroxyapatite nanoparticles. <i>Colloids and Surfaces B: Biointerfaces</i> 112, 204 - 212. | Cytotoxicity and oxidative damage. | L929 fibroblast cells (no information on source given) for cytotoxicity determination; rat liver homogenate from Wistar rats (no information on sex) for determination of oxidative stress. | Synthesized, needle-shaped nano-HYAP particles characterised by TEM, FT-IR, XRD, Zeta sizing. Particles size: < 50 nm, Zeta potential -13.4 mV in buffer, Zeta potential < 3.19 mV in water. | Cytotoxicity: MTT assay and cell morphology at 10, 25, 50, 100, 200, 400, 600 and 700 µg/mL, incubation time: 24±1 hr. Oxidative stress: photometric determination of total protein (TP), lipid peroxidation (malondialdehyde (MDA) formation), glutathione reductase (GR), GSH, glutathione peroxidase (GPx), superoxide dismutase (SOD) and 8-hydroxyl-2-deoxyguanosine (8-OHdG) after 3 hr incubation with 12.5, 25, 50 and 100 mg/ml nano-HYAP. | Cytotoxicity: some decrease of viability at the highest concentration in MTT assay, cell morphology remained normal. Oxidative damage: significantly increased MDA at 100 µg/ml, non-significantly increased GPx at 100 µg/ml; no significant change in GR, SOD, 8-OHdG. |
| Han., Y., Wang, X., Dai, H. and Li., S. (2012): Nanosize and Surface Charge Effects of Hydroxyapatite Nanoparticles on Red Blood Cell Suspensions. <i>ACS Applied Materials and Interfaces</i> 4, 4616 - 4622. | Effects of micro- and nano-HYAP (surface-modified and non-modified) on red blood cells. | Red blood cell (RBC) suspension from one male rabbit (strain not specified). | Synthesized non-modified and surface-modified nano-HYAP; non-modified nano-HYAP: needle-shaped, 18-30 nm x 45-120 nm; SSA: 139.7 m ² /g, Zeta potential: -7.3 mV; heparin-modified nano-HYAP: needle-shaped, 10-20 nm x 40-100 nm; SSA: 30.0 m ² /g, Zeta-potential: -50 mV; micro-HYAP: spherical shape, diameter: 0.15 - 0.3 µm, SSA: 10.9 m ² /g, Zeta-potential: -17.7 mV. Non-modified nano-HYAP and micro-HYAP form aggregates in aqueous solution. Zeta potential determined in aqueous solution. | Investigation of RBCs by haemolysis assay (4 hr incubation in concentration range 0.056 - 0.28 mg/ml), optical microscopy (after 1 and 1 hr 15 min incubation), TEM observations, spectrophotometric determination of adsorption on Sialic acid (SA, a membrane component of RBCs). | No haemolysis, however increase erythrocyte sedimentation induced by nano-HYAP. TEM reveals RBC aggregation after 1 hr incubation with non-modified nano-HYAP, no aggregation with micro-HYAP, lower aggregation with surface-modified nano-HYAP. Non-modified nano-HYAP interacts with RBC and finally leads to membrane-bound localization. This was not observed with micro-HYAP and to a lower extent with surface-modified nano-HYAP. However, surface-modified nano-HYAP does not belong to the materials of the submission. |
| Huang, J., Best, S., Bonfield, W., Brooks, R.A., Rushton, N., Jayasinghe, S. and Edirisinghe, M. (2004): In vitro assessment of the biological response to nano-sized hydroxyapatite. <i>J. Mater. Sci. Mater. Med.</i> 15, 441-445. | Cytotoxicity, cell growth, cytokine release. | Human monocyte-derived macrophages (HMM, no information on source) and bone-forming HOB cells (commercially obtained). | Synthesized nano-HYAP (precipitation method), characterized by TEM and XRD: rod-like nano-HYAP, size: about 50 - 80 nm in length. | Cytotoxicity determined in HMM by LDH release (commercial kit; concentration range: 1 - 100 mio particles per 0.5 mio cells; incubation time 24 hr). TNF-α-release determined in the same cultures by ELISA. Growth of bone-forming HOB cells determined on glass substrates with electrosprayed nano-HYAP on it. Growth assessed by Alamar Blue assay, cytoskeletal assessment and SEM. | Some evidence for LDH release in HMM cells, no significant release of TNF-α. Good attachment and growth of BOB cells on glass substrates with electrosprayed nano-HYAP on it. Authors conclude that material is suited for biomedical applications. |
| Li., G., Huang, J., Li, Y., Zhang, R., Deng, B., Zhang, J. and Aoki, H. (2007): In vitro study on influence of a discrete nano-hydroxyapatite on leukemia P388 cell behaviour. <i>Bio-Medical Materials and Engineering</i> 17, 321-327. | Cytotoxicity, cell proliferation, apoptosis. | Leukemia P388 cells from BALB mice (unclear, whether prepared or from commercial source). | Synthesized nano-HYAP, characterised by XRD, IR, TEM. The crystal size was estimated less than 20 nm by Scherrer's equation. Material did not aggregate in saline. | Cell viability assessed by MTT (concentration range: 8 - 40 µg/ml; incubation time: 48 hr) and TEM (concentration: 35 µg/ml, 24 hr incubation). Apoptosis: concentration: 35 µg/ml, incubation: 24, 48 and 72 hr, Annexin - propidium iodide method. | Cell viability: IC ₅₀ = 37.79 µg/ml; dose-dependent decrease in cell viability for the concentration range investigated. TEM: expansion of endoplasmatic reticulum in cells, swelling of mitochondria, further changes in cell structure. Apoptosis: compared to untreated cells, treated cell exhibited highest apoptosis rates at 24 and 48 hr, G1 phase arrest. |

Revision of opinion on hydroxyapatite (nano)

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| Liu, X. and Sun, J. (2014): Potential proinflammatory effects of hydroxyapatite nanoparticles on endothelial cells in a monocyte-endothelial cell coculture model. <i>International Journal of nanomedicine</i> 9, 1261-1273. http://dx.doi.org/10.2147/IJN.S56298 | Cell viability, cellular uptake, cytokine production, cell adhesion molecule (CAM) expression, proinflammatory effects, stimulation of mitogen-activated protein kinases (MAPKs) and factor-kappa B (NF-κB). | HUVECs (Human umbilical vein endothelial cells (EC cells)) from freshly obtained human umbilical cord; human monocytes (THP-1 cells) from commercial sources. Cells were either used as cocultures or monocultures. | Synthesized material, characterised by TEM, XRD, Energy-dispersive X-ray spectroscopy, hydrodynamic size, surface charge (zeta potential), specific surface area. Form: near spherical or slightly elongated shape, on average 15 nm in diameter; cells have a negative surface charge in culture medium; hydrodynamic size in cell culture medium: 248 nm; specific surface area: 109 m ² /g. Zeta potential: -8.89 mV in cell culture medium. | Cell viability: mitochondrial function assessed by MTS assay; cellular uptake: TEM; cytokine production (interleukin [IL]-6, IL-8, IL-1β, and tumor necrosis factor-alpha [TNF-α]); immunoassay kits; surface markers: flow cytometry with immunofluorescence detection; protein expression (MAPKs and NF-κB) by Western Blotting. Concentrations tested: 0, 25, 50, 100, 200 and 400 µg/ml. | At concentrations ≥ 100 µg/mL: decreasing cell viability. Uptake of Nano-HYAP in monocytes and ECs; phagocytosis caused an inflammatory response in monocytes, but not in ECs. No direct effect of Nano-HYAP on endothelial inflammation; indirect activation of ECs by nano-HYAP, resulting in increased IL-6 production and elevated adhesion molecule expression after coculture with monocytes. The potential proinflammatory effect of Nano-HYAP is primarily mediated by the release of soluble factors from activated monocytes, which leads to an inflammatory response of the endothelium that is possibly dependent on p38/JNK MAPK and NF-κB signaling activation. The in vitro monocyte-EC coculture model revealed potential proinflammatory effects of Nano-HYAP on ECs, suggesting that exposure to Nano-HYAP could increase the risk of cardiovascular disease. |
| Liu, X., Zhao, M., Lu, J., Ma, J., Wie, J. and Wie, S. (2012a): Cell responses to two kinds of nanohydroxyapatite with different sizes and crystallinities. <i>Int. J. Nanomedicine</i> 7, 1239-1250. | Cell viability/ proliferation / morphology, apoptosis, alkaline phosphatase (AP), inflammatory response, type I collagen and osteopontin expression. | Human osteoblast-like MG-63 and human macrophage U937 cell lines (commercially available). | Two types of synthesized (precipitation method), rod-shaped nano-HYAP materials, characterised by X-ray diffraction, X-ray, FT-IR and TEM. Nano-HYAP-1: diameter 23 ±5 nm, length 47±14 nm; nano-HYAP-2: diameter 16 ±3 nm, length 40±10 nm. Crystallinity of nano-HYAP-1: 85% ± 5%; crystallinity of nano-HYAP-2: 65% ± 3%, respectively. | Viability: MTT (only MG-63 cells) at 1 concentration (200 µl of a 100 µg/ml solution of nanomaterial was added to incubation mixture), incubation for 1, 3 and 5 days. Morphology: Field emission SEM; apoptosis: Annexin V-FITC kit and FACScan (only MG-63 cells); total protein and alkaline phosphatase (AP): 5 d culture, only in MG-63 cells by commercially available kits and photometric detection; osteopontin and type I collagen by Western Blotting (5d incubation) (only MG-63 cells), inflammatory response by TNF-α (ELISA) (18 hr incubation) in U937 cells. Morphology: TEM. | MTT: Time-dependent increase in cell viability after treatment and also in controls. Cytoskeleton of nano-HYAP-2 treated MG-63 cells more diffuse than in controls of after nano-HYAP-1. Apoptosis of MG-63 cells higher with nano-HYAP-2 compared to nano-HYAP-1. Cell uptake, especially for nano-HYAP-2. AP in MG-63 higher compared to controls, but of statistical significance only for nano-HYAP-2. Compared to controls, significantly increased TNF-α in U937 cells. Increased expression (statistically not significant) of type I collagen and osteopontin in MG-63 cells after treatment with both types of nanomaterials. |
| Liu, Z., Tang, S. and Ai, Z. (2003): Effects of hydroxyapatite nanoparticles on proliferation and apoptosis of human hepatoma BEL-7402 cells. <i>World J. Gastroenterol</i> 9, 1968-1971. | Cytotoxicity, cell proliferation, apoptosis. | Human hepatoma BEL-7402 cells (commercially available). | Synthesized nano-HYAP (sol-gel method) with uniform particle size of 50 nm (no further information). | Viability: MTT (0, 12.5, 25, 50, 75, 100, 150 and 200 mg/l for 48 hr) and TEM (0, 50 and 100 mg/l for 48 hr); Apoptosis (50, 75, 100, 150 and 200 mg/l for 48 hr), Hoechst 33258 staining and analysis by flow cytometry. | nano-HYAP inhibited the growth of hepatoma cells in a dose-dependent manner, with IC ₅₀ values of 29.30 mg/L. Treated with 50-200 mg/L for 48 hr, nano-HYAP induced apoptosis with nuclear chromatin condensation and fragmentation as well as cell shrinkage and the formation of apoptotic bodies. Apoptotic rates at 50, 75, 100, 150 and 200 mg/L were 20.35±2.23 %, 25.35±1.92 %, 29.34±4.61 %, 44.92±3.78 % and 53.64±3.49 %, respectively, which were all significantly higher compared to control (2.23±0.14 %). There was a significant correlation between nano-HYAP and apoptotic rate (r=0.994, P<0.01). |
| Liu, Z.S., Tang, S.L., and Ai, Z.L. (2003): Effects of hydroxyapatite nanoparticles on proliferation and apoptosis of human hepatoma BEL-7402 cells. <i>World J. Gastroenterol</i> 9, 1968 - 1971. | Cytotoxicity, cell proliferation and apoptotic alterations. | Human hepatoma BEL-7402 cells (commercially available). | Hydroxyapatite nanoparticles synthesized by sol-gel method, uniform particle size of 50 nm (no further characterization given). | Cytotoxicity/proliferation measured by MTT assay at 0, 12.5, 25, 50, 75, 100, 150 and 200 mg nano-HYAP/ml and 48 hr incubation; staining of apoptotic cells and flow cytometric analysis after 48 hr incubation with 50, 75, 100, 150 and 200 mg nano-HYAP/ml. | Cell proliferation significantly inhibited by nano-HYAP (IC ₅₀ : 29.3 mg/l; no significant difference between different treatment groups); dose-dependent increase in apoptosis. |

Revision of opinion on hydroxyapatite (nano)

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| <p>Mestres, G., Espanol, M., Xia, W., Persson, C., Ginebra, M., Ott, M.K. (2014): Inflammatory Response to Nano- and Microstructured Hydroxyapatite. PLoS One. 2015 Apr 2;10(3):e0120381. doi: 10.1371/journal.pone.0120381</p> | <p>Macrophage activation, cell proliferation, ROS formation.</p> | <p>RAW264.7 cells (commercially available murine macrophages).</p> | <p>Coarse (C) and fine (F) nano-HYAP substrates; C-nano-HYAP: plate-like morphology, specific surface area: 19.85 m²/g. F-nano-HYAP: needle-like structure, specific surface area: 41.72 mg/m².</p> | <p>Cell proliferation: LDH assay and quantification by fluorescence after calcein/propidium iodide staining; cell morphology: SEM; ROS formation: luminol amplified chemiluminescence assay . Two types of contact between nano-HYAP and cells: (a) direct (cells seeded on HYAP surfaces) (b) indirect (cells cultivated in medium previously incubated with nano-HYAP). Strong ROS signal for cells in contact with C-nano-HYAP, weak ROS signal for cells in contact with F-nano-HYAP.</p> | <p>Compared to negative control, significantly reduced cell number after growth of cells on F- and C-nano-HYAP substrate surfaces (apart from C-nano-HYAP on day 7). A similar pattern was observed when using extracts. This study shows that different topological textures of nanomaterials can indirectly affect macrophage proliferation. The plate-like materials used in this study have a similarity with the submitted materials, but not the needle like structures.</p> |
| <p>Motskin, M., Müller, K.H., Genoud, C. Monteith, A.G., and Skepper, J.N. (2011): The sequestration of hydroxyapatite nanoparticles by human monocyte-macrophages in a compartment that allows free diffusion with the extracellular environment. Biomaterials 32, 9470 - 9482.</p> | <p>Formation of a surface connected compartment (SCC, patocytosis) by nano-HYAP. SCC = formation of a membranous branched labyrinth repository with lumen directly open to the extracellular space.</p> | <p>(i) Human monocyte-derived macrophages (HMM) cultures, derived from human buffy coat residues; (ii) commercially available epithelial lung carcinoma cells (A549).</p> | <p>(i) nano-HYAP (gel): long axis: 49 ± 22 nm, short axis: 18 ± 5 nm; aspect ratio 2.9 ± 1.4, Zeta potential -10.28 ± 0.86 mV at pH 7, surface area 65 ± 16 m²/g; (II) nano-HYAP (colloidal): long axis: 50 ± 17 nm, short axis: 18 ± 5 nm; aspect ratio 2.9 ± 1.0, Zeta potential -31.15 ± 0.58 mV at pH 7, surface area 69 ± 22 m²/g; (iii) micro-HYAP from gel: diameter: 2151 ± 1250 nm; aspect ratio 1.05 ± 0.04, Zeta potential -16.3 ± 0.26 mV at pH 7, surface area 93.271 ± 0.05 m²/g; material becomes rod-shaped after autoclaving.</p> | <p>multi-photon live imaging; cryo-immobilisation; SEM, TEM, confocal microscopy; Bright-field transmission electron microscopy (BF-TEM).</p> | <p>SCC formation in both cell types by nano-HYAP but not by micro-HYAP; i.e. SCC formation not limited to macrophages; discussion whether SCC formation is dependent on zeta potential (aggregation).</p> |
| <p>Motskin, M., Wright, D.M., Muller, K., Kyle, N., Gard, T.G., Porter, A.E. and Skepper, J.N. (2009): Hydroxyapatite nano and microparticles: Correlation of particle properties with cytotoxicity and biostability. Biomaterials 30, 3307 - 3317.</p> | <p>Cytotoxicity of colloid, gel and rapidly sedimenting HYAP particles (5 different HYAP materials investigated)</p> | <p>Human monocyte-derived macrophages (HMM) cultures, derived from human buffy coat residues.</p> | <p>(i) nano-HYAP (gel): long axis =49±22 nm, short axis = 18±5 nm; aspect ratio 2.9±1.4, Zeta potential -10.28±0.86 mV at pH 7, surface area 65±16 m²/g; (II) nano-HYAP (colloidal): long axis =50±17 nm, short axis = 18±5 nm; aspect ratio 2.9±1.0, Zeta potential -31.15±0.58 mV at pH 7, surface area 69±22 m²/g; (iii) micro-HYAP from gel: diameter = 2151±1250 nm; aspect ratio 1.05±0.04, Zeta potential -16.3±0.26 mV at pH 7, surface area 93.271±0.05 m²/g; (iv) micro-HYAP from colloid: diameter = 1657±778 nm; aspect ratio 1.12±0.8, Zeta potential -29.56±1.1 mV at pH 7, surface area 60.3±1.4 m²/g; (v) commercially available nano-HYAP from Sigma: diameter = 170±100 nm; aspect ratio 1.05±0.03, Zeta potential -9.59±0.81 mV at pH 7, surface area 7.362±0.02 m²/g. Material becomes rod-shaped after autoclaving.</p> | <p>Cell viability: MTT assay, LDH assay and confocal live-dead assay at HYAP concentrations of 31, 62, 125, 250 and 500 µg/ml (24 hr). Analysis of cells by SEM, TEM and stereology.</p> | <p>The gel exhibited the greatest toxicity and was toxic at all concentrations tested in all three assays. The cytotoxicity of nanoparticles was greatly reduced after they were spray dried to form microparticles. The degree of toxicity correlated well with the degree of uptake indicating that cellular particle load is the main cause of cytotoxicity, probably due to the release of calcium. The uptake of all HYAP particles studied was governed by a combination of particle characteristics rather than one dominant parameter. The majority of sequestered nanoparticles and microparticles ends up in the phagocytic pathway and are dissolved over time within lysosomes. Some particles escape this pathway and end up in the cytoplasm and a few even translocate to the nucleus.</p> |

Revision of opinion on hydroxyapatite (nano)

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| Müller, K.H., Motskin, M., Philpott, A., Routh, A., Shanahan, C., Duer, M. and Skepper, J. (2014): The effect of particle agglomeration on the formation of a specific surface-connected compartment induced by hydroxyapatite nanoparticles in human monocyte-derived macrophages. <i>Biomaterials</i> 35, 1074-1088. | Influence of particle agglomeration on cellular uptake, cytotoxicity and formation of a surface-connected compartment (SCC). | In vitro cultures of mature human macrophages (HMM) isolated from human buffy coat residues. | 5 different nano-HYAP materials (4 synthesized, 1 commercially available). All synthesized materials were fine needle-like to plate-like and became longer, thicker and more lozenge-shaped after autoclavation. Characterized by BF-TEM (bright-field TEM), BET, Zeta-potential (in the presence or absence of the dispersant D7) , DLS, SEM. Synthesized material: large, round particles. Material 1: non-autoclaved, non citrated (NANC): long axis: 30 ± 5 nm, short axis: 8 ± 2 nm; aspect ratio: 3.9 ± 1.1; zeta potential (-D7): -17 ± 1.42 mV; (+D7): -36.11 ± 2.98 mV; surface area: 121.4 ± 0.6 m ² /g. Material 2: non-autoclaved, citrated (NAC): long axis: 27 ± 7 nm, short axis: 8 ± 2 nm; aspect ratio: 3.6±1.2; zeta potential (-D7): -14.48 ± 0.92 mV; (+D7): -24.08 ± 1.29 mV; surface area: 156.4 ± 1.5 m ² /g. Material 3: autoclaved, non-citrated (ANC): long axis: 54 ± 19 nm, short axis: 18 ± 5 nm; aspect ratio: 3.2 ± 1.2; zeta potential (-D7): -10.56 ± 0.84 mV; (+D7): -55.84 ± 1.2 mV; surface area: 64.9 ± 0.35 m ² /g. Material 4: autoclaved, citrated (AC): long axis: 46± 18 nm, short axis: 17± 5 nm; aspect ratio: 32.8±0.95; zeta potential (-D7): -24.29±0.75 mV; (+D7): -43.6±2.48 mV; surface area: 52.8±0.3 m ² /g. I.e. NAC and AC as uncoated materials relevant for the opinion. | Experiments were performed either in the presence or in the absence of dispersant D7. Cellular uptake: determination of calcium in lysed cells by ICP-MS after 1 hr incubation with 125 µg/ml nano-HYAP. Cytotoxicity: MTT assay, concentrations: 0, 31.25, 62.5, 125, 250 and 500 µg/ml, incubation time 24 hr. Agglomeration: DLS; SCC formation after 2 hr incubation with 30, 60 or 125 µg/ml nanomaterial. Imaging: BF-TEM. | In the absence of disperser, formation of large agglomerates in NANC and ANC with NANC forming larger agglomerates. Statistically significant reduction in viability after NANC and ANC, citration and dispersion reduced cytotoxicity. SCC formation: Formation of large, interconnected vacuoles lining the membrane and occupying larger parts of the cells with increasing concentration; SCC open to extracellular space. SCC formation/particle internalization reduced by D7 or citration. Change in particle morphology in the SCC (from needle to plate, considered as sequestration). High cellular uptake of NANC and ANC in contrast to citrated or dispersed materials. Agglomerate dispersion prevented the SCC from forming, but did not completely inhibit nanoparticle uptake by other mechanisms. |
| Opačić-Galić, V., Petrović, V., Zivković, S., Jokanović, V., Nikolić, B., Knežević-Vukčević, J. and Mitić-Čulafić, D. (2013): New nanostructural biomaterials based on active silicate systems and hydroxyapatite: characterization and genotoxicity in human peripheral blood lymphocytes. <i>Int. Endod. J.</i> 46, 506-516. doi: 10.1111/iej.12017 | Cell viability and genotoxicity (Comet Assay). | Human lymphocytes from 3 donors. | Not given. | The genotoxic potential of HA was tested in the concentration range 0.05–50 mg/ml, using lymphocytes from three donors. | Equivocal results were observed: after the treatment of lymphocytes originating from donor 1, nano-HYAP exhibited statistically significant genotoxicity (P < 0.05) at all tested concentrations in comparison with negative control. However, nano-HYAP did not induce DNA-damage in the two other donors; moreover, the percentage of DNA damage in nano-HYAP-treated cells was decreased compared to controls. |
| Palanivelu, R. and Kumar, A. (2014): Synthesis, characterization, in vitro anti-proliferative and hemolytic activity of hydroxyapatite. <i>Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy</i> 127, 434-438. | Viability, morphology, haemolytic activity. | A549 cells for MTT assay; fresh human red blood cells for haemolytic activity (no information on donor(s)). | Synthesized nano-HYAP (precipitation method), characterised by XRD, FT-IR, FT-Raman spectroscopy, SEM and TEM. According to SEM: Particles have uniform rod-like structure with pores in between; size: 35 - 65 nm in length. According to TEM: size: 65 x 21 nm; agglomeration. | Viability: MTT assay (concentration range 100 - 1000 µg/ml, incubation time 24 hr). Haemolytic assay: photometric assay (concentrations: 50, 100, 150 and 200 µg/ml; incubation time 1 hr). | Dose-dependent increase in cell death and haemolytic activity (no statistical analysis). |

Revision of opinion on hydroxyapatite (nano)

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| Qing, F., Wang, Z., Hong, Y., Liu, M., Guo, B., Luo, H. and Zhang, X. (2012): Selective effects of hydroxyapatite nanoparticles on osteosarcoma cells and osteoblasts. <i>J. Mater. Sci: Mater. Med.</i> 23, 2245-2251. | Cell viability and apoptosis. | Human osteosarcoma MG63 cells (commercially available) and normal human osteoblasts (from a 12 year old female traumatic patient). | Synthesized nano-HYAP (hydrothermal process); characterisation by XRD, TEM and SEM. Nano-HYAP particles of spherical shape and average particle size of about 40 nm. | Viability: MTT assay and TEM analysis after fluorescent staining (concentrations: 100, 250 and 500 µg/ml; incubation time: 1, 3 and 5 days). Apoptosis: TEM. | MTT assay: inhibition of cell growth in MG63 cells; stimulation of cell growth in osteoblasts. TEM: uptake of nano-HYAP into MG63 cells, mostly in lysosomes, indication of apoptosis, mitochondrial swelling, extreme vacuolation at the highest dose tested (500 µg/ml); formation of myelinosomes; after 5 d: nano-HYAP enters nucleus. No apoptotic changes observed in osteoblasts, less changes in osteoblasts; nano-HYAP entered cytoplasm and lysosomes. In summary, nano-HYAP had different effects to the two different kinds of cells investigated. |
| Remya, N.S., Syama, S., Gayathri, V., Varma, H.K. and Mohanan, P.V. (2014): An in vitro study on the interaction of hydroxyapatite nanoparticles and bone marrow mesenchymal stem cells for assessing the toxicological behaviour. <i>Colloids and Surfaces B: Biointerfaces</i> 117, 389 - 397. | Cytotoxicity, Cellular uptake, oxidative stress, apoptosis, cytoskeletal arrangement. | Mesenchymal stem cells isolated from pooled bone marrow of 6 mice (no further information on animals (e.g. sex or strain). Abbreviated: BMSC cells. | Synthesized nano-HYAP (precipitation method), characterised by XRD, FT-IR, TEM, SEM, zeta potential and energy dispersive spectroscopy (EDS). Nano-HYAP of rod-shaped structure, size below 50 nm, agglomeration observed. Zeta-potential in buffer: -14.4 mV (i.e. stable in buffer); Zeta potential in water 3.19 mV (i.e. unstable in water). | Cell viability: MTT assay and microscopic evaluation, concentrations: 10, 50, 100, 500 and 1000 µg/ml; incubation time 24 hr. Cellular uptake: light microscopy after Giemsa staining and fluorescence staining and confocal microscopy after incubation of cells with fluorescence-labelled nano-HYAP (concentration: 100 µg/ml; incubation time: 0, 2, 4, 12 and 24 hr). Apoptosis: Caspase assay after overnight incubation with 10, 50, 100, 500 and 1000 µg/ml nano-HYAP and annexin/propidium iodide staining and flow cytometry after 24 hr incubation with 50, 100, 500, 800 and 1000 µg/ml nano-HYAP; oxidative stress: formation of reactive oxygen species by DCF-DA assay. | Cytotoxicity: impairment of cell viability at the highest concentration; uptake of nano-HYAP into cells starting at 2 hr; occurrence of nano-HYAP in cytoplasm, as agglomerates/aggregates. Statistically significant increase in ROS formation at the highest dose. No indications of apoptosis by Caspase assay; annexin/propidium iodide staining: presence of early apoptotic cells after incubation with 50, 100 and 500 µg/ml, but not at higher concentrations. No late apoptotic or necrotic cells at all concentrations tested, i.e. no apoptosis. |
| Sánchez Lafarga, A., Pacheco Moisés, F., Gurinov, A., Ortiz, G., and Arizaga, G. (2015): Dual responsive dysprosium-doped hydroxyapatite particles and toxicity reduction after functionalization with folic acid and glucuronic acids. <i>Materials Science and Engineering C</i> 48, 541-547. | Ex vivo determination of different biochemical parameters (membrane fluidity, endproducts of lipid peroxidation, nitrite and nitrate values, glutathione peroxidase activity, ATPase activity). | Homogenates from kidneys, livers and lungs of Sprague-Dawley rats prepared 7 days after i.p. treatment with 10 or 20 mg nano-HYAP (untreated nano-HYAP, nano-HYAP doped with dysprosium cations, nano-HYAP functionalized with folic acid or glucuronic acid); 4 animals per dose group. | Only untreated nano-HYAP described here as modified nano-HYAP does not belong to the materials of the submission. Synthesized material characterised by XRD, thermogravimetric analysis, fluorescence. No information on size given. | Membrane fluidity: fluorimetric method; end-products of lipid peroxidation (malondialdehyde (MDA) and 4-hydroxyalkenals (4-OHA)): photometric assay kit. Nitrite and nitrate levels: Griess reaction; glutathione peroxidase activity: photometric method. ATPase activity: method not described. | Only untreated nano-HYAP results reported here. Compared to untreated controls, dose-dependent increase of products of lipid peroxidation in liver, kidney and lung; significantly increased nitrate and nitrite levels in kidneys, livers and lungs; significantly reduced membrane fluidity in liver, kidney and lung; increase in ATPase activity in liver, kidney and lung (statistically significant after 20 mg in liver and after 10 and 20 mg in kidneys); statistically significantly increased glutathione peroxidase activity in erythrocytes from rats treated with 20 mg nano-HYAP. |
| Shi, Z., Huang, X., Cai, Y., Tang, R., and Yang, D. (2009): Size effect of hydroxyapatite nanoparticles on proliferation and apoptosis of osteoblast-like cells. <i>Acta Biomaterialia</i> 5, 338-345. | Cell proliferation and apoptosis. | Human osteoblast-like MG-63 cells; commercially available. | Synthesized nano-HYAP of 2 different sizes characterised by TEM, XRD, DLS, field electron scanning microscopy (FESEM), FT-IR. Sphere-like nano-HYAP of 20 ± 5 nm (np20) and 80 ± 12 nm (np80) diameter and weak crystallinity; micro-sized HYAP used for comparison. | Cell proliferation: acridine orange staining and fluorescence microscopy; FESEM, TEM; Apoptosis: propidium iodide staining and FACScan; analysis of apoptosis-related genes by RT-PCR. Analyses were performed on glass films coated with 1% suspension of HYAP materials; incubation was up to 5 days. | Cell proliferation: compared to untreated controls, proliferation was decreased with all HYAP materials investigated; the most profound effect was observed with microsized material. Morphological changes of cells observed after treatment with np80 and microsized HYAP, but not with np20. Cellular uptake - on the other hand - only observed with np20. Apoptosis: increased rate of apoptosis with increasing particle size; decreased Bcl-2 levels with increasing particle size. |

Revision of opinion on hydroxyapatite (nano)

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| Shi, Z., Huang, X., Liu, B. and Tao, H. (2010): Biological response of osteosarcoma cells to size-controlled nanostructured hydroxyapatite. <i>Journal of Biomaterials Applications</i> 25, 19-37. | Cell proliferation and apoptosis. | Human osteoblast-like MG-63 cells; commercially available. | Synthesized nano-HYAP of 2 different sizes characterised by TEM, XRD, DLS, field electron scanning microscopy (FESEM), FT-IR. Sphere-like nano-HYAP of 20 ± 6 nm (NanoHA-S) and 80 ± 14 nm (NanoHA-L) diameter and weak crystallinity; micro-sized HYAP used for comparison. | Cell proliferation: modified MTT assay and acridine orange staining and fluorescence microscopy. Apoptosis: Annexin V and propidium iodide staining and FACScan; Western Blot analysis of caspase-8 and caspase-9. Analyses were performed on glass films coated with 1% suspension of HYAP materials; incubation was up to 5 days. | Cell proliferation: compared to untreated controls, cell growth was reduced after nano-HYAP treatment. Compared to controls, increased apoptosis rates observed in particle treated cells (larger particles induced higher apoptosis rates). Activation of procaspase-9 into caspase-9 by nano-HYAP, but not in untreated controls. Caspase-8 detected in none of the samples. |
| Sun, J. and Ding, T. (2009a): p53 Reaction to apoptosis induced by hydroxyapatite nanoparticles in rat macrophages. <i>J. Biomed. Mater. Res. A.</i> 88, 673-679. doi: 10.1002/jbm.a.31892 | Cytotoxicity, Apoptosis, Morphological changes. | Macrophages from male Sprague-Dawley rats. | Commercially available nano-HAYP, sonicated. Diameter between 30 and 80 nm (no further information given). | Cytotoxicity: determined by response index zone/lysis and microscopy; concentrations: 10, 20, 100 and 200 µg/ml, incubation time: 24 hr. Morphological changes determined by TEM; Apoptosis analysed by p53 measurements using semiquantitative RT-PCR and Western Blotting. | The results showed a dose-dependent proliferative inhibition of macrophages by nano-HYAP at concentrations between 20 and 200 µg/ml. The characteristic morphological changes of apoptosis were observed in macrophages after treatment with nano-HYAP for 24 hr, p53 expression was induced after treatment with nano-HYAP. |
| Sun, J. and Ding, T. (2009b): Differences in DNA Damage Pathways Induced by Two Ceramic Nanoparticles. <i>IEEE Transactions on Nanobioscience</i> 8, 78-82. | Expression of p53, p21 and heat shock protein 70. Growth arrest, DNA damage 45 (Gadd45). | Macrophages prepared from male Sprague-Dawley rats | Commercially available nano-HAYP, sonicated. Diameter between 30 and 80 nm (no further information given). | RT-PCR after incubation of macrophages for 24 hr with 10, 20, 100 and 200 µg/ml nano-HYAP. | Dose-dependent increase in p53 expression; dose-dependent increase in p21 expression up to 100 µg/ml, then decrease to negative control levels at 200 mg/ml; no effect on Gadd45 expression; dose-dependent increase in HSP70 expression. |
| Sun, W., Chu, C., Wang, J. and Zhao, H. (2007): Comparison of periodontal ligament cells responses to dense and nanophasse hydroxyapatite. <i>J. Mater. Sci: Mater. Med.</i> 18, 677-683. | Cell proliferation and differentiation. | Human periodontal ligament fibroblast-like cells (PDLC) prepared from patients undergoing surgical extraction of teeth. | Synthesized nano-HYAP (sol-gel method), characterised by TEM and energy-dispersive X ray (EDAX); particle size in average less than 50 nm, spontaneous congregation (no further information on shape or size dimensions). Commercially available micro-sized HYAP was used for comparison. | Cell proliferation: incubation with 1 mg/ml HYAP materials, cultivation for 5 days and analysis by SEM, TEM and EDAX. MTT: after cultivation for 4 days. Cell differentiation: alkaline phosphatase activity after cultivation for 5 and 8 days; flow cytometric analysis after cultivation for 5 days. | Uptake of nano-HYAP into cells, but not of micro-sized material. Alkaline phosphatase not statistically significantly increased after nano-HYAP when compared to micro-sized material and controls. Cell proliferation increased by nano-HYAP. |
| Tang, W., Yuan, Y., Liu, C., Wu, Y., Lu, X. and Qian, J. (2014): Differential cytotoxicity and particle action of hydroxyapatite nanoparticles in human cancer cells. <i>Nanomedicine</i> 9, 397-412. | Cytotoxicity, Apoptosis, cellular uptake, oxidative stress. | Human gastric cancer cells (MGC80-3); human cervical adenocarcinoma cells (HeLa); human hepatocellular carcinoma cells (HepG2); normal human liver cells (L-02). | Synthesized (aqueous precipitation method) nano-HYAP; characterized by TEM and XRD. Rod-shaped; length x width 50 x 20 nm (no further information). FITC-labelled nano-HYAP prepared and used for cellular uptake. | Cytotoxicity: MTT Assay ; concentrations: 62, 125, 250, 500, 750 and 1000 µg/ml; incubation time: 24, 48 and 72 hr; nuclear morphology: fluorescence microscopy after treatment with 500 µg/ml nano-HYAP for 72 hr. Apoptosis: AnnexinV/propidium iodide assay after treatment with 250 and 500 µg/ml for 48 and 72 hr; caspase 3, 8 and 9 determination by fluorimetric assay kits after treatment with 250 or 500 µg/ml for 72 hr; oxidative stress: fluorescence assay (DCFH-DA) for intracellular ROS and GSH assay kit for intracellular GSH after treatment with 250 or 500 µg/ml for 72 hr. Cellular uptake: flow cytometry and fluorescence microscopy of FITC-labelled nano-HYAP; fluo-3 labelling for intracellular calcium. | nano-HYAP significantly inhibited cell proliferation and induced apoptosis in cancer cells with an order of MGC80-3 > HepG2 > HeLa, but had no impact on normal hepatic cells (L-02). The increase in apoptosis was accompanied by activation of caspase 3 and 9, but not caspase 8. nano-HYAP induced formation of reactive oxygen species and decreased intracellular glutathione in cancer cells (highest reactive oxygen species burst in HeLa cells). No correlation observed between degree of cytotoxicity and cellular uptake. After 48 hr incubation, nano-HYAP was detected in all three cancer cell lines both in cytoplasm and nucleus. Highest uptake in nucleus found in MGC80-3 cells. In normal cells, nano-HYAP was not located in nucleus. An increase in the intracellular calcium level was observed in all cells (highest level in MGC80-3). The study authors conclude that various types of cancer cells react differently to nano-HYAP and they hypothesize that elevated calcium concentration and nuclear localization of the particles might be the main mechanism of growth inhibition by nano-HYAP. |

Revision of opinion on hydroxyapatite (nano)

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| Tay, C., Cai, P., Setyawati, M., Fang, W., Tan, L., Hong, C., Chen, X., and Leong, D. (2014a): Nanoparticles Strengthen Intracellular Tension and Retard Cellular Migration. <i>Nano Lett.</i> 14, 83-88. | Cell migration. | TR146 oral mucosa cells. | Commercial grade nano-HYAP (no information on source), spherical shape, characterised by TEM; primary particle diameter: 49 ± 14 nm; hydrodynamic diameter in cell culture medium: 236 ± 9 nm (i.e. agglomeration); Zeta potential (determined in serum containing cell culture medium): -6.5 ± 2 mV. | Combination of different culture experiments and visualization techniques. | nano-HYAP inhibits cell migration; internalisation as prerequisite for inhibition of migration; interference of nano-HYAP with microtubule assembly. |
| Turkez, H., Yousef, M.I., Sönmez, E., Togar, B., Bakan, F., Sozio, P. and Di Stefano, A. (2014): Evaluation of cytotoxic, oxidative stress and genotoxic responses of hydroxyapatite nanoparticles on human blood cells. <i>Journal of Applied Toxicology</i> 34, 373 - 379. | Cytotoxicity, Oxidative stress, Genotoxicity. | Human peripheral blood lymphocyte cultures prepared from blood samples from 6 healthy, non-smoking men. | Synthesized needle-like nano-crystals; average diameter 10 - 50 nm (TEM). | Cytotoxicity: MTT and LDH assay. Total antioxidant capacity (TAC) and Total oxidative stress (TOS) determined by commercially available kits. Chromosome aberration (CA), Micronucleus (MN) formation (Cytochalasin B addition after 44 hr of incubation with nanomaterial), Sister Chromatide-Exchange (SCE) for assessment of genotoxicity. 8-OH-dG formation determined by commercially available kit. Concentrations tested: 5, 10, 20, 50, 75, 100, 150, 300, 500 and 1000 ppm; incubation time: 72 hr. | Cytotoxicity: statistically significantly decreased cell viability at concentrations ≥ 150 ppm (MTT) and ≥ 300 ppm (LDH). Antioxidant capacity: change in TAC levels at concentrations ≥ 150 ppm and in TOS levels at concentrations ≥ 300 ppm. Increase in 8-OH-dG at concentrations ≥ 300 ppm. Statistically significant differences of SCE (at 150, 300 and 500 ppm), MN (at 500 ppm) and CA (at 300 and 500 ppm) compared to control . |
| Wang, L., Zhou, G., Liu, H., Han, J., Zheng, L. and Fan, Y. (2012): Nano-hydroxyapatite particles induce apoptosis on MC3T3-E1 cells and tissue cells in SD rats. <i>Nanoscale</i> 4, 2894-2899. | Cell proliferation and apoptosis. | MC3T3-E1 cells (commercially available osteoblast cell line). | Synthesized nano-HYAP (precipitation method) characterised by TEM, XRD and FT-IR. Shape: rod, mean size: 28.47 ± 0.4 nm. | Cell proliferation: MTT assay, concentrations: 0, 10, 100, 1000 and 10000 $\mu\text{g/ml}$, incubation time: 24 hr. Apoptosis: TEM (concentrations unclear; incubation time: 24 hr). | Dose-dependent and statistically significant decrease in cell viability in the concentration range observed. Apoptosis: after incubation with 10000 $\mu\text{g/ml}$ (highest dose), chromatin condensation, chromosomal DNA-fragmentation, membrane shrinking and formation of apoptotic bodies were observed. |
| Xu, J., Xu, P., Li, Z., Huang, J. and Yang, Z. (2011): Oxidative stress and apoptosis induced by hydroxyapatite nanoparticles in C6 cells. <i>J. Biomed. Mater. Res. A.</i> 100, 738-745. doi: 10.1002/jbm.a.33270 | Cytotoxicity, oxidative stress, apoptosis. | C6 cells (commercially available rat glioma cell line). | Synthesized nano-HYAP (precipitation method); size: 50 - 80 nm (no further information). | Cytotoxicity: MTT assay (concentrations: 10, 100, 250 and 500 $\mu\text{g/ml}$, incubation time: 24, 36 and 48 hr). Apoptosis: nuclear staining by Hoechst 33342 and by flow cytometry after Annexin-V-FITC and propidium iodide staining. Oxidative stress: DCFH-DA-assay and T-SOD (superoxide dismutase) assay. | Compared to untreated controls, concentration- and time-dependent significant decreases in cell viabilities which could be prevented by using a ROS scavenger. Compared to controls, dose-dependent increase in apoptosis which could be reduced by using a ROS scavenger. Apoptosis was confirmed by morphology. Concentration-dependent significant increase in ROS formation by nano-HYAP. Dose-dependent decrease (statistically significant from 100 $\mu\text{g/ml}$) in SOD. Authors conclude that nano-HYAP reduces growth of C6 cells and levels of SOD in these cells. It increases ROS levels and induces apoptosis. |

Revision of opinion on hydroxyapatite (nano)

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| Xue, Y., Wu, J. and Sun, J. (2012): Four types of inorganic nanoparticles stimulate the inflammatory reaction in brain microglia and damage neurons in vitro. <i>Tox. Lett.</i> 214, 91-98. | Release of proinflammatory cytokines and chemokines. | Primary microglia cells prepared from brains of Sprague-Dawley rats and commercially available PC12 cell line. | Nano-HYAP obtained from a university, characterized by TEM, zeta potential, XRD. Needle-shaped material, length 60 nm, diameter 12 nm, Zeta-potential (in medium) 17.8 mV | Microglia cells were incubated with 0.25 and 50 mg/ml nano-HYAP for 24 hr. Supernatants were used to stimulate PC12 cells. Lipopolysaccharide was used as positive control. In microglia, production of nitrite was determined by photometry. Nitric acid synthase, MCP-1 and MIP-1 α were determined by RT-PCR, nitric acid synthase also by Western blotting; NF-kB activation in microglia by electrophoretic mobility shift assay; cytokines (TNF- α , IL-1 β and IL-6) in microglia supernatants were determined by ELISA, cytotoxicity in PC12 cells after incubation with microglia supernatant was determined by MTT assay. | Compared to negative controls, significantly increased nitrite and nitric acid synthase. Significantly upregulated MCP-1 and MIP-1 α production. Increased expression of NF-kB; significantly increased levels of cytokines (TNF- α , IL-1 β and IL-6). Effects in PC12 cells incubated with supernatants from nano-HYAP treated microglia cells: significantly reduced viability. |
| Yin, M., Han, Y., Bauer, I.W., Chen, P. and Li, S. (2006): Effect of hydroxyapatite nanoparticles on the ultrastructure and function of hepatocellular carcinoma cells in vitro. <i>Biomed. Mater.</i> 1, 38-41. | Cytotoxicity, cellular uptake and morphology, cell cycle analysis | Bel-7402 human hepatocellular carcinoma cells. | Synthesized nano-HYAP (precipitation method), analyzed by TEM. Rod- and ball-shaped, average size about 15 x 60 nm (apparently superficial positive charge, see section 3.2). | Cytotoxicity: MTT assay (concentrations: 0, 0.14, 0.28 and 0.56 mmol/l; incubation time: 1, 2, 3, 4, 5, 6, 7 days). Cell morphology: after incubation with 0.56 mmol/l. Apoptosis: propidium iodide staining of 3, 4 and 5 day incubations. Cell counts: all 1-5 day incubations. Influence of a not further specified stabilizer on cellular uptake was also investigated. | Cellular adherence affected by nano-HYAP due to attachment of nano-HYAP on cell membrane followed by internalisation (uptake in membrane, endocytosis). After 4 days, structural changes of cells (e.g. mitochondrial vacuolization, swell of endoplasmic reticulum, nuclear blebs. Dose- and time-dependent decrease in cell number and cell growth. Time-dependent increase of cells in G1 phase versus decrease of cells in S and G2/M phase. The authors conclude that nano-HYAP easily enters human hepatocellular carcinoma cells, change their ultrastructure and suppress their proliferation. |
| Yuan, Y., Liu, C., Qian, J., Wang, J. and Zhang, Y. (2010): Size-mediated cytotoxicity and apoptosis of hydroxyapatite nanoparticles in human hepatoma HepG2 cells. <i>Biomaterials</i> 31, 730 - 740. | Cell viability, apoptosis, cellular uptake | Human hepatocellular carcinoma HepG2 cells and normal human liver L-02 cells. | Synthesized nano-HYAP particles of different size (26 nm; 45 nm, 78 nm, 175 nm) and FITC-labelled nano-HYAP particles. Characterization: X-ray diffraction, Dynamic light Scattering, morphology analysis by Nanoscope \emptyset AFM. Particle shape only for 45 nm material explicitly mentioned: spherical. | Cellular uptake and nuclear morphology: microscopy. Cell viability: MTT assay. Apoptosis: FITC Annexin staining (flow cytometry). Caspase activities (fluorescence); PARP, pro-caspase 3, Bax, Bcl-2, Bid and cytochrome c by Western Blotting. Concentration: 50, 100 and 200 μ g/ml; incubation time: 24, 48 and 72 hr. Cell viability measured in both cell types; all other tests performed in HepG2 cells only. | Cell viability decreases with decreasing particle size; HepG2 cells more affected than L-02 cells. In HepG2 cells, apoptosis strongly depended on the size (45-nm > 26-nm > 78-nm > 175-nm). Particles ranging from 20 - 80 nm in size activate caspase 3 and 9, decrease Bcl-2 and increase levels of Bax, Bid and the release of cytochrome c from mitochondria into cytoplasm, with the best efficiency from 45-nm nano-HYAP. Correlating the cellular response with the cellular internalization, it can be inferred that the size of HYAP and thereby the cellular localization had a predominant effect on the HAYP-induced cytotoxicity, apoptosis, and the levels of the apoptotic proteins in HepG2 cells. |
| Zhang, M., Liu, S., Xu, G., Fu, J. and Zhang, D. (2014): Cytotoxicity and apoptosis induced by nanobacteria in human breast cancer cells. <i>International Journal of Nanomedicine</i> 9, 265-271. | Cell viability, apoptosis. | MDA-MB-231 human breast cancer cell line (commercially available). | Commercially obtained nano-HYAP, particle size determined by SEM revealed a size range from 50 to 200 nm (no further information on characterization). | Viability determined by CCK-8 kit, exposure concentrations unclear, duration of incubation: 72 hr. Apoptosis determined by (FITC)-annexin V and propidium iodide staining and flow cytometry. Cell morphology investigated by phase contrast and electron microscopy after 72 hr incubation with nano-HYAP. | Uptake of nano-HYAP into cells and morphological changes in cells. Compared to controls, significantly decreased cell viability; induction of early apoptosis but not of late apoptosis or necrosis. Mitochondrial swelling. |

Revision of opinion on hydroxyapatite (nano)

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| <p>Zhao, X., Heng, B.C., Xiong, S., Guo, J., Tan, T.T., Boey, F.Y.C., NG, K.W. and Loo, J.S.C. (2011): In vitro assessment of cellular responses to rod-shaped hydroxyapatite nanoparticles of varying lengths and surface areas. <i>Nanotoxicology</i> 5, 182 - 194.</p> | <p>Cell viability, oxidative stress, cellular uptake.</p> | <p>BEAS-2B (an immortalized human bronchial epithelial cell line), RAW264.7 (an immortalized mouse monocyte/macrophage cell line) and HepG2 cells.</p> | <p>Synthesized rod-shaped nano-HYAP of different sizes; characterized by FT-IR, X-ray diffraction, BET analysis, TEM, DLS. Agglomeration observed in PBS, therefore medium containing SHMP (sodium hexametaphosphate) was used. Characterisation: nHA60: average length 60 ± 10 nm; average width 25 ± 5 nm (TEM); average size in 0.1% SHMP: 79 nm; zeta potential -55.4 mV in SHMP and -0.4 mV in water; specific surface area 47.02 cm²/g. nHA120: average length 120 ± 15 nm; average width 30 ± 5 nm (TEM); average size in 0.1% SHMP: 96 nm; zeta potential -36.3 mV in SHMP and -5.7 mV in water; specific surface area 23.33cm²/g. nHA240: average length 240 ± 30 nm; average width 20 ± 5 nm (TEM); average size in 0.1 % SHMP: 112 nm; zeta potential -45.8 mV in SHMP and -5.3 mV in water; specific surface area 46.12 cm²/g.</p> | <p>Cell viability: WST-8 assay, concentration range: 10 - 300 µg/ml, incubation time: 24 hr. Intracellular ROS formation: DFDA assay (a fluorescence method), concentration range: 10 -300 µg/ml, incubation time: 4 hr. Alizarin S-assay (for calcium mineral histochemistry), concentration: 100 µg/ml, incubation time: 4 hr.</p> | <p>Cell viability: compared to positive controls, only minor impairment of cell viability in BEAS-2B and HepG2 cells, in RAW264.7 cells, viability decreased with increasing concentrations of nano-HYAP reaching statistical significance at 100 and 300 µg/ml (for all sizes of nano-HYAP); in general, lower cell viabilities with nHA20 compared to other sizes. ROS generation: increased ROS in all three cell lines with all sizes of nano-HYAP. Most significant increase in BEAS-2B cells with nHA 240 at 30 and 100 µg/ml. In RAW264.7 and HepG2: most significant increase with nHA60 at 300 µl. Alizarin Red-S: compared to untreated controls: increased staining for calcium in all three cell lines, comparable staining for nHA 60 and nHA240, lower staining for nHA120.</p> |
| <p>Zhao, X., Ng, S., Heng, B., Guo, J., Ma, L., Tan, T., Ng, K. and Loo, S. (2013): Cytotoxicity of hydroxyapatite nanoparticles is shape and cell dependent. <i>Arch.Toxicol.</i> 87, 1037-1052.</p> | <p>Apoptosis, DNA-content, Intracellular ROS generation, Release of IL-6 in BEAS-2B cells and of TNF-α in RAW264 cells, intracellular particle distribution, particle-cell association.</p> | <p>RAW264.7 cells (commercially available murine macrophages) and BEAS-2B cells (commercially available human bronchial epithelial cells).</p> | <p>Synthesized nano-HYAP of four different shapes (rod-like (RD), needle-shaped (DN), spherical (SP) and plate-shaped (PL)), characterised by BET, XRD, TEM, DLS, zeta potential, FT-IR. Nano-HYAP-ND: average size 74.9 nm (in 0.1 % SHMP), Zeta potential 4.8 ± 0.3 mV (in deionised water), specific surface area 74.88 cm²/g. Nano-HYAP-RD: average size 96 nm (in 0.1 % SHMP), Zeta potential -5.7 ± 0.6 mV (in deionised water), specific surface area 23.33 cm²/g. Nano-HYAP-SP: average size 92.8 nm (in 0.1 % SHMP), Zeta potential -14.1 ± 0.3 mV (in deionised water), specific surface area 32.823 cm²/g. nano-HYAP-PL: average size 108.7 nm (in 0.1 % SHMP), Zeta potential -9.7 ± 0.3 mV (in DI water), specific surface area 61.75 cm²/g.</p> | <p>DNA-content: pico-green assay after 24 hr incubation in concentration range 10 - 300 µg/ml. Apoptosis/necrosis: Annexin-V/propidium iodide staining and flow cytometry/ FACScan after 24 hr incubation. Intracellular ROS generation: dihydrofluorescein diacetate (DFDA) assay after 4 hr incubation and photometric reading. Release of IL-6 in BEAS-2B cells and TNF-α in RAW264 cells: ELISA after 24 hr incubation in concentration range 10 - 300 µg/ml. Particle-cell association: Alizarin red S staining after 4- and 24 hr incubation with nano-HYAP.</p> | <p>Viability and DNA-content: compared to the positive control nano-ZnO, only minor concentration-dependent decreases in viability observed for all materials in BEAS-2B cells, viability not changed in RAW264.7 cells. No significant ROS formation in both cell types; highest cytotoxicity observed for needle- and plate-shaped nano-HYAP. Cytokine production: induction of IL-6 in BEAS-2B cells comparable to positive control (Lipopolysaccharide); highest induction for needle- and platelet shaped material. TNF-α in RAW264 cells: dose-dependent increase, but no difference between shapes. Cellular uptake: higher uptake in RAW264.7 cells, incubation-time dependent increase in uptake; rod-shaped particles generally exhibited higher levels of cell-particle association than rod-shaped material. Conclusion: cytotoxicity of nano-HYAP first dependent on cell type, then on shape. ROS generation in this study most probably not the cause for cell death. Role of inflammatory cytokines in cell death to be further elucidated; particle agglomeration influences cellular uptake. Higher uptake does not necessarily lead to higher cytotoxicity, as uptake was highest for rod-shaped material.</p> |

Information on solvent used for determination of Zeta potential was given when available (it was not always given in the publications)

Abbreviations used in Table 2 (if not explained in Table 2):

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| BET: | Brunauer, Emmett and Teller |
| EDX: | Energy Dispersive X-Ray |
| FT-IR: | Fourier Transform Infrared Spectroscopy |
| HYAP: | Hydroxyapatite |
| IR: | Infrared Spectroscopy |
| RT-PCR: | Real-Time Polymerase Chain Reaction |
| ROS: | Reactive Oxygen Species |
| SEM: | Scanning Electron Microscopy |
| SSA: | Specific Surface Area |
| TEM: | Transmission Electron Microscopy |
