Scientific Committee on Consumer Safety

SCCS

OPINION ON

Hydroxyapatite (nano)

The SCCS adopted this document
at its plenary meeting on 30-31 March 2021

ACKNOWLEDGMENTS

Members of the Working Group are acknowledged for their valuable contribution to this Opinion. The members of the Working Group are:

For the preliminary and the final version

The SCCS members:
Dr U. Bernauer (Chairperson)
Dr L. Bodin
Prof. Q. Chaudhry
Prof. P.J. Coenraads
Prof. M. Dusinska
Dr E. Gaffet
Prof. E. Panteri
Dr C. Rousselle (Rapporteur)
Dr M. Stepnik
Dr S. Wijnhoven

The SCHEER members
Dr W.H. de Jong

The External expert(s)
Dr N. von Goetz

All Declarations of Working Group members are available on the following webpage:

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This Opinion has been subject to a commenting period of eight weeks after its initial publication (from 9 November 2020 until 4 January 2021). Comments received during this time were considered by the SCCS. For this Opinion, comments received resulted in the changes related to impurities and genotoxicity.

1. ABSTRACT

The SCCS concludes the following:

(1) In view of the above, and taking into account the scientific data provided, does the SCCS consider the nanomaterial Hydroxyapatite safe when used in leave-on and rinse-off dermal and oral cosmetic products according to the maximum concentrations and specifications reported in the attached list, taking into account reasonably foreseeable exposure conditions?

Having considered the data provided, and other relevant information available in scientific literature, the SCCS cannot conclude on the safety of the hydroxyapatite composed of rod–shaped nanoparticles for use in oral-care cosmetic products at the maximum concentrations and specifications given in this Opinion. This is because the available data/information is not sufficient to exclude concerns over the genotoxic potential of HAP-nano.

(2) Does the SCCS have any further scientific concerns with regard to the use of Hydroxyapatite in nano form in cosmetic products?

Although the particle shape in the HAP-nano considered in this Opinion is reported as rod-shaped, the SCCS is aware that, depending on the manufacturing process, needle-shaped HAP nanoparticles may also be produced. The available information indicates that HAP-nano in needle-shaped form is of concern in relation to potential toxicity. Therefore, needle-shaped HAP-nano should not be used in cosmetic products.

As detailed in Annex I, the SCCS has concluded that there is a basis for concern that the use of HAP-nano in cosmetic products can pose a risk to the consumer. The SCCS will be ready to assess any evidence provided to support safe use of the materials in cosmetic products.

Keywords: SCCS, scientific opinion, Hydroxyapatite (nano), CAS No 1306-06-5, EC No. 215-145-20, Regulation 1223/2009

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on Hydroxyapatite (nano), preliminary version of 27-28 October 2020, final version of 30-31 March 2021, SCCS/1624/2020

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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.).

Scientific Committee members

Contact

European Commission
Health and Food Safety
Directorate C: Public Health
Unit C2: Health information and integration in all policies
L-2920 Luxembourg
SANTE-SCCS@ec.europa.eu

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2. MANDATE FROM THE EUROPEAN COMMISSION

Background

Article 2(1)(k) of Regulation (EC) No 1223/2009 (Cosmetics Regulation) states 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. some metals, metal oxides, carbon materials, etc.). 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 cosmetic products containing nanomaterials other than colorants, preservatives and UV-filters and not otherwise restricted by the Cosmetics Regulation to be notified to the Commission six months prior to being placed on the market. Article 19 of this Regulation requires nano-scale ingredients to be labelled (name of the ingredient, followed by 'nano' in brackets). If there are concerns over the safety of a notified nanomaterial, according to Article 16 of the Regulation, the Commission shall refer it to the Scientific Committee on Consumer Safety (SCCS) for a full risk assessment.

The Commission services received 17 notifications under Article 16 of the Cosmetics Regulation *via* the Cosmetic Product Notification Portal (CPNP) for cosmetic products containing Hydroxyapatite (CAS No 1306-06-17 and EC No. 215-145-20) in nano form. Hydroxyapatite without any reference to the nano form is reported in the CosIng database as an abrasive, bulking, oral care and skin-conditioning agent. It is not regulated under the Cosmetic Regulation (EC) No 1223/2009.

According to the notifications submitted, this ingredient is used in both leave-on and rinse-off dermal and oral cosmetic products, including skin (skin care) and oral hygiene (toothpaste, mouthwash) products, with different concentrations and specifications as reported in the attached list.

The Commission has concerns on the use of Hydroxyapatite in nano form because of the potential for nanoparticles to be absorbed dermally or across a mucous membrane and to enter cells. Therefore, we request the SCCS to carry out a safety assessment of the nano form of Hydroxyapatite reported in the notifications listed in the annex to this mandate.

Terms of reference

(1) In view of the above, and taking into account the scientific data provided, does the SCCS consider the nanomaterial Hydroxyapatite safe when used in leave-on and rinse-off dermal and oral cosmetic products according to the maximum concentrations and specifications, taking into account reasonably foreseeable exposure conditions?

(2) Does the SCCS have any further scientific concerns with regard to the use of Hydroxyapatite in nano form in cosmetic products?

3. OPINION

Preamble

A first Opinion on safety assessment of nanohydroxyapatite (HAP-nano) was published by the SCCS in October 2015 and revised in March 2016 (SCCS/1566/15). The assessment done in this first Opinion was based on the data provided in the 35 CPNP notifications at that time for the cosmetic products containing hydroxyapatite (CAS No 1306-06-5) in nano form. The overall Opinion was inconclusive, as the SCCS considered that the safety of HAP-nano materials, when used in oral cosmetic products up to a concentration of 10%, could not be decided on the basis of the limited data submitted by the Notifiers and that retrieved from literature search. In this Opinion, 15 notifications have been assessed (after the withdrawal of 2 notifications) under Article 16 of the Cosmetics Regulation for the use of hydroxyapatite (CAS No 1306-06-17 and EC No. 215-145-20) in nano form in cosmetic products.

Following initial assessment of the data provided, the SCCS sent a request to the manufacturer of HAP-nano for some clarifications and additional data/information. This led to the provision of results from some *in vitro* assays to the SCCS along with other information on specific aspects. This Opinion has taken into account all the information that was available to the SCCS up through October 2020.

3.1 CHEMICAL AND PHYSICAL SPECIFICATIONS

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

IUPAC: Pentacalcium hydroxide triphosphate
INCI: Hydroxyapatite(nano)

3.1.1.2 Chemical names

Hydroxyapatite
Hydroxylapatite
Calcium Phosphatetribasic
Calcium Hydroxyphosphate
Pentacalcium hydroxide tris(orthophosphate)

3.1.1.3 Trade names and abbreviations

Trade name: nanoXIM •CarePaste
Abbreviations: nanoXIM

3.1.1.4 CAS / EC number

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

Synonym
CAS number: 12167-74-7

EC number: 235-330-6

3.1.1.5 Structural formula

Hydroxyapatite nanoparticles are **nanorod** or **nanothumb** shaped.

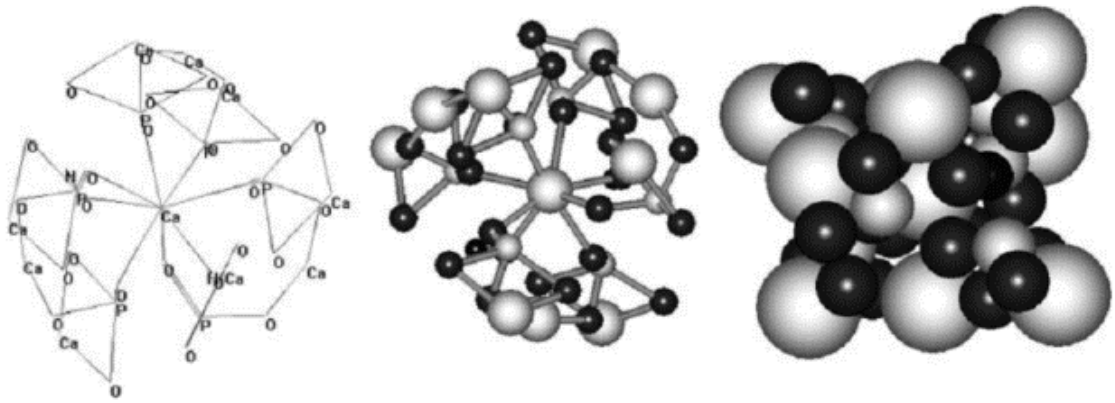

Figure 1: Spatial model of the hydroxyapatite molecule with calcium as the central atom (Ca, big white; O, small black; P, small white; H, smallest black).

3.1.1.6 Empirical formula

Formula: $Ca_{10}(PO_4)_6(OH)_2$

3.1.2 Physical form

3.1.3 Molecular weight

Molecular weight: 1004.6 g/mol

3.1.4 Purity, composition and substance codes

nanoXIM.CarePaste is composed of synthetic and inorganic hydroxyapatite in water, as indicated in the following table:

Table 1 – nanoXIM•Care Paste composition specifications

Substance	CAS No EC No	Function	Concentration (wt%)
Hydroxyapatite	1306-06-5 215-145-7	main component	15.5 ± 0.5 %
Potassium Chloride (KCl)	7447-40-7 231-211-8	preservative	4.5% ± 0.5%
Water	7732-18-5 231-791-2	excipient	80.0% ± 1.0%

Hydroxyapatite (nano) is fully synthetic and inorganic.

3.1.5 Impurities / accompanying contaminants

This product (nanoXIM.CarePaste) contains no residues from solvents but it contains the following impurities:

Table 2 – nanoXIM•Care Paste impurities specifications

Substance	Concentration
Total Heavy Metals (as Pb)*	< 20 ppm

* Ph. Eur. 7th Ed. 2.4.8. Heavy metals

KCl is also an impurity of nanoXIM.CarePaste.

The origin of impurities (heavy metals) comes from the reactants used in the manufacturing of the product. Results of heavy metals content are in accordance with allowable quantities for hydroxyapatite for medical devices uses and for dentifrice applications, including ISO11609:2010 Dentistry -- Dentifrices -- Requirements, test methods and marking.

Table 3: Content of heavy metal impurities in nanoXIM.CarePaste

Arsenic (As)	mg/kg	SM	<0.3	0.48	0.74
Barium (Ba)	mg/kg	SM	1.4	1.4	0.6
Lead (Pb)	mg/kg	SM	0.24	0.12	0.13
Iron (Fe)	mg/kg	SM	38	39	15
Potassium (K)	mg/kg	SM	11	9.9	7.5
Copper (Cu)	mg/kg	SM	0.43	0.44	0.32
Magnesium (Mg)	mg/kg	SM	160	160	90
Manganese (Mn)	mg/kg	SM	1.7	1.7	0.16
Sodium (Na)	mg/kg	SM	82	86	110
Nickel (Ni)	mg/kg	SM	1.1	1.1	0.27
Strontium (Sr)	mg/kg	SM	43	44	24

SCCS comment
The Notifiers should provide detailed information on the level of any significant impurities other than heavy metals and provide an upper limit of total impurities in the raw material. The content of heavy metal impurities should be expressed in % [w/w].

3.1.6 Solubility

Insoluble (or slightly soluble) in water (0.0065 g/L at 20 °C – EU method A.6, GLP), soluble at low pH.

3.1.7 Partition coefficient (Log P_{ow})

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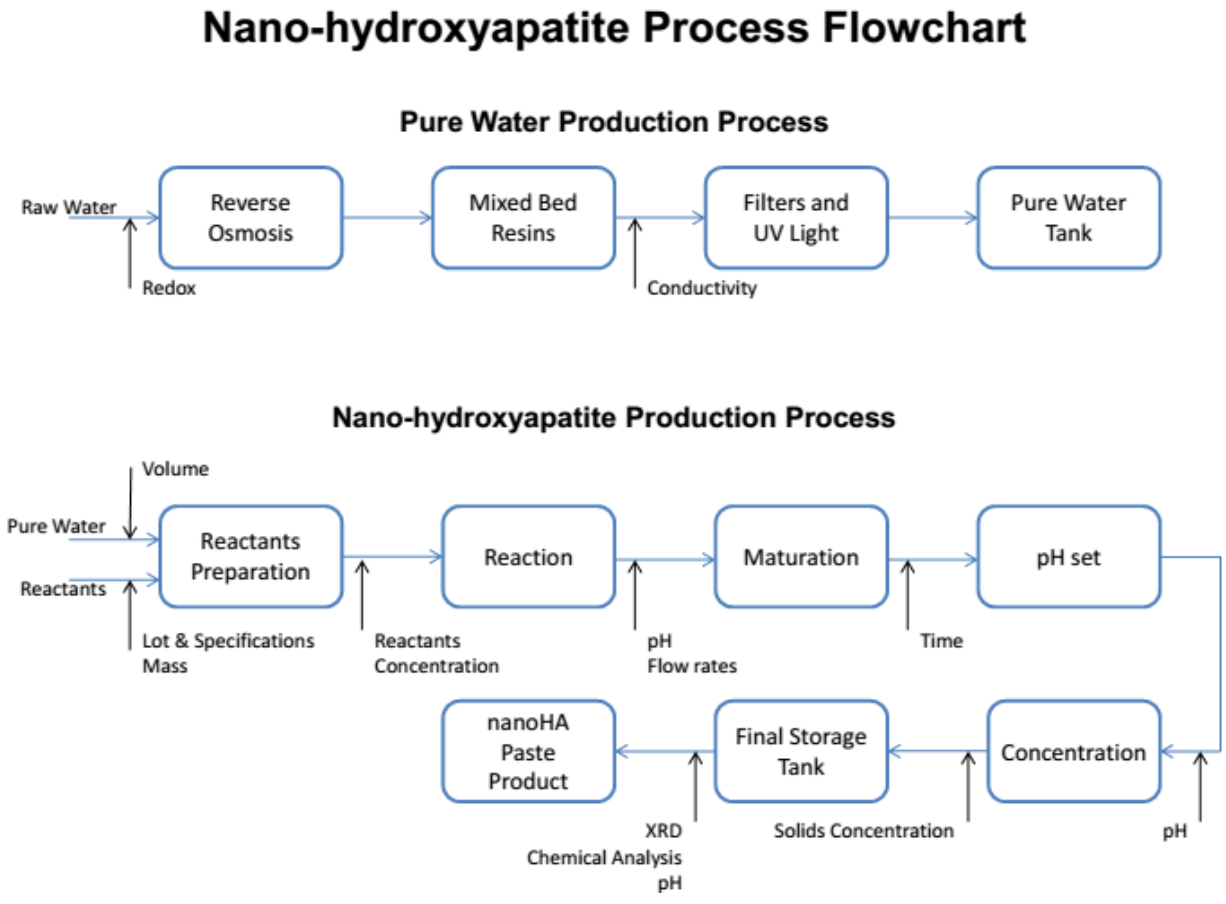
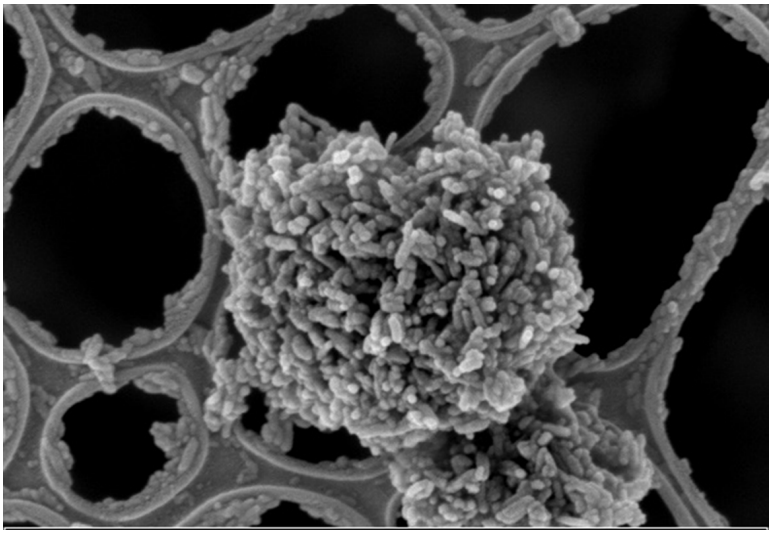
3.1.8 Additional physical and chemical specifications

According to the information provided, the nanoparticle form of hydroxyapatite is fully synthetic and inorganic. It is a white, odourless paste. The manufacturing process for HAP-nano (shown below) involves continuous wet chemical precipitation carried out close to room temperature, which results in a diluted slurry. This is then concentrated to 15.5% wt paste. As such, the process does not involve any calcination step.



Quoting a number of studies (Santos *et al.*, 2018; Ryabenkova *et al.*, 2017; Hruschka *et al.*, 2017; Salaie *et al.*, 2019), the Notifier has stated that the process is designed to produce only rod shape particles and that no needle-like nanoparticles have been observed in the products produced in this manner (TEM image is shown below).



Following SCCS request, the manufacturer provided additional information:

HAP-nano is obtained continuously by wet chemical precipitation, resulting in a diluted slurry that is then concentrated to its final value of 15.5 %wt. The product is not obtained by diluting nano-powder in water. It is a slurry from the beginning of reaction to the final product concentration. All the reaction and manufacturing process is done close to room temperature and below 60 ºC.

Basically, the reactants (calcium and phosphorous salts) are prepared separately by dissolving these salts in purified water in individual stirred tanks. Then, separately, these reactants solutions are both fed continuously to a NETmix reactor where the precipitation reaction of the HAP-nano occurs instantaneously. The precise control of mixing provided by the reactor technology makes it possible to obtain a highly pure and stoichiometric HAP-nano with the morphology of nano-rods. The residence time in this continuous reactor is just of a few seconds. From there, the slurry flows to a reception tank and is allowed to rest overnight at room temperature. Next day, this slurry is fed to a continuous centrifuge to be concentrated to the final desired value of 15.5% HAP-nano wt, where pH is adjusted to the final value of 10.0±0.5, if needed. All process variables are monitored and registered in the computer (volumes, flowrates, pH, temperatures,…).

The process was, therefore, designed to produce only particles with rods shape. No needle-like nanoparticles are observed in the product that was produced in this manner.
The TEM analysis shown in the reports and papers were done with samples being prepared just by placing the HAP-nano suspension on the TEM grid and let it dry. No calcination step of the materials was applied.

More details about the NETmix technology are available here:

https://www.fluidinova.com/index.php/company-nano-hydroxyapatite-manufacturer-and-supplier/#technology.

Besides these, several papers by other researchers have been published which state that the HAP-nano produced following the above-described manufacturing process is characterized as nano rod-like particles.

SCCS comment
From the additional information and clarification provided, the SCCS acknowledges that the HAP-nano for which notification was sent and which is intended for these cosmetic uses are rod shaped. Therefore, this Opinion relates only to rod-shaped nano HAP and will not be applicable to any needle-shaped HAP-nano.

3.1.9 Particle size

The typical particles found in nanoXIM®CarePaste are micron size agglomerates built from nanoparticles of hydroxyapatite. Particle size distribution (in number) of the product agglomerates has been determined directly by Malvern Mastersizer 2000 (dispersion unit Hydro 2000s, see Figure 2).

It is also possible to observe that no agglomerates with particle size below 100 nm are detected. The smaller agglomerates detected present a size bigger than 600 nm.

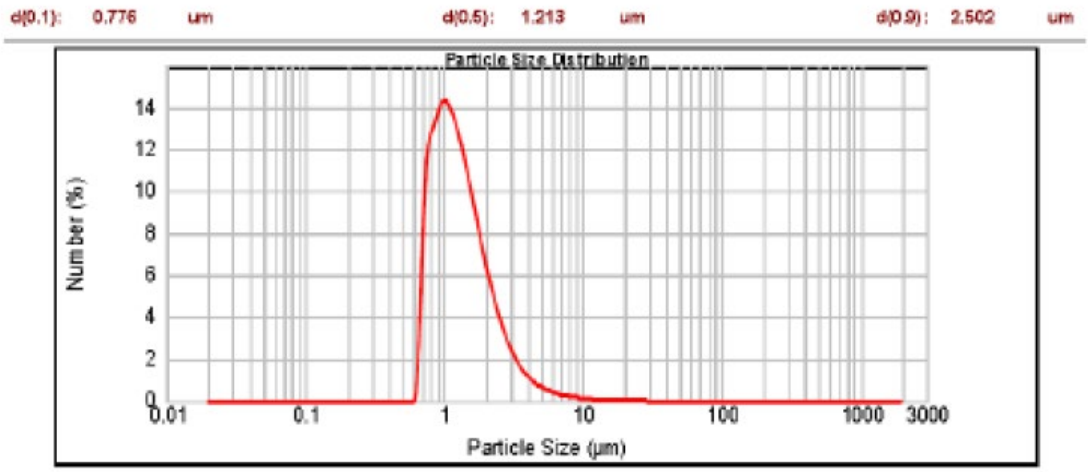


Figure 2 – nanoXIM•CarePaste agglomerates particle size distribution by number

However, it is known from scanning electron microscopy (SEM) and transmission electron microscopy (HRTEM) that this material is nanostructured. TEM images (Figure 3) show nanorod shaped entities with size below 100 nm. Structures larger than 100 nm can be observed due to the agglomeration of the particles.

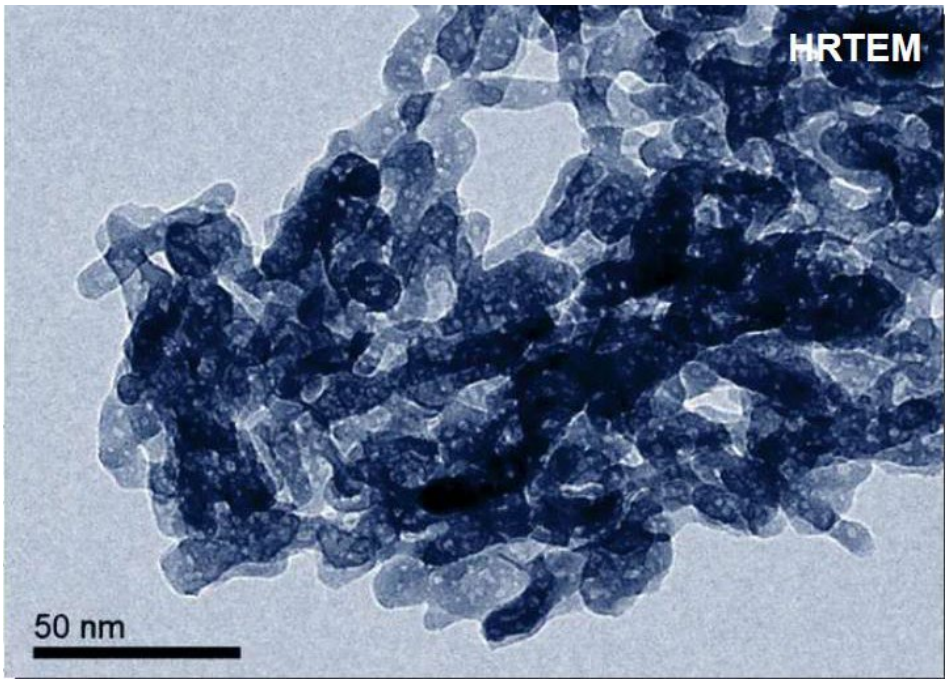


Figure 3: TEM image of a sample of nanoXIM.CarePaste

If a particular preparation of the sample is applied for the particle size distribution analysis using a dispersant and stressing the sample with ultra-sounds, it is possible to detect individualised nanoparticles and determine its size distribution.

As it can be observed, the nanoXIM hydroxyapatite nanoparticles possess a rod-like morphology and a particle size < 100 nm. Particularly, the particles morphology show a width between 5–20 nm (typically close to 10 nm) and length below 50 nm (typically between 20 to 40 nm).

Figure 4 presents the particle size distribution for this sample preparation. Mean particle size of individualised particles (per particle number) was 64 nm

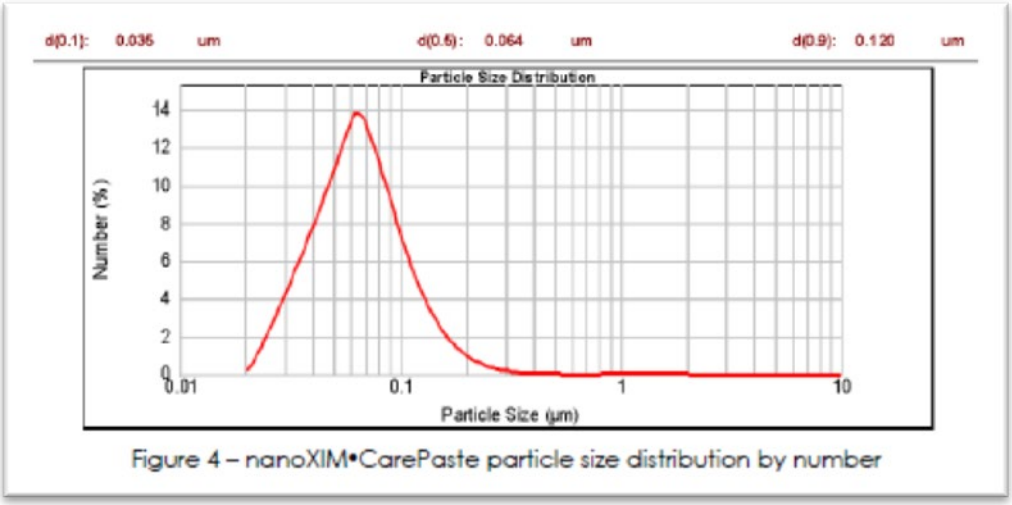


Figure 4 – nanoXIM•CarePaste particle size distribution by number

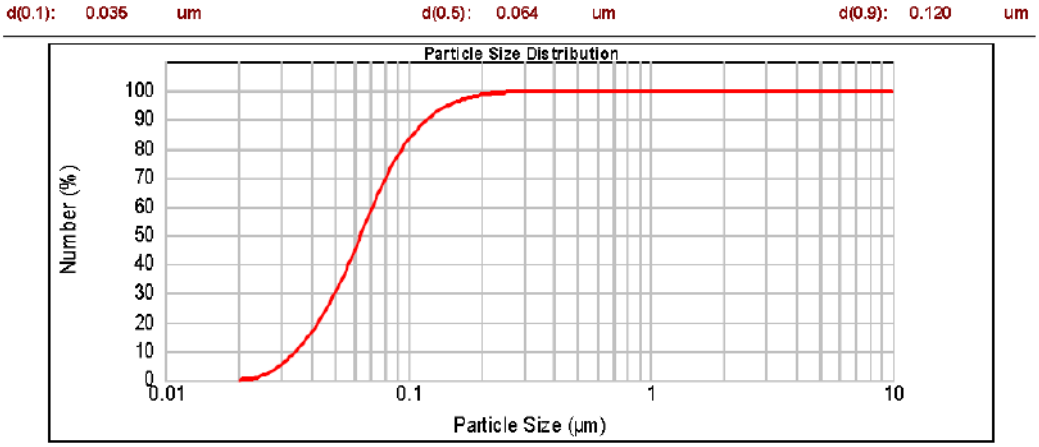


Figure 4 – nanoXIM•CarePaste cumulative particle size distribution by number

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3.1.10 Microscopy

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3.1.11 Crystal structure

Structure – hexagonal, space group P63/m

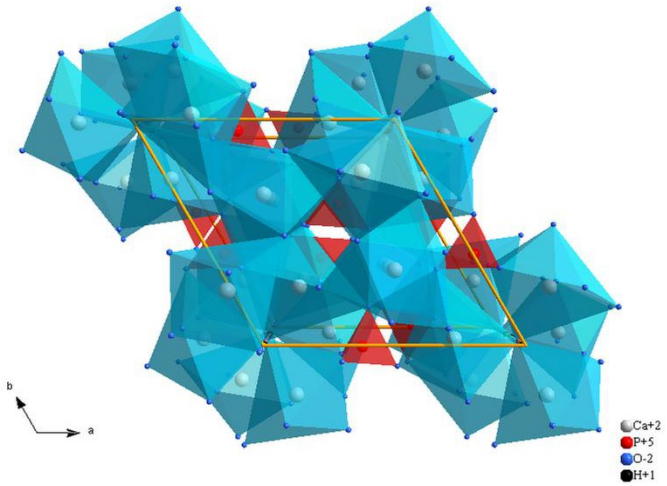

Figure 5: Unit cell projection

3.1.12 UV absorption

No information provided in the Dossier.

3.1.13 Surface characteristics

Surface characteristics

Specific surface area	: 80 - 100 m^2/g*
Surface charge (zeta potential)	: +30 (±1) mV
Surface modifications or functionalization	: No
Coating	: Uncoated

* assessed by BET Nitrogen Adsorption

3.1.14 Droplet size in formulations

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3.1.15 Homogeneity and stability

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 HAP-nano -nanoXIM·CarePaste - it should be stirred before every use. This 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.

Microbiological assays reflecting the total viable aerobic count, pH, organoleptic characteristics such as aspect, colour and odour and concentration determinations were performed ensuring nanoXIM·CarePaste specifications for 18 months. The shelf life of this product is 18 months.

3.1.16 Other parameters of characterisation

Density – 3.14 g/cm^3
Melting temperature – > 1100°C

Organoleptic characteristics	: White paste, odourless.
Density	: 1.1 – 1.2 g/cm^3
pH limits	: 10.0 ± 0.5 (measured directly on the product)
Solids content (wt %)	: 20.0 ± 1.0 %
Viscosity	: 50 – 400 cP at 25°C (Brookfield DVERV – Spindle RV2 – 100 rpm)
Porosity	: Not applicable
Flammability	: Not considered as flammable
Explosive properties	: There are no chemical groups present in the molecule which are associated with explosive properties. Not considered as an explosive material.

3.2 FUNCTION AND USES

The following information was provided by the Notifiers:

Hydroxyapatite as an ingredient is reported in the CosIng database without any reference to the nano form with the function of abrasive, bulking and emulsion stabilising.

HAP-nano is intended to be used in the following categories of cosmetic products:

- Oral hygiene products > Tooth care products >Toothpaste at concentrations up to 10%
- Skin products > Skin care products > Other skin care at concentrations up to 5%
- Oral hygiene products > Mouth wash / breath spray >Mouth wash at concentrations up to 0.465%

The following information was provided in the notification files

nanoXIM•CarePaste is intended to be incorporated in oral care products. Manufacturer recommended use concentrations for this application are generally between 3-15%, but it can be used in concentrations up to 90% which corresponds to 13.95% of HAP-nano (w/w) in the final product. To insure homogeneity of nanoXIM.CarePaste it should be stirred before every use.

During manufacture of oral care products, it can be easily included in water-based products and it is stable at high temperatures. In emulsion-type products, it should be added after emulsion formation, during cooling-process with continuous mixing.

Hydroxyapatite can be found in teeth and bones within the human body. Thus, it has been used as a biocompatible ceramic in many medical applications. It has been widely used in orthopaedics, mainly for bone reparations and osseous implants, and in dentistry for dental reparations and implants.

Hydroxyapatite is also used in aesthetic surgery (mainly in fillers) and cosmetics, namely in dermocosmetics products.

According to the Cosmetics Database of Environmental Working Group's Skin Deep, hydroxyapatite may be used as an abrasive, a bulking agent, as an oral care agent or as a stabilising emulsion.

Eye and lip cosmetic products and facial cosmetic products containing microparticles of hydroxyapatite are available on the market.

In vitro studies evidenced that hydroxyapatite stimulates skin fibroblasts proliferation (Ninomiya, 2001) and several clinical assays prove hydroxyapatite implants efficacy on wrinkle filling and also on the correction of facial lipoatrophy in HIV patients (BioForm Medical, Stupak, 2007 and Hamilton, 2007).

Oral care products containing nanoparticulate hydroxyapatite used for teeth remineralisation are also available on the market.

3.3 SAFETY EVALUATION

The SCCS published an Opinion on HAP-nano on 16.10.2015 (revised on 16.03.2016) in regard to application in cosmetic products. The opinion was based on 35 notifications of cosmetic products containing HAP-nano. The SCCS also reviewed and assessed a large body of literature (65 publications and reports). However, it was concluded that in many cases, the studies were not performed according to the OECD or the EU guidelines, and that important information on the nature of the particles (as outlined below) was missing. Therefore, no definite conclusions on safety could be reached and the Opinion was inconclusive. The Opinion, however, highlighted that needle-shaped HAP-nano 'is of concern in relation to potential toxicity [and] should not be used in cosmetic products'.

In particular, in the previous Opinion on HAP-nano, the SCCS concluded that:

"only a limited amount of data was provided by the Notifiers 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 nanohydroxyapatite when used in oral cosmetic products.

This is because:

- There was a lack of information on the characterisation of the test materials used in toxicological studies, or they were poorly described, or were different from those under evaluation (Materials 1 and 2). It is not clear in most cases if and to what extent the investigated materials correspond to the materials under evaluation.

- Hardly any of the toxicological studies provided were 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 Notifiers 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 nanohydroxyapatite 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 nanohydroxyapatite 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 Notifiers 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).

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

In the current submission, some information on nanoXIM® ingredient (Material 1 referred in SCCS/1566/15) was provided, but not on Material 2. Therefore, this Opinion has only assessed the safety of HAP-nano resembling Material 1 (i.e. composed of rod shaped nanoparticles, not needle-shaped).

SCCS General Approach for the Safety Assessment
The approach followed in this Opinion to assess the safety of HAP-nano is based on the SCCS Notes of Guidance (10th edition, 2018) and the Guidance on the Safety Assessment of Nanomaterials in Cosmetics. As a first step, systemic exposure of the HAP-nano has to be explored (see figure 1 of the Guidance), and in case of significant exposure, some specific toxicity tests on the HAP-nano would be required. If significant systemic exposure to the HAP-nano may be excluded, then only local toxicity and genotoxicity of the nanoforms will be assessed and the risk following systemic exposure will only consider the non-nanoform of HAP. This assessment is described below.

3.3.1 Exposure assessment considering possible routes

As nanoXIM® ingredient is only intended to be used in oral cosmetic products (toothpastes, mouthwashes…), only exposure *via* oral route has to be considered. After entering into the mouth, part of the cosmetic formulation will enter into contact with the buccal mucosa and part may be ingested. Therefore systemic exposure to the HAP-nano may occur either *via* uptake by mucosal cells or by crossing the intestinal tract. Both routes have been assessed by the Notifier.

3.3.1.1 Penetration of HAP-nano via the buccal mucosa

Cell internalization of HAP-nano in the mucosa

In the study from Ramis *et al.* (2018) described below, the presence or absence of HAP-

nano in the gingival mucosa was assessed by observation with Transmission Electron Microscopy (TEM).

Method:
For each of the tested products or controls, at the end of the incubation period, one culture was fixed in a balanced 2.5% glutaraldehyde solution, dehydrated and later embedded in resin. Vertical sections of 0.3 µm were photographed under a TEM microscope.

Results:
TEM analysis (figure below) did not show HAP-nano nanoparticles internalized into the cells after treatment in any of the cell layers (only the superficial layer is shown in Figure 6). None of the sections analysed contained cells with nanoparticles internalized. Some nanoparticles were observed outside the most superficial cell layer in the samples treated with 3.1% HAP-nano after 1h of exposure, which is more likely to be related to insufficient washing after treatment for this time point, since this did not occur after a longer exposure time of 3h.

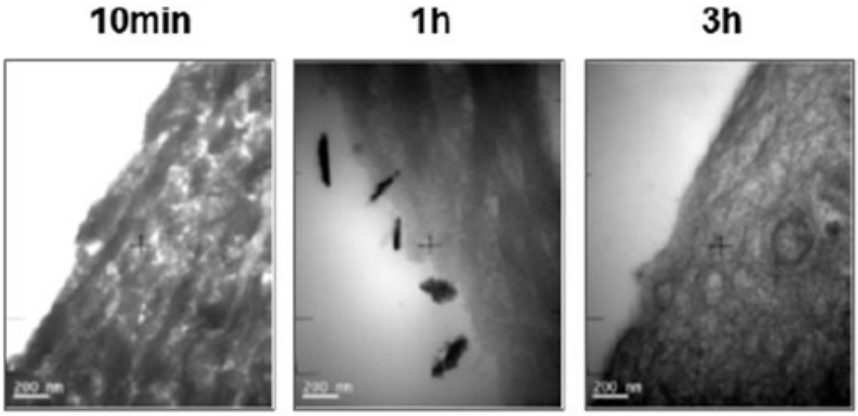


Figure 6: Transmission electron microscopic detection of HAP-nano after 10 minutes, 1 h, and 3 h incubation of HGE tissues. Images were taken from the most superficial layer at a magnification of 50,000x. A negative and a positive control after 3 h of incubation were also included in the TEM analyses (data not shown)

Conclusion:
The results of this study confirmed that at 3.1% and up to 3 hours after exposure, there is no cellular uptake of HAP-nano by Human Gingival Epithelium.

SCCS comment
The 3h timeframe is considered too short to properly evaluate the uptake of HAP-nano by Human Gingival Epithelium.

Further information available in the literature
A recently published study (Komiyama *et al.*, 2019) has investigated histologically the ability of two types of HAP-nano, SKM-1 and Mi-HAP, to permeate oral epithelium, using two types of three dimensional reconstituted human oral epithelium, SkinEthic HGE and SkinEthic HOE respectively with and without a *stratum corneum*. Both types of HAP-nano formed aggregates in solution, but both aggregates and primary particles were much larger for SKM-1 than for Mi-HAP. Samples of each tissue model were exposed to SKM-1 and Mi-HAP for 24h at concentrations ranging from 1,000 to 50,000 ppm. After treatment, paraffin sections from the samples were stained with Dahl or Von Kossa stains. The study also used OsteoSense 680EX, a fluorescent imaging agent, to test for the presence of HAP in paraffin tissue sections. The results for both types of HAP-nano showed that the nanoparticles did

not penetrate the stratum corneum in SkinEthic HGE samples and penetrated only the outermost layer of cells in SkinEthic HOE samples without stratum corneum, and no permeation into the deeper layers of the epithelium in either tissue model was observed. In the non-cornified model, OsteoSense 680EX staining confirmed the presence of HAP-nano particles in both the cytoplasm and extracellular matrix of outermost cells, but not in the deeper layers. The results also suggest that the *stratum corneum* may act as a barrier to penetration of HAP-nano into the oral epithelium. Moreover, the authors consider that since oral epithelial cell turnover is around 5–7 days, superficial cells of the non-keratinized mucosa, in which nanoparticles are taken up, are likely to be deciduated within that period. Their findings suggest that HAP-nano is unlikely to enter systemic tissues *via* intact oral epithelium.

SCCS comment
Based on the available data, it is not possible to confirm that the two-types of HAP-nano studied by Komiyama *et al.* (2019) are equivalent to the ones used in cosmetic products.

Furthermore, new studies were provided by the Notifier following SCCS request on local toxicity that have investigated penetration into cells after 48 hours (described below in section 3.3.2). From the results, it seems that even if there was a penetration of HAP-nano into the cells layers of the oral mucosa, it would be limited to the superficial layers and NPs would be present mostly as agglomerates and, as such, any significant systemic exposure is not expected through this route.

3.3.1.2 Oral ingestion of HAP-nano

Absorption by gastric compartment

The stability of nanoXIM.CarePaste HAP-nano was assessed in a stability study in simulated gastric fluid (SGF) by determination of calcium content at different time points (7.5, 15 and 30 mins). A different batch was used for each time point evaluated. The stability was tested in the mouthwash conditions of exposure as the maximum daily exposure is higher than for the toothpaste. The maximum incorporation of nanoXIM in a mouthwash is 10% but as a precaution, 20% of nanoXIM was used. This maximum dose of nanoXM was added to 250 ml of SGF, following the recommendations of the FDA (Guidance for the Dissolution testing of Immediate Release Solid Oral Dosage Forms, 1997).

SGF was prepared according to the United State Pharmacopeia XXII, containing 0.2% (w/v) NaCl and 0.7% (v/v) HCl. The pH of the SGF solution was 1.2. The SGF did not contain pepsin since it does not play an active role in the solubilisation of the HAP-nano.

Digestions were carried out in a closed flask with a magnetic stirrer at 100 rpm on a heating plate with temperature control at 37°C, using a Sensoterm II temperature gauge in a thermostated bath (JP Selecta, Barcelona, Spain).

The Ca^{2+} concentration in the digestion media was determined by Inductivey Coupled Plasma-Atomic Emission Spectroscopy. Blank samples containing SGF were also analysed and their mean intensity subtracted from the sample values.

The nanoXIM 20% suspension was completely soluble in SGF at the concentration tested and thus not stable in these conditions. After 7.5 min of incubation at 37°C, the solution was clear and no particles were observed. Moreover after centrifugation, there was no sediment observed, which reinforces that 20% nanoXIM was fully dissolved in SGF. The concentration of Ca^{2+} in the digestion media was 104.2 ± 3.0 mg/L, which corresponded to the maximum expected concentration. If the total amount of hydroxyapatite nanoparticles were dissolved during the digestion, the concentration of Ca^{2+} in the digestion medium

would be 102.7 mg Ca^{2+}/L.

t (min)	[Ca^{2+}] mg/L
7.5	104.2 ± 3.0
15	103.8 ± 2.1
30	100.2 ± 4.2

Table 4: Calcium concentration in the digestion media determined by ICP-AES after digestion in SGF of 2.16 g of NANOXIM 20% at different time-points. Values ± SEM (n=3 replicates). The calculated maximum Ca^{2+} concentration in the digestion fluid if all the hydroxyapatite nanoparticles were dissolved would be 102.7 mg Ca^{2+}/L

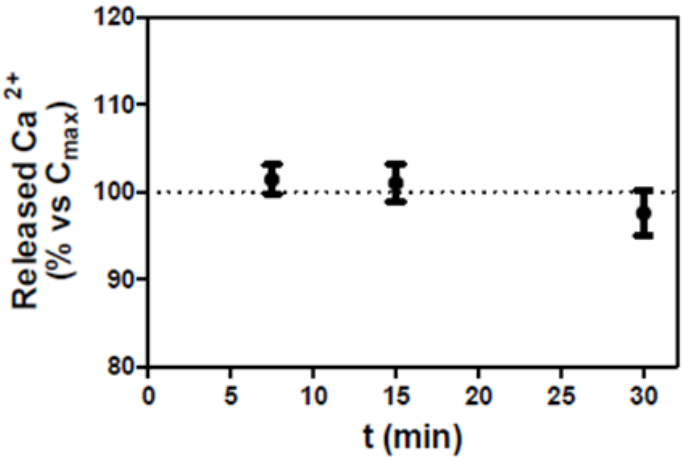


Figure 7: Released calcium ions to the digestion media from 2.16g nanoXIM 20% in 250 ml of SGF for different time-points. The calcium concentration is represented in relation to the maximum Ca^{2+} concentration expected if the totality of the nanoparticles were dissolved.

The study concluded that nanoXIM.CarePaste HAP-nano is completely degraded in the stomach fluid after 7.5 minutes of digestion. Therefore, it is expected that in case a quantity of nanoXIM.CarePaste HAP-nano should be ingested and reach the gastrointestinal tract, it would be degraded in the stomach, and would not become systematically available through this path.

SCCS comment

Although the study did not strictly follow EFSA *in vitro* solubility testing and used an FDA method instead, the test carried out is, however, valid and clearly shows that the material would solubilise in the gastric fluid if ingested. Therefore, there should not be nano-related concerns over safety following ingestion of the type of HAP-nano considered in this Opinion.

Further information available in the literature

Following a bibliographic search, the SCCS identified three *in vivo* studies that have been recently published by the same group (Mosa *et al.*, 2019a, 2019b and 2020). The studies investigated inflammatory responses of HAP-nano in rats' gastric or renal tissue following oral uptake. The authors reported that at the dose of 300 mg/kg/bw administered orally for

45 days (the only dose tested in these studies), a decline in stomach antioxidant enzymes, reduction in glutathione level, induction in lipid peroxidation and nitric oxide as well as interference with various pro-inflammatory gene products were observed. Moreover, HAP-nano administration was associated with intense histological changes in kidney architecture and immunoreactivity.

SCCS comment
The SCCS has noted that the authors of these studies considered, based on XRD analysis, that the HAP-nano tested in their studies had needle-like crystal morphology. However, some of the images in the publications and in the notifications dossiers under evaluation seem quite similar and, therefore, the SCCS cannot exclude the possibility that the material used in Mosa *et al.* (2019a, 2019b and 2020) studies also bears resemblance to the HAP-nano covered by this Opinion. However, no conclusion on human safety can be drawn from these studies because of severe limitations including those related to material characterisation. In one of the studies (Mosa *et al*., 2019b), HAP allegedly in a nanoform was obtained from crushed bone using alkaline hydrolysis, and no proof was provided that this material was indeed in a nanoform. In another study (Mosa *et al*., 2019a) AP-nanos were prepared by an in-lab chemical synthesis, but no description of the method was provided. High resolution TEM was used to characterise the NPs. In another study (Mosa *et al*. 2020), according to the description in the materials and methods section, hydroxyapatite was prepared using NaOH and Na_2CO_3, apparently without using any calcium salt. XRD analysis was conducted to confirm the skeleton structure. In all three studies, the nanoform characterisation was not sufficiently documented either in the pristine form or after preparing suspensions to check for stability.

3.3.2 Assessment of the local toxicity by oral route

***Taken from previous opinion* SCCS/1566/15**

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

New submission

Ocular irritation

An assessment of the ocular irritation potential after application to the embryonic Hen`s Egg Chorioallantoic membrane was performed (Inovapotek Final Report FR02B/P118B13, December, 2013) using HET-CAM test according to Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) guidelines. The product nanoXIM was tested at a concentration of 25%, diluted in water. Three fertilized hen's eggs were incubated with each substance (the test substance nanoXIM and positive and negative controls) for nine days, and on the 10^{th} 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 Mili-Q water. The final result was based on the observation of the irritant effects that could occur within the 0.5, 2, and 5 minutes after rinsing-off the test substance.

According to the HET-CAM, nanoXIM 25% was considered weakly or slightly irritant with an irritation index of 2.8 on the CAM.

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. However, considering the cosmetic uses of HAP-nano (oral products), it could be reasonably anticipated that ocular exposure will not be of concern.

Buccal mucosa irritation

***A. In vitro* study on a reconstructed gingival epithelium model**

A study was performed on a reconstructed gingival epithelium model to assess the buccal mucosa primary irritation potential of the test item (Buccal Mucosa Primary Irritation study performed on a reconstructed gingival epithelium model. IEC France, report nº 140733RD4 of 6 June 2014). The study was conducted in compliance with GLP.

The objective of the study was to determine the T_{50} contact time point that causes 50% of mortality in a reduction test of MTT.

Test system:
Reconstructed gingival epithelium model of 0.5 cm^2 (5 days of culture), from gingival human normal epithelial cells, cultivated in chemically defined medium supplied by STERLAB (Vallauris, France).

Reconstructed epithelia preparation:
- After receipt: checking of the date of sending, the temperature and the colour of the agar medium
- Transfer of each reconstructed epithelium, using a tweezer, into a 24 wells plate containing 500 µl of maintenance medium (balanced at room temperature)
- Incubation of the plate at 37° ± 1°C , 5 ± 1% CO2, 95 ± 5% humidity (CO_2 incubator) until the following day

Validation of the trial:
- Negative control: Sodium chloride to 0.9% (w/v): the mean O.D. (Optical Density) of the negative control was superior than 0.600
- Positive control: Sodium Dodecyl Sulfate to 1.5 % (w/v): the T50 was included between 10 and 60 minutes
- Per each time point, C.V. (coefficient of variation) of the O.D. must be < 20%.

Test item:
As supplied by the sponsor – Desensin repair colutrio (ref C/TRS-07-04- batch n° H-006) – dental solution for adult.

Protocol:
- Deposit of 30 µL of each control and the test item, in duplicate on the epithelia using a micropipette with a positive displacement.
- Incubation of the epithelia during the following contact time points (CO_2 incubator):

	CONTACT TIMEPOINT
Test item	10 ± 1 minutes, 1 hour ± 5 minutes and 3 hours ± 10 minutes, in duplicate
Positive control	10 ± 1 minutes and 1 hour ± 5 minutes, in duplicate
Negative control	3 hours ± 10 minutes, in duplicate

- MTT test performed
- Calculation of the mean percentage of cellular viability for each contact time point
- Determination of T50 and classification

Results:

- Negative control : O.D. (570 nm) : 0.832
- Positive control : T50: 37.9 min
- Test item: T50 > 180 min : non irritant

The trial could be validated based on the results of the negative and positive control.
Conclusion from the Notifier
From the results obtained under the experimental conditions adopted and taking into account the target, the test item designated as "DESENSIN REPAIR COLUTORIO" applied as supplied on a reconstructed gingival epithelium model, **can be considered as well tolerated at the buccal mucosa level.**

B. Reconstructed Human gingival epithelium cells (Ramis *et al.*, 2018)

SkinEthic™ model of human gingival epithelium was used to assess the biocompatibility of HAP nano to human oral gingiva. MTT activity, LDH activity and IL-1alpha production was measured. To assess the systemic bioavailability of HAP nano through absorption in the gingival tissue, transmission electron microscopy (TEM) was performed after different exposure times (Ramis *et al.*, 2018).

Method:
The HAP nano (CAS 12167-74-7 also referred as 1306-06-5) used in this study was provided by one Notifier as a commercial formulation (nanoXIM). This formulation consisted of 15.5% HAP nano suspension in water.

HAP-nano characterization was done (size and morphology).

The SkinEthic™ reconstructed human gingival epithelium (HE) tissue model was used (EpiSkin, yon, France) as an *in vitro* model to perform the biocompatibility and irritation tests. The HE model is composed of normal human gingival cells cultivated on an inert polycarbonate filter at the air liquid interface in a chemically defined medium.

A dilution 40% of HAP-nano aqueous suspension in ultrapure Mili-Q water diluted 1/1 in PBS resulting in final concentration of 20% of the HAP-nano aqueous suspension was tested, which corresponds to 3.1 % HAP-nano (as the maximum concentration in a toothpaste).
As a negative control, 1/1 Mili-Q water dilution in PBS was used, and as a positive control 0.5 % SDS was used. Incubations were done at different times: 10 min, 1h and 3h.

At the end of the incubation period, LDH activity as an indicator of cytotoxicity was measured in the culture media. An MTT test, as a marker of cell viability, was performed

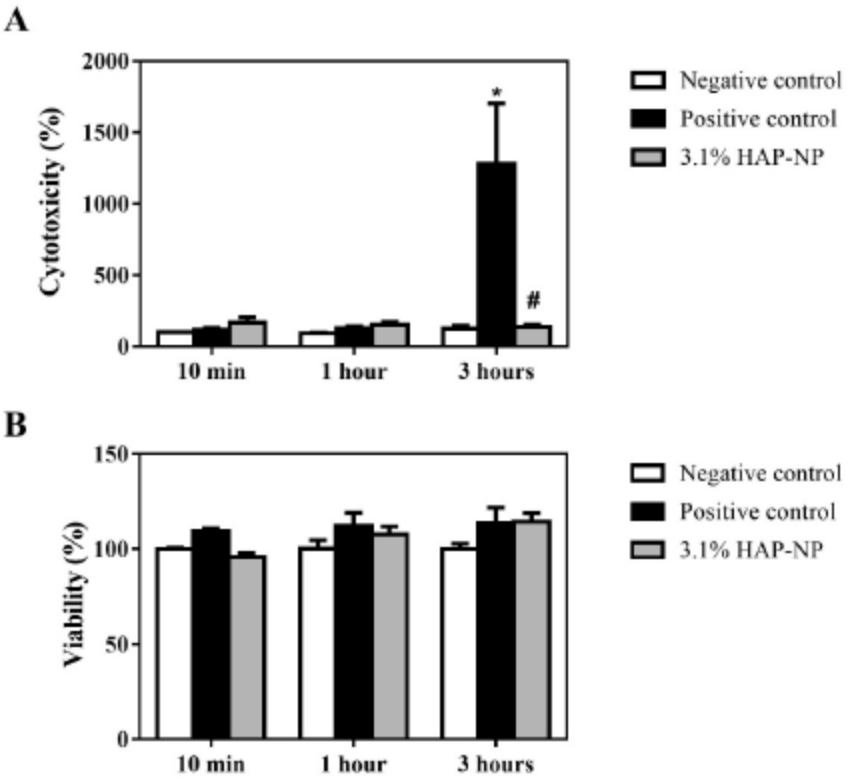
and IL-alpha – a pro-inflammatory cytokine that is released after cell membrane damage - was measured in the media. Histopathology scores were also determined based on a histopathology scale.

Results:
The HAP-nano used in this study possess a rod-like morphology and a particle size < 100 nm (width between 5-20 nm and length below 50 nm). The HAP-nano was phase-pure and the specific surface area was 103 m^2/g.

The LDH assay shows that HAP-nano did not induce cytotoxicity at any of the incubation times evaluated. Positive control only showed cytotoxicity after 3h incubation.

As shown in figure 8B, 3.1% HAP-nano showed no effect on cell viability at any of the incubation times tested. No significant deleterious effect was observed with the positive control on the HE tissue. This may be explained by the relatively low SDS concentration and the lack of sensitivity of the test, probably due to the external keratinized layer.

The release of IL-1alpha was only increased with the positive control over time but not with 3.1% HAP-nano, indicating the absence of acute irritation. In agreement with the cytotoxicity and IL-1alpha levels results, there was no sign of tissue changes after incubation with the negative control and the 3.1% HAP-nano solution. Tissues changes were only observed after treatment with the positive control SDS 0.5% after 1 and 3h of exposure.



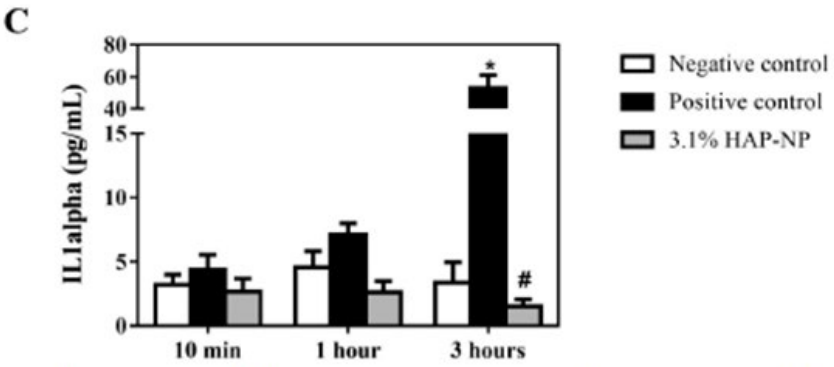


Figure 8: Biocompatibility in HE after different exposure times with the treatments. (**A**) Lactate dehydrogenase (LDH) activity, an indicator of cytotoxicity, measured in culture media at the end of the different exposure times human gingival epithelium (HGE) with the sample and controls; (**B**) MTT, an indicator of the number of viable cells, measured in the HGE tissues at the end of the different exposure times with the sample and controls; (**C**) IL-1alpha, a pro-inflammatory cytokine that is released after cell membrane damage, measured in culture media at the end of the different exposure times of HGE with the sample and controls. Negative control was obtained from culture media treated with milliQ water: PBS (1:1), for LDH and MTT negative control was set at 100% at each time point. Positive control was obtained from culture media treated with 1% SDS diluted in PBS (1:1). Test sample was 3.1% HAP-NP in PBS. Values represent the mean ± SEM. Mann-Whitney test: * $p < 0.05$ versus Negative control; # $p < 0.05$ versus Positive control.

Conclusion from the Notifier
Based on the results of this study, exposure to HAP-nano at 3.1% up to 3 hours does not induce cytotoxicity or IL-1alph release in a reconstructed human gingival epithelium model.

C. *In vitro* cytocompatibility of nanoXIM hydroxyapatite nanoparticles

In vitro cytocompatibility of hydroxyapatite nanoparticles towards human gingival fibroblasts (HGF) was assessed. Cytotoxicity was evaluated in terms of metabolic activity, reactive oxygen species (ROS) production and cell death.

Materials and Methods

Sample preparation
Test item: 20 % nanoXIM suspension was made by diluting 0.2 grams of nanoXIM per millilitre of Alpha Minimum Essential Medium (α-MEM) without phenol red (Gibco). Afterwards, the suspension was vortexed at 25 Hz during 5 seconds. In addition, the 20% nanoXIM suspension was centrifuged at 4000 g during 10 minutes and the supernatant was spared. Both nanoXIM samples (suspension and supernatant) were freshly prepared just before the in vitro test. The times and methods were chosen in an attempt to mimic the tooth brushing procedure.

Cell culture
Human gingival fibroblast (HGF) were maintained in α-MEM (Sigma-Aldrich) supplemented with 10% (v/v) foetal bovine serum (FBS, Gibco), 2.5 µg/mL amphotericin B (Gibco), 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco). The cells were incubated in a humidified atmosphere at 37°C and 5% CO_2. Medium was changed 2 times a week and cell growth was monitored using an inverted phase contrast microscope (Nikon TMS, Nikon). At about 70-80% confluence, cells were enzymatically detached using a trypsin – EDTA

solution. For experiments on cell metabolic activity, ROS production and live/dead cells straining, cells were seeded on 96 –well culture plates at a density of 1 x 10^5 cells/mL 24 hours before the experiments.

Metabolic activity

Metabolic activity was assessed using Alamar Blue® assay. This assay is based on the capacity of living cells to reduce resazurin (a blue and non-fluorescent ingredient) to resorufin (a fluorescent and pink-colored compound), which made it possible to perform a quantitative measure of the metabolic activity. Twenty-four hours after cell seeding, the medium was removed and cells were washed with phosphate buffered saline (PBS). Afterwards, 100 µL of nanoXIM suspension and supernatant were incubated with the cells and 10% (v/v) of resazurin (0.1 mg/mL, Sigma-Aldrich) was added to the medium.

Positive control was obtained by treating cells with 2% Triton X-100 and cells in culture medium were considered as the negative control. Cells were incubated at 37 °C and 5% CO_2 and the metabolic activity was assessed after 2, 3, 4 and 24 hours of incubation. Fluorescence was measured at 530 nm excitation and 590 nm emission using a microplate reader (SynergyMix, BioTek) with Gen5 1.09 Data Analysis Software. Results are expressed as percentage of viability in comparison to negative control (non-treated cells), for each time point.

ROS production

ROS production was performed using DCFDA Cellular Reactive Oxygen Species Detection Assay Kit (Abcam) according to the protocol supplied by the manufacturer. This kit contains a cell permeant reagent 2´,7´- dichlorofluorescin diacetate (DCFDA), which is a fluorogenic dye that measures hydroxyl, peroxyl and other ROS activity within the cell. After the diffusion of DCFDA into the cell, this dye is hydrolyzed by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS to a highly fluorescent compound 2´,7´- dichlorofluorescin (DCF) that can be detected using fluorescence spectroscopy. Briefly, 24 hours after cell seeding, the medium was removed and cells were washed with a specific buffer from the kit. Afterwards, buffer was removed and cells were incubated with DCFDA solution for 45 minutes in the dark. After incubation, the DCFDA solution was removed, cells were washed with PBS and the nanoXIM suspension and supernatant were added. Tert-butyl hydrogen peroxide (TBHP) in a concentration of 500 µM was used as positive control and non-treated cells were used as negative control. Fluorescence was measured in a microplate reader (SynergyMix, BioTek) after 2, 3 and 4 hours at 485 nm excitation and 528 nm emission. Results are expressed in terms of relative fluorescence units (RFU).

Live/dead cells staining

Live/dead cell staining was performed to evaluate cell viability in a shorter time point. This staining is a two-colour fluorescence assay that simultaneously determines live cells and dead cells. The live cells are able to convert the non-fluorescent calcein acetoxymethyl (calcein AM) to a highly fluorescent calcein. The calcein is retained in the cells and these are marked in green. As dead cells have damaged membranes, propidium iodide (PI) is able to enter the damaged cells and become fluorescent when bounded to nucleic acids, producing a bright red fluorescence. For that purpose, 24 hours after cell seeding, cells were washed 1x with PBS and then incubated with nanoXIM suspension and supernatant for 10 minutes.

Non-treated cells were used as a control. After incubation, the nanoXIM suspension and supernatant were removed and cells were washed 2x with PBS. Calcein AM (Sigma-Aldrich) was diluted in cell culture medium without phenol red to obtain a solution with a final concentration of 2 µl/mL. PI (BD Biosciences) solution was used as supplied. Each solution was added to cells and incubated for 30 min at 37°C, protected from light. Fluorescence was observed using an inverted fluorescence microscope (Axiovert 200M, Zeiss) with green (488 nm) and red (594 nm) filters. Image processing was performed using AxioVision Software from Zeiss.

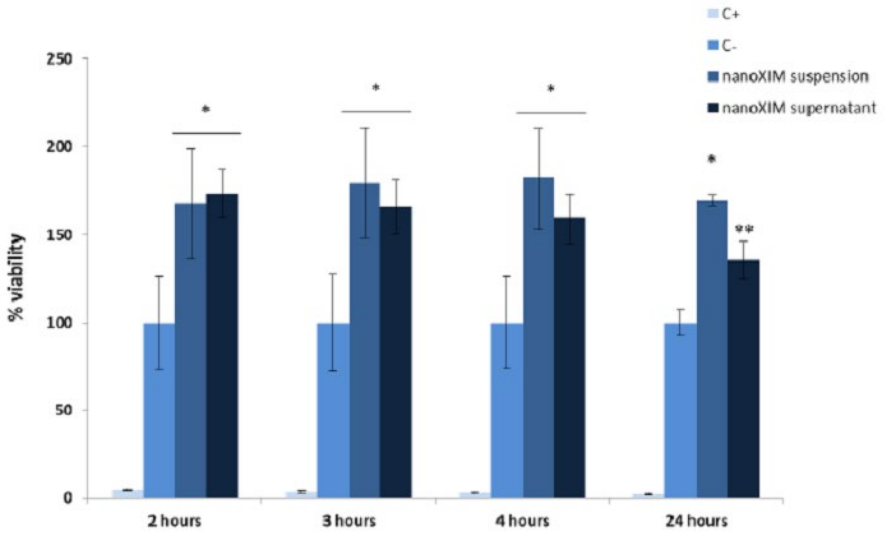
Statistical analysis

The statistical analysis was performed using GraphPad Prism Software version 5.01 (GraphPad Software, Inc., USA). The data analysis was done using the one-way analysis of variance (One-way ANOVA) followed by post hoc Tukey test with a significance level of $p < 0.05$.

Results

The cytocompatibility of nanoXIM nanoparticles was first evaluated using Alamar Blue® assay. For all of the time points tested, it can be observed that nanoXIM nanoparticles were not toxic to HGF (Figure 9). In fact, there was a significant increase of metabolic activity for cells incubated with both nanoXIM suspension and supernatant, providing evidence that nanoXIM hydroxyapatite nanoparticles induce a positive stimulus on these cells.

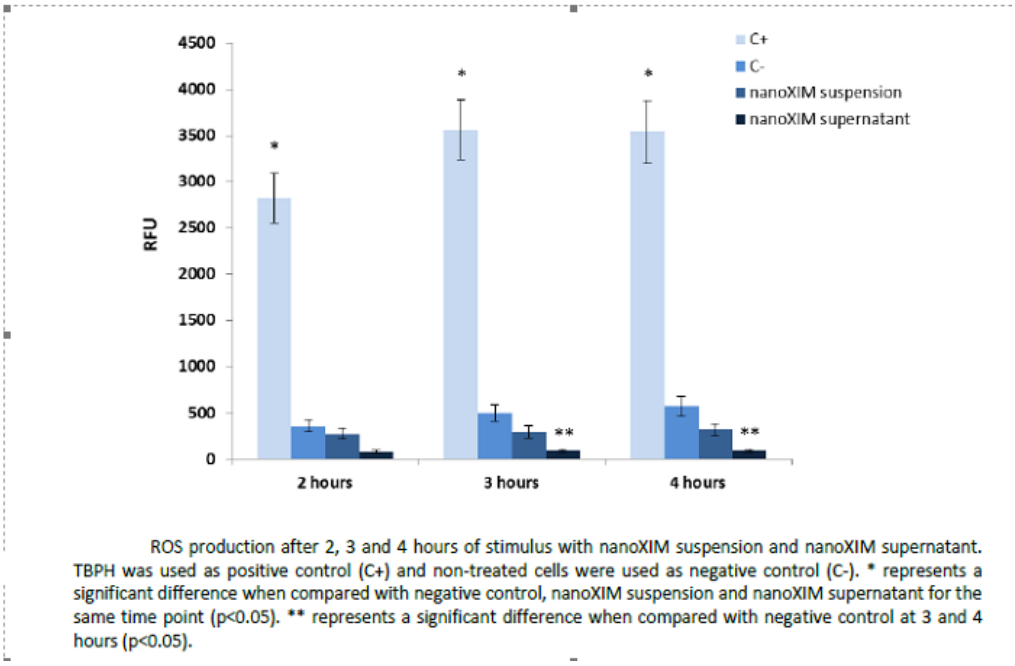
Figure 9:



Metabolic activity of HGF after 2, 3, 4 and 24 hours of incubation with nanoXIM suspension and nanoXIM supernatant. * represents a significant difference when compared with cells treated with 2 % Triton X-100 (C+) and non-treated cells (C-) for all the time points ($p<0.05$); ** represent a significant difference when compared with cells treated with 2 % Triton (C+), non-treated cells (C-) and with nanoXIM suspension at 24 hours ($p<0.05$).

In addition, the cytotoxic potential of nanoXIM was also evaluated in terms of ROS production. For all the time points tested, nanoXIM suspension and supernatant did not induce the production of ROS in HGF cells. As demonstrated in Figure 10, fluorescence of cells treated with nanoXIM was always significantly lower than the positive control (cells treated with TBHP) and equal or lower than the negative control (non-treated cells).

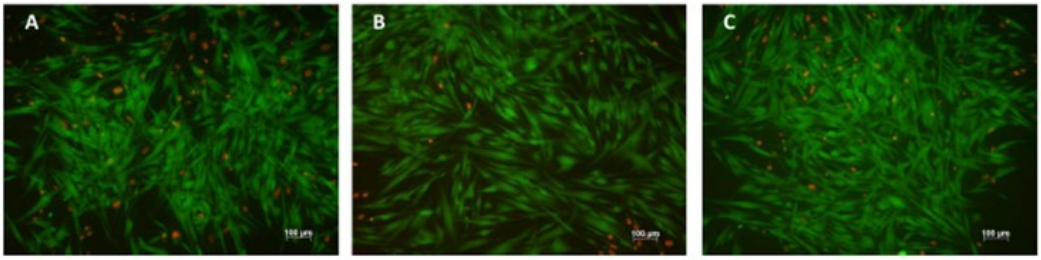
Figure 10:



ROS production after 2, 3 and 4 hours of stimulus with nanoXIM suspension and nanoXIM supernatant. TBPH was used as positive control (C+) and non-treated cells were used as negative control (C-). * represents a significant difference when compared with negative control, nanoXIM suspension and nanoXIM supernatant for the same time point ($p<0.05$). ** represents a significant difference when compared with negative control at 3 and 4 hours ($p<0.05$).

Finally, live/dead cell staining was performed to access cell viability after a shorter time point, i.e. 10 minutes exposure. This assay evidences that nanoXIM suspension (Figure 11B) and nanoXIM supernatant (Figure 11C) do not induce cell death for the time point tested, as the amount of green and red cells is very similar to the non-treated cells (Figure 11A). In addition, cells treated with nanoXIM are well adhered and spread, presenting their characteristic elongated morphology.

Figure 11:



Live/dead cell staining after incubation with nanoXIM suspension and nanoXIM supernatant during 10 minutes. The green-labelled cells correspond to live cells (stained with calcein) and the ones in red correspond to death cells (stained with PI). A) Control (non-treated cells); B) Cells treated with nanoXIM suspension; C) Cells treated with nanoXIM supernatant. Scale bar: 100 µm.

Conclusion from the Notifier

This study revealed that a 20% nanoXIM suspension and its supernatant were not cytotoxic towards HGF for the time points tested. In fact, in terms of metabolic activity, both nanoXIM suspension and supernatant were able to induce a positive effect on HGF cells only after 2 hours of exposition. Moreover, nanoXIM suspension and supernatant did not induce the formation of ROS after 2, 3 and 4 hours of exposition. It was not possible to evaluate ROS production after 24 hours because the DCFDA kit only guarantees fluorescence signal until 6 hours of incubation. A Live/dead assay was performed to visualize the effect of nanoXIM on HGF cells in a shorter time point (10 minutes). It was concluded that a 20% nanoXIM suspension and the corresponding supernatant did not induce cell death after 10 minutes of exposure.

For the time points tested, it can be concluded that 20% nanoXIM suspension and the correspondent supernatant were not cytotoxic to HFG, as nanoXIM was able to increase metabolic activity and did not induce both ROS production and cell death.

Ref: Coelho C., 2017

SCCS comments on the local toxicity assessment
There are a number of shortcomings concerning the *in vitro* studies used for the assessment of local toxicity of nano-HAP in a cosmetic formulation, in particular, with respect to the study on a reconstructed gingival epithelium model to assess the buccal mucosa primary irritation potential of Desensin repair Colutorio (IEC, 2014).

a. The study report is not detailed and does not specify the application of nano-HAP. Also, no reference is provided to indicate whether the study followed any of the existing OECD guidelines.

b. The number of replicates for the test material and controls is not adequate (at least 3 replicates should have been used). The study used no post-treatment incubation.

In another study, SkinEthic™ model of human gingival epithelium was used to assess biocompatibility of nano-HAP with human oral gingiva. In this study, MTT reduction, Lactate dehydrogenase (LDH) activity and IL-1alpha production by the exposed cells were determined. Transmission electron microscopy (TEM) at different exposure times was used to assess absorption of nano-HAP by the gingival tissue (Ramis *et al.*, 2018). In this model, nanoXIM™ nano-HAP was shown to be not cytotoxic. Another study by Coehlo *et al.* (2019) concluded that a 20% nanoXIM™ nano-HAP suspension and its supernatant were not cytotoxic towards human gingival fibroblasts (source not specified) when investigated for up to 4 hours. However, the SCCS has concerns about the validity of the conclusions, because:

a. normally (i.e. for non-nano materials), the viability measurement is not performed immediately after short exposure (e.g. 3-4 hours) to the test substance. It is done after a sufficiently long (e.g. 48 hours) post-treatment incubation period of the rinsed tissue in fresh medium. This allows for both the recovery from weak cytotoxic effects and for the appearance of clear cytotoxic effects. However, measurements in these studies were carried out after only 3 hours (Ramis *et al.*, 2018) or 4 hours (Coehlo *et al.*, 2019) and were not followed at later time points. There are several studies in the published literature that have reported effects of nano-HAP on cells after 48 hours or 72 hours.

b. the studies used gingival epithelium model, which is a keratinising epithelium, i.e. it is lined with a few layers of cells that are already dead, or are in the final stage of cell death (eventually forming keratin layers, stratum corneum). On the other hand, the stratified squamous epithelium in the superficial layers of the lining mucosa, such as in the labial mucosa, buccal mucosa and the mucosa lining the floor of the mouth, the ventral surface of the tongue, and the soft palate is non-keratinised. Thus, non-keratinised epithelium is the most common form of epithelium in the oral cavity. The presence of layers of stratum corneum in the gingival epithelium model used in the provided studies might have hampered penetration of HAP-nano into deeper cell layers. Therefore, the SCCS considers that, for a worst-case scenario, the studies should have used an appropriate non-keratinised epithelium model. Models of non-keratinised stratified squamous epithelium are commercially available.

In view of these concerns, the SCCS asked the Notifier to provide an appropriate *in vitro* study of the toxicity of nanoXIM•CarePaste on cellular models that are representative of the non-keratinised oral epithelium with sufficient time of exposure (48 hours as a minimum) with TEM analysis. The following new data were provided by the Notifier (July 2020) in response to the SCCS request:

New data in response to SCCS request:

***In Vitro* Cytotoxicity test of nanoXIM Hydroxyapatite Nanoparticles on Human Oral Epithelium**

To determine the *in vitro* cytotoxicity on human oral epithelium of nanoXIM hydroxyapatite nanoparticles (HAP-nano), an *in vitro* model of reconstructed human oral epithelium was used after exposure to nanoXIM NPs. LDH activity released to the media of the exposed tissues was performed.

Material and methods

- Reconstructed Human Oral Epithelium

The SkinEthic reconstructed Human Oral Epithelium (HOE) (EpiSkin, Lyon, France) was used as an *in vitro* model to perform the cytotoxicity test. The HOE consists of an airlifted, living, multi-layered tissue construct, produced in polycarbonate inserts in serum-free and chemically defined medium. The HOE feature normal ultra-structure and functionality similar to human tissue *in vivo*.

The inserts containing the HOE (size 0.5 cm^2) were shipped at room temperature in a multiwell plate filled with an agarose-nutrient solution in which they were embedded.

Upon arrival, the tissues were processed following the manufacturer's protocol. Briefly, the HOE tissues were removed from the agarose-nutrient solution and were placed in a plate previously filled with the SkinEthic Maintenance Medium (EpiSkin, Lyon, France) at room temperature. The tissues were incubated in a cell incubator at 37°C, 5% CO2 and saturated with humidity overnight.

- Treatment with the nanoXIM nanoparticles

The HOE tissues were dosed with 20% nanoXIM (3.1% HAP-NP) for 48 h. The dose of nanoXIM was calculated with the assumption that adults use 1 g of toothpaste in one brushing and that the maximum concentration of nanoXIM (15.5% wt. HAP-NP) in a toothpaste is 20%. Then, the net amount of exposure to HAP-NP would be 31 mg of HAP-nano per brushing. Using the areas for the tissues used in this study (0.5 cm^2) and the surface area of the adult oral cavity (214.7 ± 12.9 cm^2), this results in a concentration of 0.144% HAP-NP, which we roundup to 0.155% HAP-NP, i.e. 1% nanoXIM. In order to have a large safe margin test above the maximum usage level, we multiplied this value by 20 and therefore selected a 20% nanoXIM concentration (3.1% HAP-NP) to perform these tests.

Evaluation was done after 48 hours of incubation. Triplicate tissues were assessed for LDH activity analysis. Three different treatment groups were assessed: 3.1% HAP-NP, negative control (Phosphate buffer saline, PBS) and positive control (1% X-100 Triton).

- LDH activity

LDH activity in the culture media was used as an index of cytotoxicity. LDH activity was determined spectrophotometrically after 30 min incubation at 25 °C of 100 µl of culture and 100 µl of the reaction mixture by measuring the oxidation of NADH at 490 nm in the presence of piruvate, according to the manufacturer's kit instructions (Roche Diagnostics, Mannheim, Germany). Results were presented relative to the LDH activity in the medium of negative control group (Phosphate buffer saline, PBS; low control, 0 % of cell death) and on tissue culture plastic (TCP) adding triton X-100 1% (high control, 100 % of death), using the equation:

Cytotoxicity (%) = (exp. value – low control)/ (high control – low control) * 100

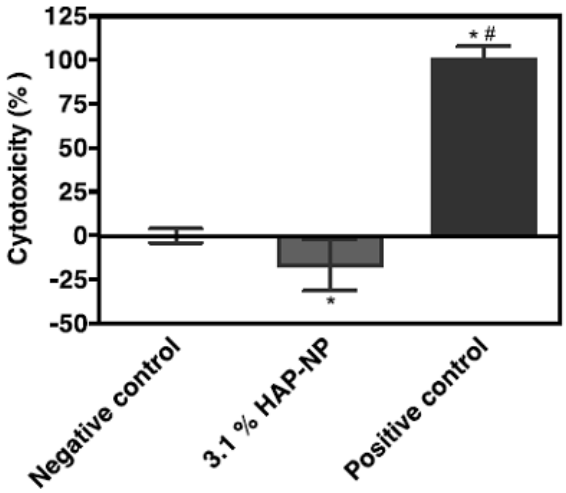
- Statistics

All data are presented as mean values ± SD. The Kolmogorov-Smirnov test was done to assume parametric or non-parametric distributions for the normality tests. Differences between groups were assessed by Student t-test. Results were considered statistically significant at p-values <0.05. SPSS® program for Windows, version 17.0 (SPSS Inc, Chicago, IL, USA) was used.

Results

The LDH assay was used as an indicator of cytotoxicity, as this enzyme leaks out through the plasma membrane of damaged cells. Consequently, lower LDH activity means higher biocompatibility. As shown in Figure 12, after 48 h incubation, the positive control showed significantly higher cytotoxicity than the other groups. Tissues treated with 3.1 % HAP-nano showed significantly lower cytotoxicity than the negative control, thus confirming lack of cytotoxicity for the tissues.

Figure 12:



LDH activity of HOE after 48h exposure with the treatment. Lactate dehydrogenase (LDH) activity, an indicator of cytotoxicity, measured in the HOE tissues after 48 h exposure with the sample and controls. Low control (negative control, 0% toxicity) of HOE treated with milliQ water: PBS (1:1). Positive control was obtained from culture media of HOE treated with 2% X-100 Triton diluted in PBS (1:1). 3.1% HAP-NP corresponds to HOE treated with 40% nanoXIM diluted in PBS (1:1). Values represent the mean ± SD. Student-t-test: * p<0.05 *versus* Negative control; # p<0.05 *versus* 3.1 % HAP-NP.

Conclusion from the Notifiers

After an incubation time for 48 hours, the 3.1 % HAP-nano group showed significantly lower LDH activity values compared to the negative control. Therefore, it can be concluded that 3.1 % HAP-NP were not cytotoxic for the tissues.

SCCS comment

The Notifier used only one SkinEthic reconstructed Human Oral Epithelium (HOE) (EpiSkin, Lyon, France) as *in vitro* model to perform the cytotoxicity test. Most probably it was a non-keratinizing model, which represents the worst-case scenario, i.e. if no toxicity in this test model is observed, it could be expected that there would be no toxic effects in a keratinized model, which has an additional barrier of stratum corneum.

In the test, a very high and single concentration of 3.1% (i.e. 31 mg HAP-nano / 1000 µL), was used. At high concentrations, more agglomeration of NPs is likely but no data have been provided to indicate if and how agglomeration was prevented or minimised in the test suspension.

It is not also certain if the NPs interfered with the activity of LDH (an important drawback considering the negative result), as no appropriate control (i.e. HAP-nano incubated in the presence of known activity of LDH) was conducted. It has indeed been reported that many characteristics of NPs (composition, size, coatings, and agglomeration) can interfere with different *in vitro* cytotoxicity assays (WST-1, MTT, LDH, neutral red, propidium iodide, 3H-thymidine incorporation, and cell counting), pro-inflammatory response evaluation (ELISA for GM-CSF, IL-6, and IL-8), and oxidative stress detection (monoBromoBimane, dichlorofluorescein, and NO assays) (Guadagnini *et al.*, 2013).

In Vitro Biocompatibility and Irritation test of nanoXIM Hydroxyapatite Nanoparticles on Human Oral Epithelium

To determine the biocompatibility / oral irritation test on human oral epithelium of HAP-NP, an *in vitro* model of reconstructed human oral epithelium was used after exposure to nanoXIM nanoparticles. MTT viability test and histological analysis of the exposed tissues followed by microscopic observations were performed.

Material and methods

- Reconstructed Human Oral Epithelium

The SkinEthic reconstructed Human Oral Epithelium (HOE) (EpiSkin, Lyon, France) was used as an *in vitro* model to perform the biocompatibility and irritation tests. The HOE consists of an airlifted, living, multi-layered tissue construct, produced in polycarbonate inserts in serum-free and chemically defined medium. The HOE feature normal ultrastructure and functionality similar to human tissue *in vivo*. The SkinEthicTM HOE model is composed of TR146 cells (derived from a squamous cell carcinoma of the buccal mucosa) cultivated on an inert polycarbonate filter at the air liquid interface in a chemically defined medium. This model forms an epithelial tissue devoid of *stratum corneum*, histologically resembling the mucosa of the oral cavity.

The inserts containing the HOE (size 0.5 cm^2) were shipped at room temperature in a multiwell plate filled with an agarose-nutrient solution in which they are embedded. Upon arrival, the tissues were processed following the manufacturer's protocol. Briefly, the HOE tissues were removed from the agarose-nutrient solution and were placed in a plate previously filled with the SkinEthic Maintenance Medium (EpiSkin, Lyon, France) at room temperature. The tissues were incubated in a cell incubator at 37°C, 5% CO2 and saturated humidity overnight.

- Treatment with the nanoXIM nanoparticles.

The HOE tissues were dosed with 20% nanoXIM (3.1% HAP-NP) for 48 h. The dose of nanoXIM was calculated with the assumption that adults use 1 g of toothpaste in one brushing and that the maximum concentration of nanoXIM (15.5% wt. HAP-NP) in a toothpaste is 20%. Then, the net amount of exposure to HAP-NP would be 31 mg of HAP NP per brushing. Using the areas for the tissues used in this study (0.5 cm^2) and the surface area of the adult oral cavity (214.7 ± 12.9 cm^2), this results in a concentration of 0.144% HAP-NP, which we round up to 0.155% HAP-NP, i.e. 1% nanoXIM. In order to have a large safe-margin test above the maximum usage level, we multiplied this value by 20 and

therefore selected a 20% nanoXIM concentration (3.1% HAP-NP) to perform these tests.

Evaluation was done after 48 hours of incubation. Triplicate tissues were assessed for tissue viability (MTT assay) and for histological analysis. Six samples were used for LDH activity.

Three different treatment groups were assessed:

- 3.1% HAP-NP;
- Negative control: Phosphate buffer saline, PBS;
- Positive control: 1% X-100 Triton for cytotoxicity determination or 5% SDS (sodium dodecyl sulphate) for the rest of tests.

- LDH activity

Lactate dehydrogenase (LDH) activity in the culture media was used as an index of cytotoxicity. LDH activity was determined spectrophotometrically after 30 min incubation at 25 °C of 100 µl of culture and 100 µl of the reaction mixture by measuring the oxidation of NADH at 490 nm in the presence of piruvate, according to the manufacturer's kit instructions (Roche Diagnostics, Mannheim, Germany). Results were presented relative to the LDH activity in the medium of negative control group (Phosphate buffer saline, PBS; low control, 0 % of cell death) and on tissue culture plastic (TCP) adding triton X-100 1% (high control, 100 % of death), using the equation:

Cytotoxicity (%) = (exp.value – low control)/ (high control – low control) * 100

- MTT test

At the end of the incubation period, the tissues treated with HAP-NP or controls were rinsed with PBS and placed on 300 µl of 0.5 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (ThermoFisher Scientific, MA, USA). After 3 hours of incubation at 37 °C, 5% CO2, cultures were placed in 2 ml of isopropanol. Extraction was performed overnight at room temperature. Optical density was measured on 200 µl of extracts at 570 nm (reference filter: 690 nm). Results are expressed as a percentage of viability compared to negative control for each time point (mean +/- SD), using the following equation:

% of viability = [OD(570 nm - 690 nm) test sample / OD(570 nm - 690 nm) negative control] x 100.

- Histology

For both HAP-NP and controls, at the end of the incubation period, each tissue was divided into 2 parts. One part was used for histological analysis and the other for observation with transmission electron microscopy (TEM) (see next section). For histological analysis, one half of the tissue was fixed in a balanced 10% formalin solution and later embedded in paraffin. Four microns vertical sections were stained with hematoxylin/eosin (H/E) or using the von Kossa staining kit (Abcam, Cambridge, UK) following the manufacturer instructions and photographed under a microscope. H/E results obtained with the test sample were compared to the positive (damaged tissue) and to the negative (regular tissue) control, and scored according to the following scale:

Score	Description of observations
0	Absence of, or minor epithelium changes
1	Slight epithelium changes: Slight edema and/or slight cellular changes and/or upper cell layer disintegration
2	Moderate epithelium changes: Moderate edema and/or moderate cellular alterations and/or presence of significant number of necrotic cells and/or disintegration of superficial cell layers
3	Marked to severe epithelium changes: Marked edema and/or cellular alterations and/or partial tissue necrosis and/or disintegration of supra basal cell layers
4	Total tissue necrosis and/or tissue disintegration

- Transmission electron microscopy

At the end of the incubation period, the other part of each tissue treated with HAP-NP or controls (see previous section) was fixed in a balanced 2.5% glutaraldehyde solution (Sigma- Aldrich, St. Louis, MO), dehydrated and later embedded in resin. Vertical sections of 0.3 µm were photographed under a TEM microscope.

- Statistics

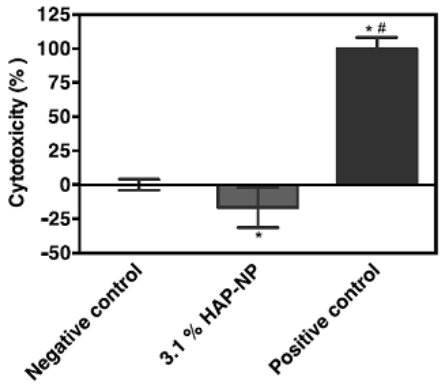
All data are presented as mean values ± SD. The Kolmogorov-Smirnov test was done to assume parametric or non-parametric distributions for the normality tests. Differences between groups were assessed by Student t-test. Results were considered statistically significant at p-values <0.05. SPSS® program for Windows, version 17.0 (SPSS Inc, Chicago, IL, USA) was used.

Results

- LDH activity

As shown in Figure 13, after 48 h incubation, the positive control showed significantly higher cytotoxicity than the other groups. Tissues treated with 3.1 % HAP-nano showed significantly lower cytotoxicity than the negative control. A different positive control was used for the cytotoxicity test (1% X-100 Triton), since it has been reported that high concentrations of SDS inhibit the LDH enzyme.

Figure 13:

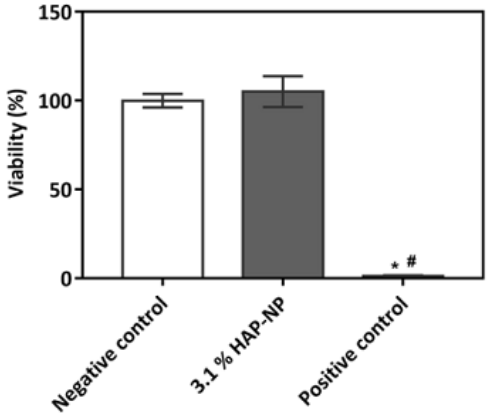


LDH activity of HOE after 48h exposure with the treatment. Lactate dehydrogenase (LDH) activity, an indicator of cytotoxicity, measured in the HOE tissues after 48 h exposure with the sample and controls. Low control (negative control, 0% toxicity) of HOE treated with milliQ water: PBS (1:1). Positive control was obtained from culture media of HOE treated with 2% X-100 Triton diluted in PBS (1:1). 3.1% HAP-NP corresponds to HOE treated with 40% nanoXIM diluted in PBS (1:1). Values represent the mean ± SD. Student-t-test: * $p<0.05$ versus Negative control; # $p<0.05$ versus 3.1 % HAP-NP.

- MTT assay

The MTT assay was used as a marker of cell viability, as it measures the MTT reduction by mitochondrial reductase enzymes. As shown in Figure 14, a complete reduction of cell viability was found for the positive control treated with 5% SDS for 48h, compared to the negative control. The group treated with the 3.1% HAP-NP showed similar viability compared to the negative control.

Figure 14:

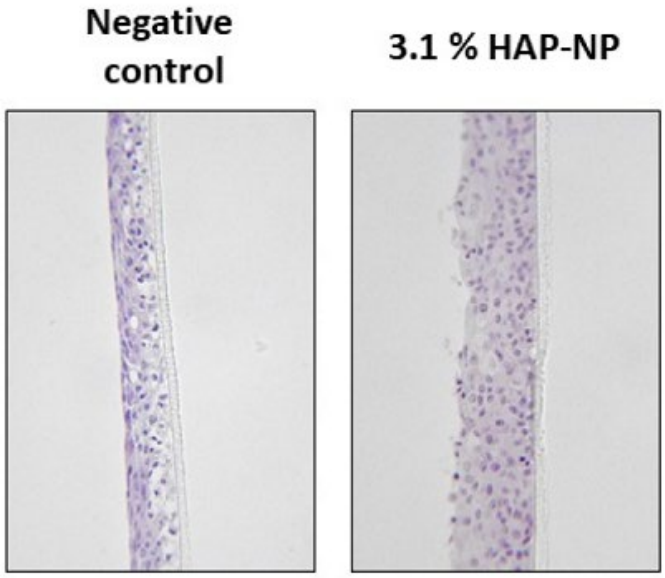


MTT viability of HOE after 48 h exposure with HAP-NP. MTT, an indicator of the number of viable cells, measured in the HOE tissues after 48 h exposure with the sample and controls. Negative control (that was set to 100%) was obtained from culture media of HOE treated with milliQ water: PBS (1:1). Positive control was obtained from culture media of HOE treated with 10% SDS diluted in PBS (1:1). 3.1% HAP-NP corresponds to HOE treated with 40% nanoXIM diluted in PBS (1:1). Values represent the mean ± SD. Student-t-test:* $p<0.05$ *versus* Negative control; # $p<0.05$ *versus* 3.1 % HAP-NP.

- Histology

There was no sign of tissue changes after incubation with 3.1% HAP-NP (Figure 15 and Table 5). Total tissue disintegration (image not shown) was observed after treatment with 5% SDS after 48 hours of exposure and no pictures could be taken, as only paraffin was present.

Figure 15:



Histology of HOE after 48 h exposure with HAP-NP. Negative control corresponds to HOE treated with mQ water: PBS (1:1). 3.1% HAP-NP corresponds to HOE treated with 40% nanoXIM diluted in PBS (1:1). Paraffin slices were stained with hematoxylin/eosin. x200 magnifications are shown.

Table 5:

Results with Histopathological scores of HOE tissues after treatment with HAP-NP or controls.

Group	Score
Negative control	0
3.1% HAP-NP	0
Positive control	4

The oral mucosa consists of a stratified squamous epithelium, which may or may not be keratinized depending on its location in the mouth and an underlying connective tissue layer, the lamina propria. The lining mucosa is a non-keratinized stratified squamous epithelium, which is found almost everywhere in the oral cavity and similar to the 3D HOE *in vitro* tissue model used in the present study.

Some particle internalization was observed through von Kossa staining. However, most of the particles were seen outside the outer layer of the tissue. Additionally, it seemed that HAP-NP formed agglomerates, since they were easily observed with an optical microscope.

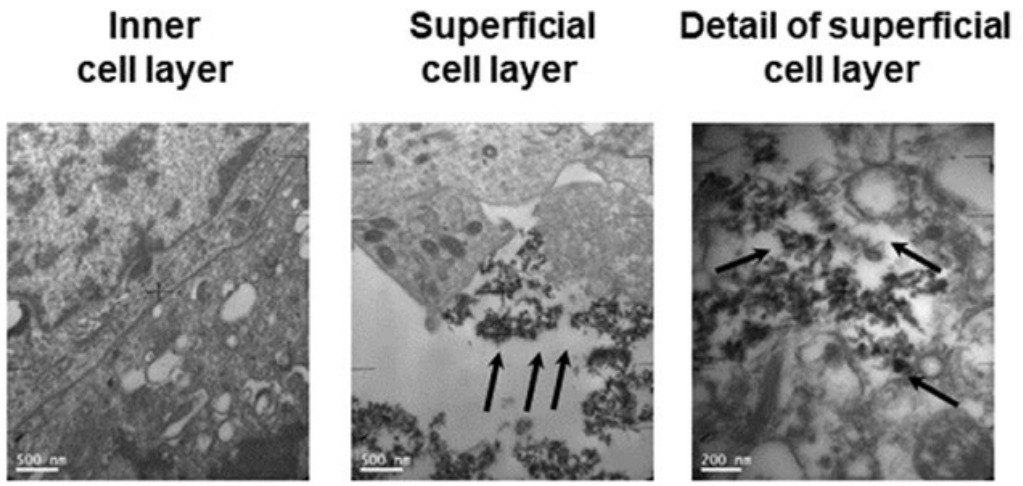
- TEM examination

Finally, TEM analysis was performed on the same tissues to inspect particle internalization in the group treated with HAP-NP (Figure 16). The observation confirmed the results obtained with von Kossa staining, with particle internalization in the superficial layers of the HOE

tissue, located in the cytoplasm compartment and also in the extracellular matrix. By TEM, it was also confirmed that HAP-NP were present mostly as agglomerates, as suspected in the histological observations with von Kossa staining.

In addition, some superficial cells showed cell death due to the HAP-NP accumulation. However, the cell death was not significant since the MTT results did not show relevant cell death when compared to the negative control, and no histological damage was observed with H/E as indicated in Table 2. Frequently, HAP-NP particles were located outside the 3D HOE tissue.

Figure 16:



TEM images of HOE after 48 h exposure with HAP-NP test product. 3.1% HAP-NP corresponds to HOE treated with 40% nanoXIM diluted in PBS (1:1). Black arrows indicate the presence of HAP-NP.

Conclusions from the Notifiers

After an incubation time of 48 hours, the 3.1% HAP-NP group showed significantly both lower LDH activity values compared to the negative control and a MTT cell viability similar to negative control, thus providing evidence that 3.1% HAP-NP were not cytotoxic to HOE tissues.

The treatment with 3.1% HAP-NP did not induce microscopic HOE tissue damage after 48 hours of incubation, as observed with the H/E staining.

HAP-NP were found only in the superficial layers of the HOE tissue as observed with von Kossa staining after 48 hours of incubation. This result was also confirmed with TEM analysis with HAP-NP found only in the outer layers. Using TEM, cellular internalization was observed with some cells containing HAP-NP in the cytoplasm and also in the extracellular matrix. No HAP-NP particles were found in the inner layers of the tissue. Frequently, HAP-NP particles were located outside the 3D HOE tissue.

Taking into account that oral epithelial cell turnover is around 5–7 days, superficial cells of the non-keratinized mucosa in which nanoparticles are taken up are likely to be eliminated within that time frame. Thus, our findings suggest that HAP-NP in the concentration used in the present study are unlikely to enter systemic tissues *via* intact oral epithelium.

SCCS comment
The TEM images are difficult to analyse. Only small fragments of the cells are presented in the images without showing a general picture of the tissues exposed.

SCCS general comments on local toxicity
In general, the cytotoxicity study, although with some limitations, has shown negative results. TEM analysis of the HAP-nano is only partially acceptable. Internalisation by the cells is documented by TEM but only in the outer layers. These findings suggest that HAP-nano at the concentration used in the present study are unlikely to enter systemic tissues *via* intact oral epithelium.

3.3.3 Genotoxicity

***Taken from previous opinion* SCCS/1566/15**

"Information on three genotoxicity studies performed with nanohydroxyapatite 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 nanohydroxyapatite 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 nanohydroxyapatite. In a study using needle-shaped nanohydroxyapatite, 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 nanohydroxyapatite. 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 nanohydroxyapatite. The available information indicated that needle-shaped nanohydroxyapatite might be of concern in relation to genotoxicity."

New submission

SCCS assessment

Even if the Notifiers now only support rod shape HAP-nano and not needle shape HAP-nano anymore, the SCCS could still not conclude on the genotoxicity of the nanomaterial nanoXIM•CarePaste (rod shape) based on the data provided in the first submission for the following reasons:

1. the bacterial gene mutation test is not considered appropriate for nanomaterial mutagenicity assessment, due to limited uptake of the nanomaterial by the bacteria. This has already been mentioned in the SCCS Guidance on the Safety Assessment of Nanomaterials in Cosmetics (SCCS/1611/19). The SCCS also explicitly noted this in the previous Opinion (SCCS/1566/15) on HAP-nano by stating that 'A gene mutation test performed in bacteria cannot be used for the assessment of genotoxicity of nanohydroxyapatite as this type of study is inappropriate for nanomaterials…..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 nanohydroxyapatite.'

2. the SCCS has noted in the previous Opinion on HAP-nano (SCCS/1566/15) that on the basis of available information needle-shaped HAP-nano is of concern in relation to genotoxicity. Further detailed analysis of the methodology, as described in two key publications (Turkez *et al.*, 2014 and Sonmez *et al.*, 2016), has indicated that the effects were observed using HAP-nano after calcination at high temperature. It is acknowledged that the material evaluated in this Opinion was not subjected to a high-temperature calcination step during manufacturing. However, analysis of TEM images of HAP-nano provided in the two publications (Turkez *et al.*, 2014; Sonmez *et al.*, 2015) indicates that the actual shape of the nano-HAP might be rod and spherical, respectively.

3. Review of the published literature indicates a potential of HAP-nano to be effectively internalised by cells of different types after the exposure. Some studies have also reported nuclear translocation of nano-HAP (Tay *et al.*, 2014; Muller *et al.*, 2014; Cui *et al.* 2016). Therefore, the SCCS considered that it is not clear if the nanomaterial nanoXIM•CarePaste, which is rod-like shape, could be regarded devoid of a genotoxic potential.

In view of the above concerns, the SCCS asked the Notifier to provide the following *in vitro* genotoxicity tests on nanoXIM•CarePaste according to relevant OECD TG:

a. a mammalian cell chromosome aberration/clastogenicity test (OECD TG 487)

b. an in vitro mammalian cell gene mutation test (OECD TG 490)

The SCCS also emphasised that the *in vitro* genotoxicity studies would need to be accompanied by an assessment of cellular (and preferably nuclear) uptake of HAP-nano to demonstrate exposure of the target cells (see OECD 2014, SCCS/1611/19). The tests should be carried out with due attention to the selection of appropriate, analysable concentrations and the proper characterisation (particle size distribution and agglomeration, e.g. by DLS) of HAP-nano in the culture media, preferably at the beginning and at the end of the incubation/exposure period. It is known that decreased stability of the NP suspension may affect bioavailability of NPs to the cells and thus lead to false negative results. In this regard, the Notifier was referred to the SCCS Guidance on the Safety Assessment of Nanomaterials in Cosmetics (SCCS/1611/19), and the SCCS Memorandum on 'Relevance, Adequacy and Quality of Data in Safety Dossiers on Nanomaterials' (SCCS/1524/13).

New data provided by the Notifier (July 2020) in response to SCCS request.

In vitro toxicity assessment on nanoXIM•CarePaste according to relevant guidelines under GLP conditions – Report 1

Materials and methods

- *nanoXIM.CarePaste material*

The nanoXIM.CarePaste material containing 15.5% ± 0.5% wt. of HAP-nano (nHA) dispersed in water was used as test material. Samples from 3 different batches of this material were used, identified here in this report as Sample 1, Sample 2 and Sample 3.

Particles hydrodynamic radius of the three nHA samples was confirmed by dynamic light scattering (DLS) measurements in the very dilute regime of parts per million (after gradual dilutions of the initial nHA suspensions) at three different angles, including 90° according to

a previous report, by means of an instrument ALV-5000 goniometer/correlator setup from the company ALV Langen.

Transmission electron microscopy (TEM) analysis was also performed for the visualization of the three nanoparticulate samples (Samples 1, 2 and 3) per se.

- *Cell cytotoxicity assessment by cell viability evaluation*

For the assessment of the cell cytotoxicity, the murine connective tissue fibroblasts cell line L-929 (DSMZ Braunschweig, Germany, ACC-2) established from the normal subcutaneous areolar and adipose tissue of a male C3H/An mouse was used. This is a relevant cell type for the biocompatibility testing of materials for the development of bone implants. Cells were grown in RPMI culture medium (biosera), supplemented with 10% v/v Fetal Bovine Serum (FBS) (Gibco), 50 IU/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA), 50 g/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA)] and 2 mM L-glutamine (Gibco) in a 5% CO2 incubator (Heal Force) at 37 °C.

Cell viability was evaluated according to ISO 10993-5 (2009) standards and measured by the PrestoBlue® viability assay (Invitrogen) and the manufacturer's instructions as previously reported in materials cytotoxicity assessment. For the assessment of the nHA samples, 10000 cells per well were seeded into 96 well plates and next day culture medium was replaced with medium containing the nHA samples at four different concentrations, 0.1, 0.25, 0.5, and 1% v/v. Cell viability was measured after 1 and 2 days in culture. The measurements were performed by means of a spectrophotometer (Synergy HTX Multi-Mode Microplate Reader, BioTek, Bad Friedrichshall, Germany) and the absorbance was measured at 570 and 600 nm. The term "control" refers to cells that are only cultured on tissue culture treated polystyrene (TCPS) without any induction materials.

- *Cells uptake assessment*

For the assessment of the samples uptake by the cells, the pre-osteoblastic MC3T3-E1 cell line was used. The murine MC3T3-E1 cell line (DSMZ Braunschweig, Germany, ACC-210) established from new-born mouse calvaria was used as a relevant cell type for the biocompatibility testing of materials for the development of bone implants. Cells were grown in alpha-MEM culture medium (biosera), supplemented with 10% v/v Fetal Bovine Serum (FBS) (Gibco), 50 IU/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA), 50 g/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA)] and 2 mM L-glutamine (Gibco) in a 5% CO2 incubator (Heal Force) at 37 °C.

Transmission electron microscopy (TEM) analysis was performed for both the visualization of (i) the three nanoparticulate samples (Samples 1, 2 and 3) per se, and (ii) their uptake by pre-osteoblastic cells, as a proven cellular system for nanoparticles uptake by cells [10], at a concentration of 0.25% v/v by means of a high resolution transmission electron microscope according to the methodology described earlier Briefly, the cells incubated for 24 h with the three nHA samples (and the control cells without samples) were detached from the culture flask, washed with phosphate buffer saline (PBS), and fixed in 2% (vol/vol) glutaraldehyde, 2% (vol/vol) paraformaldehyde in 0.1 M sodium cacodylate buffer (SCB), pH 7.4, for 24 h at 4 °C. The samples were washed three times for 5 min each in 0.1 M SCB, postfixed in 1% aqueous OsO4 for 12 h at room temperature, and then washed again. After the last washing step, the samples were stained with 2% (vol/vol) uranil acetate for 1 h. Then, the samples were rinsed with SCB, dehydrated through an ascending acetone gradient of 30, 50, 70, 90, 100% (vol/vol), infiltrated with Durcupan ACM Fluka resin [3:1 propylene oxide: resin mixture for 1 h followed by a 1:1 and a 1:3 propylene oxide:resin mixture for 1 h each and finally 100% (vol/vol) resin for 16 h], and embedded in flat molds. The resin was cured in a drying oven at 60 °C for 48 h. The samples were trimmed, thin-sectioned, and absorbed onto 300-mesh copper grids. Observation was carried out using a high-resolution transmission electron microscope JEOL JEM-2100HR at an operating voltage

of 80 kV.

- *Genotoxicity assessment according to OECD 487 – Micronucleus formation assay*

For the assessment of the cell genotoxicity by means of the micronucleus formation, the V-79 cell line was used. The Chinese hamster (*Cricetulus griseus*) cell line V-79 (lung fibroblasts) (DSMZ Braunschweig, Germany, ACC-335) was used for the vitro mammalian cell micronucleus test according to the OECD 487. Cells were cultured in RPMI culture medium (biosera), supplemented with 10% v/v Foetal Bovine Serum (FBS) (Gibco), 50 IU/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA), 50 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA)] in a 5% CO2 incubator (Heal Force) at 37 °C.

For the genotoxicity assessment, 30.000 cells per well (V-79) were seeded on 48 well plates followed by 24 h incubation at 37° C with 5% CO_2. The genotoxic agent methyl methanesulfonate (MMS) was used as the positive control, as suggested by the Organization of Economic Cooperation and Development (OECD) 487 guidelines. Cells only cultured on TCPS without any induction materials were used as negative control. Afterwards, fresh culture medium was added with the nHA samples at a concentration of 0.25% v/v, in a total volume of 300 µl per well. After 24 h incubation, the fixation and staining were performed as follows: the culture medium was removed and the samples were washed twice with PBS. Fixation was achieved with 4% w/v paraformaldehyde (PFA) for 20 min. Then, after two washes with PBS, staining of the DNA was performed with a Giemsa solution (Giemsa 3% in Na2HPO4 and K2HPO4 0.6 M each, pH 6.8) for 40 min and washed twice with PBS. Finally, the number of micronuclei was counted under an optical microscope (2000-3000 cells per measurement).

The concentration of the samples used was 0.25% v/v, which corresponds to 2.5 µl/ml, and this is in line with the OECD 487 guidelines (2019) based on the statement described in page 7: 'If no precipitate or limiting cytotoxicity is observed, the highest test concentration should correspond to 10 mM, 2 mg/mL or 2 µl/mL, whichever is the lowest'.

- *Genotoxicity assessment according to OECD 490 – Mouse lymphoma assay*

For the assessment of the cell genotoxicity by means of the mouse lymphoma assay (MLA), the L-5178-Y cell line recommended in the OECD 490 protocol was used. The L-5178-Y cells were cultured in Fischer's (F10P) growth medium containing 0.22 mg/mL sodium pyruvate, (10 ml of Biosera's), 1 mL of penicillin–streptomycin (10,000 units/mL), 5 mL of pluronic F68, 5 mL of L-glutamine (200 mM), 438.5 mL of Fischer's medium, 50 mL of horse serum. Additionally, a cloning Medium (R10P) was used; this contained 0.22 mg/mL of sodium pyruvate, 1 mL of penicillin–streptomycin (10,000 units/mL), 5 mL of pluronic F68, 5 mL of L-glutamine, 438.5 mL of RPMI 1640 medium, 50 mL of horse serum.

The MLA was performed according to a protocol previously reported by Schisler *et al.* (2013). Control growth medium was used as solvent and the nHA samples were diluted in culture medium. As positive control, methyl methane sulfonate (MMS) was used.

For each treatment, $5x10^5$ cells/ml at a final volume of 12 ml were plated. First, the appropriate cell number is determined and the cells are seeded into the culture flasks and left for at least 20 min to equilibrate in an orbital shaker at 37°C at 5% CO_2. Then 8 ml of fresh F_{10p} medium at each flask (total volume = 20 ml, cell density= $0.3x10^6$ cells in medium containing 10% horse serum) containing 0.25% v/v of the nHA Samples 1, 2 and 3 were added, incubated for 24 h in a roller drum at 37°C, centrifuged for 5 min at 350 x g, resuspended in 10 ml fresh F_{10p} and gently mixed. Cells were counted and the cell number was adjusted at $3x10^5$ cells/ml in a final volume of 20 ml. Afterwards, cells were incubated for 24 h in a roller drum and this procedure was repeated several times as described in the protocol until the mutant selection occurred by means of the addition of the lethal nucleoside analogue triflurothymidine (TFT) following an incubation period of 12 days.

At the end of the 12-days incubation time, the formed colonies on all the plates were measured for the viability counts. Specifically, the lethal nucleoside analogue TFT containing plates for the mutant selection were counted manually to determine the formation of small and large colonies. For the viability count into two 96 well plates, 200 µl/well, and 1.6 cells per well were plated. For the TFT mutant selection into 96 well plates, 200 µl/well, and 2000 cells per well were plated. For the colony formation, after the incubation time of 12 days, the TFT-containing plates were counted manually using the following guidance to determine small and large colonies, namely, small colonies are defined as those covering less than 25% of the well diameter, while large colonies are defined as those covering more than 25% of the well diameter. The mutant index is calculated as the mutant frequency of the treated/average mutant frequency of the solvent control.

- *Statistical analysis*

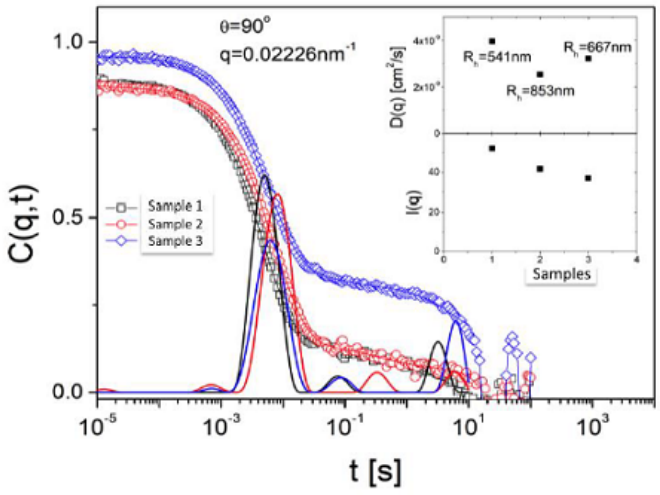
Cell viability and genotoxicity data are presented as mean values ± standard deviation of at least three independent experiments (n=9). Statistical analysis was performed using GraphPad Prism version 8 software one-way ANOVA followed by Dunnett's multiple comparisons tests to evaluate significant differences among means of values obtained from all Samples and conditions used as well as of the cells on the TCPS control at each experimental time point. A p value of <0.05 was considered significant, if no other indication is stated.

Results

- *Samples characterization by means of dynamic light scattering (DLS)*

Particles hydrodynamic radius of the three nHA samples was confirmed by DLS measurements in the dilute regime of parts per million (after gradual dilutions of the initial nHA suspensions) at an angle of 90° (for all samples) and three different angles for Sample 1. We suggest that there is some size polydispersity of the particles for all three samples.

Figure 17:



Dynamic light scattering (DLS) analysis of the samples 1, 2, 3 (measured at T=20 °C).

The DLS graph shows a comparison the authors made of the 3 samples at the measurement angle of 90°. They assume that the distribution of the relaxation times (thick solid curves main graph) is translational diffusion. They know that they measured in dilute condition and therefore it is safe to assume that the main mode follows translational diffusion (Brownian motion). The graph of the upper inset indicates an apparent hydrodynamic radius, which is related to the actual size, shape and particle-solvent interactions. From the correlation

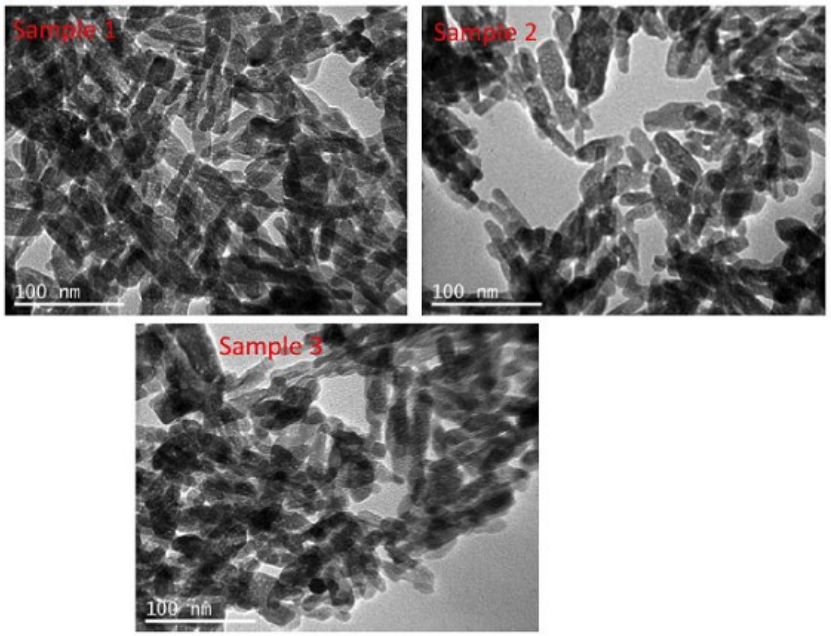
function (open symbols main plot) and its CONTIN analysis (fit and distribution) the authors consider that in the 3 different solutions, there is mainly one big species and some very large agglomerates (that are low in concentration). Actually, since for samples 2 and 3 they only have one angle, they can only say that most of the particles are apparently around 500-850 nm in size and that a few larger agglomerates are present. They do not have enough data from many different angles in DLS for samples 2 and 3 to conclude that size distribution of the main mode is polydisperse, although it is very likely that they are polydisperse given that the main mode is already an agglomerate of cylinders/rods.

The polydispersity of all three samples as well as their tendency to form big agglomerates were visualized in the confocal microscope time-lapse photos. Based on the fact that all three samples form big agglomerates, the authors consider that any further investigation by means of DLS is not necessary.

- Sample characterization by means of transmission electron microscopy (TEM)

The following figures show representative TEM images from the Samples 1, 2 and 3. Representative TEM images show that all three nHA samples (Samples 1, 2 and 3) have a rod-like shape with an average length of approximately 20 nm (Figure 18).

Figure 18:

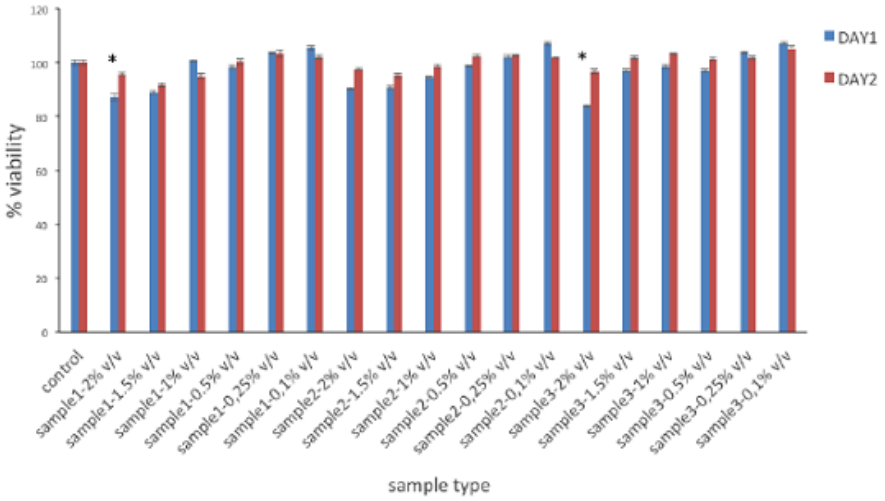


Transmission electron microscopy images from the Samples 1, 2 and 3 indicating a rod-like shape of an average length of 20 nm. The scale bar represents 100 nm.

- *Cytotoxicity assessment*

The cell viability, quantified using six different concentrations (0.1, 0.25, 0.5, 1.0, 1.5 and 2% v/v) of the three nHA samples using L-929 murine fibroblasts after 1 and 2 days in culture, and the % viability to the control (cells only, set at 100%) are graphically presented (Figure 19).

Figure 19:



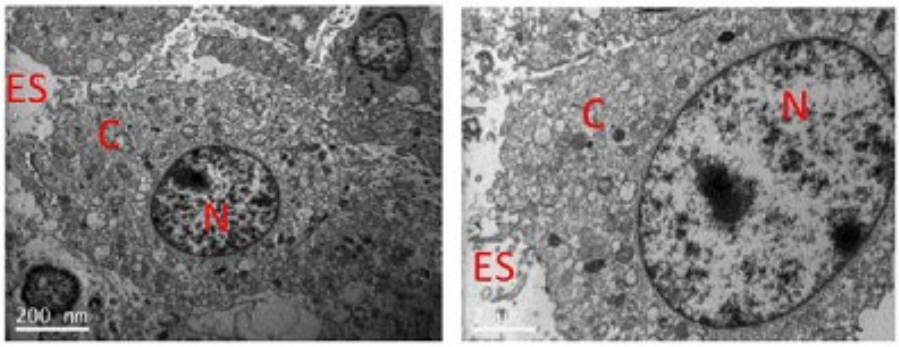
Assessment of cytotoxicity of three samples at the concentrations of 2% (corresponds to 3.1 mg/ml), 1.5%, 1%, 0.5%, 0.25%, and 0.1% v/v according to ISO 10993-5 (2009) using the resazurin-based cell viability reagent PrestoBlue®. Cell viability of L-929 fibroblasts expressed as % of the control (control was set at 100%). Initial cell number: 10000 cells/well in 96 well plates, n=9 (three independent experiments). The asterisk (*) designates significant differences compared to the control (p=0.0001)

All Samples 1, 2 and 3 present a very high cell viability of 90 to 100% compared to the control on both time points, days 1 and 2, at all six investigated concentrations, except of the samples 1 and 3, which, at the highest concentration of 2% v/v present a cell viability of 87% and 84%, respectively. According to the ISO 10993-5, a limit of 70% cell viability considers the samples cytocompatible. All the samples investigated are above 70%, therefore highly biocompatible. Statistical analysis in a Dunnett's multicomparison test reveals that significant differences (p=0.0001) are observed for samples 1 and 3 at the concentration of 2% v/v vs. control.

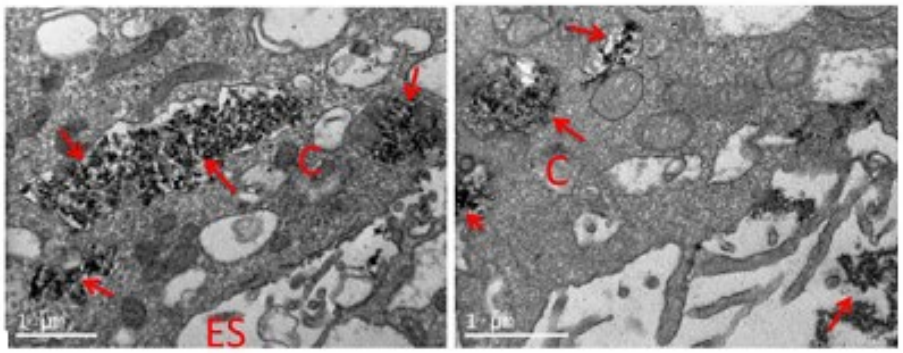
- *Uptake of nHA samples by cells*

The cells' uptake of the three nHA samples is visualized in the following Figure 20. TEM analysis reveals that all three samples can be uptaken by pre-osteoblastic cells as depicted in the TEM images showing nanoparticles of the three samples in cyst-like structures in the cytoplasm. Samples were not observed in the cell nuclei since the authors did not identify their morphology inside this compartment. In the representative TEM images, nanoparticles of all three sample types (Samples 1, 2 and 3) are shown localized in both the extracellular space outside and between cell membranes and in the intracellular space in cytoplasm within cyst-like structures.

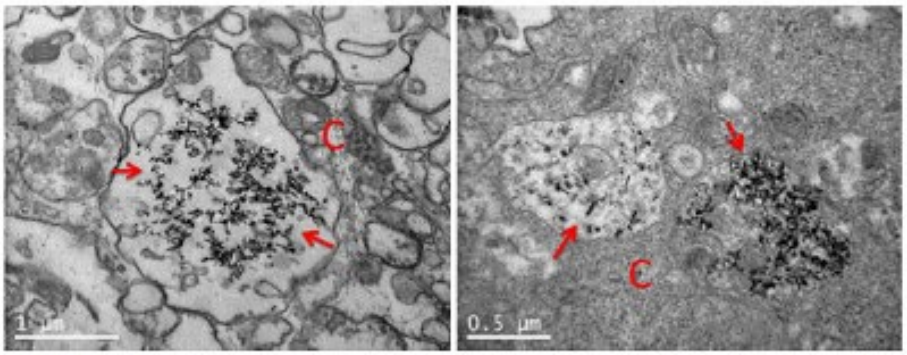
Figure 20:



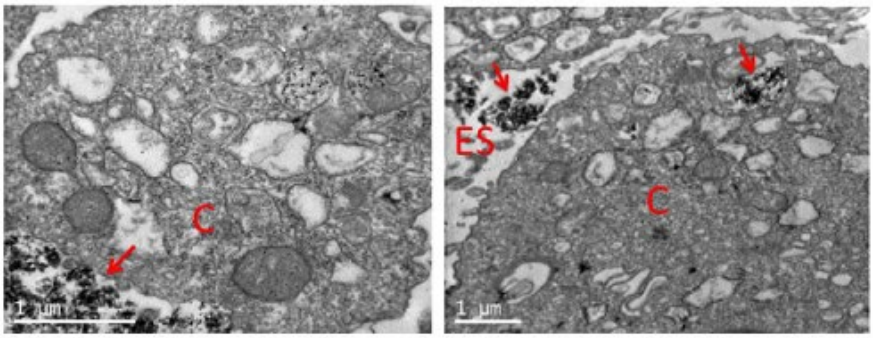
*Transmission electron microscopy images from **pre-osteoblastic cells (control)** in the absence of any nHA samples. The scale bar represents 200 nm (left) and 2 µm (right). The letter N designates the nucleus, C the cytoplasm and ES the extracellular space.*



Transmission electron microscopy images from Sample 1 uptaken by pre-osteoblastic cells. Nanoparticles of the Sample 1 are shown localized and presented in both, the extracellular and the intracellular space in cyst-like structures. The scale bar represents 1 µm. The letter C designates the cytoplasm and ES the extracellular space. The red arrows depict the nanoparticulate Sample 1.



Transmission electron microscopy images from Sample 2 uptaken by pre-osteoblastic cells. Nanoparticles of the Sample 2 are shown localized in the intracellular space in vesicles (in other images that are not shown, they appear also in the extracellular space). The scale bar represents 1 µm (left) and 0.5 µm (right). The letter C designates the cytoplasm and the red arrows depict the nanoparticulate Sample 2.

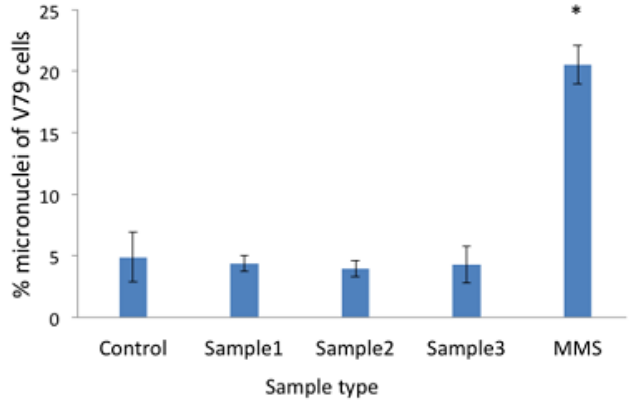


Transmission electron microscopy images from Sample 3 uptaken by pre-osteoblastic cells. Nanoparticles of the Sample 3 are shown localized in both, the extracellular and the intracellular space in cyst-like structures. The scale bar represents 1 µm. The letter C designates the cytoplasm, ES the extracellular space, and the red arrows depict the nanoparticulate Sample 3.

- *Micronucleus*

The results are presented graphically as the percentage of micronuclei in the cells (Figure 21). The three samples present a very low percentage of micronuclei, lower or similar to the control (cells only), indicating that they are not genotoxic.

Figure 21:



Genotoxicity assessment according to the OECD 487 in V-79 cells (Hamster Chinese lung cells). The graph shows the % of micronuclei formation in stained V-79 cells. The concentration of the samples used was 0.25% v/v. Positive control: 400 µM methyl methane sulphonate (MMS). Micronuclei are measured in 2000-3000 cells in each measurement (n=3). The asterisk (*) designates significant differences of all three samples and the control (cells) compared to the MMS positive control (p<0.0001).

The results show a micronuclei formation of 4.4% for sample 1, 3.9% for sample 2 and 4.2% for sample 3, while the negative control (cells only) has a value of 4.9%, and the positive control 21%. Statistical analysis according to Dunnett's multiple comparison test indicates significantly higher values of micronuclei formation among the three samples including the negative control (cells only) compared to the MMS positive control ($p<0.0001$).

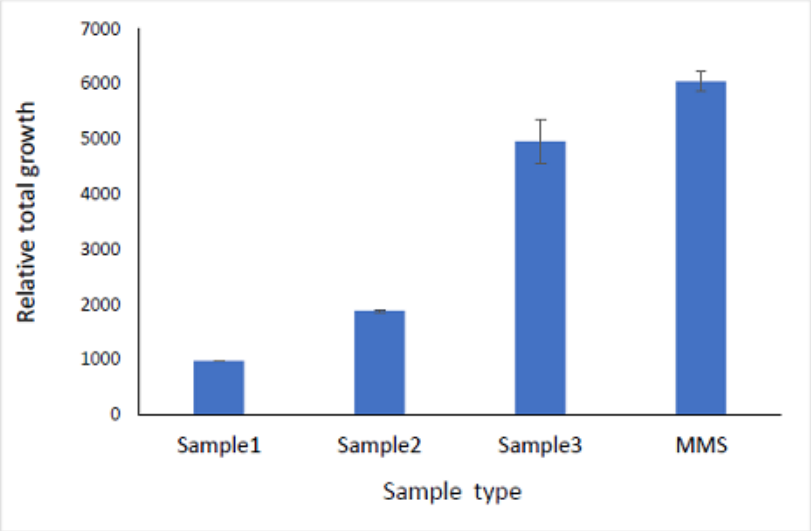
- *Mouse Lymphoma Assay*

At the end of the 12 days incubation period, several colonies were formed at all the 96 well plates for the viability counts, demonstrating that the cells were grown in a healthy environment forming colonies under physiological conditions and the three samples did not present cytotoxicity. In fact, in the wells with colonies, the colour of the culture medium

changed, as was expected according to Schisler *et al* (2013).

The relative total growth (RTG) of the cells in the presence of the samples within the first three days in culture under physiological conditions compared to the solvent control is calculated according to the MLA protocol described and is presented in Figure 22. For this, we first calculated the cloning efficiency by using the equation (CE) = -ln (number of empty wells) from the VC plates/192)/1.6, we then calculated the relative cloning efficiency (RCE) = cloning efficiency of the treated at the time of mutant selection x 100/cloning efficiency of the solvent control at the time of mutant selection. Finally, the relative total growth is given as RTG = RSG x RCE x 100, where RSG is the relative cumulative suspension growth (RSG) = cumulative suspension growth of culture x 100 / average cumulative suspension growth of the solvent control.

Figure 22:



Relative total growth (RTG) of the samples adjusted to the solvent control.

In the plates containing the TFT at a concentration of 3 µg/ml according to Schisler *et al*. (2013), the selection plates, no colonies were observed. The role of TFT is to select only the mutated cells that have undergone genotoxicity. The TFT is responsible for detecting forward gene mutations at the Tk locus, due to heterozygosity of the L5178Y cells. Induced heritable loss of thymidine kinase activity occurs because of a mutational event (from $Tk^{+/-}$ to $Tk^{-/-}$) that results from DNA damage by physical or chemical agents. $Tk^{-/-}$ mutants can be selected due to their inherent resistance to toxic thymidine analogues like TFT. This analogue is incorporated into the DNA of TK-competent ($Tk^{+/-}$) cells resulting in genotoxicity, hence, only the mutated cells survive. Forward mutations at the single functional Tk gene ($Tk^{+/-}$ to $Tk^{-/-}$) result in the loss of function of TK enzyme and as a result, the TFT is not incorporated into the cellular DNA. Consequently, no colony formation at the selection plates reflects absence of genotoxic ability.

Surprisingly, colonies were not formed in the plates containing the MMS, either. This could be explained by several controversial reports on the function of MMS, although it is still the most commonly positive control used for the continuous treatment. The different batches of MMS have been reported to present different genotoxic ability. Importantly, the IWGTP suggests that MMS should be used as positive control and it is still the most commonly used one. The MMS we used is genotoxic, as it resulted in a significantly higher number of micronuclei formation compared to the negative control when applied at a concentration of 400 µM. Another parameter is its employed concentration. For the MLA, it was used at a concentration of 3 µg/ml (27 µM), as this was the maximum concentration recommended by

Schisler *et al.* (2013). Other studies suggest higher concentrations in the range of 10 µg/ml, and even 15 µg/ml, which was applied in repetitive experiments without any changes in the mutagenic capacity of MMS. Moreover, the MMS was freshly prepared, as it can be easily hydrolyzed. As we were not able to infer any genotoxic effect from the MLA assay based on our results, another MLA experiment applying a higher MMS concentration of 15 µg/ml was performed and still no effect was observed.

Conclusion from the Notifier

All three samples present a high cell viability of 90-100% compared to the control, in both experimental time periods of 1 and 2 days in culture at all six concentrations investigated, namely at 2, 1.5, 1, 0.5, 0.25 and 0.1% v/v, except of the Samples 1 and 3 that present a cell viability of 87% and 84%, respectively, at the highest concentration of 2% v/v. It was therefore concluded that all three samples are highly biocompatible at the investigated concentrations.

None of the three samples seems to present mutagenicity according to the OECD 487 protocol.

From the MLA according to the OECD 490 protocol we have observed several colonies formed for the viability counts after 12 days incubation period, demonstrating that the cells were grown in a healthy environment and none of the three samples present genotoxicity. Surprisingly, the positive control MMS did not show any genotoxicity, as evidenced by the absence of the selected mutations colonies, and this can be attributed to the employed MMS concentration that was probably low to elicit a genotoxic effect.

The polydispersity of all three samples was shown by means of the dynamic light scattering. All three samples tend to form big agglomerates as visualized by means of a confocal microscope in time-lapse photos. Based on this data, it was considered by the Notifier that any further investigation by means of DLS is not necessary. TEM provided more information about the nanoparticulate size of the samples, indicating that all three samples have a rod-like shape with an average length of approximately 20 nm. TEM analysis also confirms the presence of agglomerates.

TEM analysis reveals that all three sample types can be uptaken by pre-osteoblastic cells as depicted in the TEM images showing nanoparticles of the samples in the cytoplasm. Nanoparticulated samples were not observed in the cell nuclei. Nanoparticles of all three sample types were observed to be localized in both the extracellular space and intracellularly in cyst-like structures in cytoplasm.

Taken together, the visualization with TEM indicates that nHA samples enter the cell without causing any cytotoxic effect. The genotoxicity assessment, according to OECD 487, depicts that no genotoxicity is evidenced, as the number of micronuclei formed is similar to the negative control (cells only).

SCCS comments

Concerning the Micronucleus test:
The study results are poorly documented. According to the Authors: 'the concentration of the samples used was 0.25% v/v, which corresponds to 2.5 µl/ml'. The description does not clearly indicate what was the final concentration of HAP-nano in the culture medium. Probably the authors used a nanoXIM.CarePaste at a concentration of 2.5 µL/1 mL of culture medium, which means 387.5 µg/mL (2.5 µg assuming d=1 g/mL X 15.5% / 100 µg = 0.3875 mg/mL). Only one concentration was used, which is not acceptable according to OECD TG 487. As a considerable precipitation was most probably observed, it cannot be excluded that the precipitate interfered with scoring of the nuclei. The spontaneous MN frequency of 4.9% in control cells is rather high (49 MN BN per 1000 BN). Usually

acceptable spontaneous frequency for an established cell line is below 2% (20/1000 BN) (Report on statistical issues related to OECD Test Guidelines (TGS) on genotoxicity (ENV/JM/MONO(2014)12). No data on MN frequency in historical controls was provided. Due to all these limitations, the study is not acceptable.

Concerning the MLA:
The study results are poorly documented and presented in an unusual way. Results on increasing Relative Total Growth (RTG) provided for all 3 samples shows a high variability between all three samples and high cytotoxicity for sample 1 and very low cytotoxicity for MMS. No mutagenic effect observed after applying MMS at two concentrations (3 and 15 µg/mL) indicates poor response of the test cells and this invalidates the whole study. Additionally, no background level of mutations in control cells is documented. Due to many limitations, this study is not acceptable.

Concerning the *samples characterisation by means of dynamic light scattering (DLS):*
The results are reported irregularly. Information on important parameters is missing.

Concerning the characterization by means of transmission electron microscopy (TEM):

Images of the nanoparticles are acceptable but size distribution was not analysed.

Concerning the Uptake of HAP-nano samples by cells investigated by TEM:

Images are of good quality proving internalisation of HAP-nano by MC3T3-E1 cells.

During the consultation period, the notifiers sent some clarification related to the genotoxicity of HAP-nano:

Concerning the Micronucleus test, the Notifiers confirm that they have used a concentration of 2.5 µL/1 ml of culture medium, which corresponds to 0.3875 mg/ml of hydroxyapatite nanoparticles. They also confirm that no precipitate was found and consequently there was no precipitate interfering with scoring of the nuclei.

New experiments on micronuclei formation were performed using 3 different concentrations as recommended in the OECD TG 487 guideline (Figure 1). They tested the following concentrations: 2, 1 and 0.5 mg/mL, using 3 distinct batches of nanoXIM (Sample 1, 2 and 3):

Regarding the MN frequency, they agree that 4.9 % in control cells is indeed high. However, that result was obtained in just one individual experiment (n=3). More experiments were performed in the meantime and the notifiers have presented the data on historical controls (positive and negative) obtain. An average value of 8.1 % was obtained for the positive control MMS and an average value of 1.1 % was obtained for the negative control.

SCCS comments on the additional data provided for the micronucleus test

SCCS considers that the two highest concentrations are too high and not recommended for testing genotoxicity of nanomaterial due to agglomeration and thus also possibly no or limited uptake by cells.

No measure for particle size distribution in the test medium was provided – for example by DLS or other methods. SCCS cannot therefore exclude that large agglomerates were formed and precipitation had also occurred.

The laboratory did not provide sufficient proof for their efficiency. According to OECD TG 487 §50: The laboratory's historical negative control database, should initially be built with a minimum of 10 experiments but would preferably consist of at least 20 experiments

conducted under comparable experimental conditions. Laboratories should use quality control methods, such as control charts (e.g. C-charts or X-bar charts (88)), to identify how variable their positive and negative control data are, and to show that the methodology is 'under control' in their laboratory. The historical control values should include at least minimal-maximal values, means, and preferably 95% control limits of the distribution of the laboratory's historical controls. Historical control values should include at least minimal-maximal values, means, and preferably 95% control limits of the distribution of the laboratory's historical controls.

Concerning the MLA, the notifiers indicate that they were able to detect living cells and colony formation was observed for all viability plates that were not treated with the selection factor trifluorothymidine (TFT). In opposition, for the plates in which TFT selector was added, no colonies were observed. The experiments were repeated several times but the same result was observed in all of them. Therefore, they assume that probably the TFT reagent was not in proper conditions and for that reason was causing toxicity to the cells. The supplier from the TFT reagent was contacted but the technical team was not helpful. To try to solve this issue, they tested different concentrations of TFT namely 0.05 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml and 3 µg/ml (being 3 µg/ml one concentration recommended in the literature). Additionally, different concentrations of the positive control methyl methanesulfonate (MMS) were also tested. Particularly, it was tested the concentrations of 15 µg/ml, 3 µg/ml and 1 µg/ml for each concentration of TFT selector factor used (e.g. for the 5 concentration of TFT mentioned above). However and once again, no colonies were observed after adding TFT.

SCCS comments on the additional data provided for the MLA

The provided data indicate that the test method (MLA) did not work properly; especially as the selection chemical TFT showed no activity and no mutagenic effect after applying MMS (positive control). This therefore do not fulfil the acceptance criteria for the results to be considered.

Concerning the samples characterisation by means of dynamic light scattering (DLS), The DLS results that were presented by the Notifiers reinforce that the nanoparticles present in nanoXIM product naturally form agglomerates. For that reason, in their opinion, DLS is not the best technique to evaluate these nanoparticle size. Therefore, they performed TEM that better show the size of the nanoparticles and also the agglomerates.

SCCS comments concerning the samples characterisation by means of dynamic light scattering (DLS)

SCCS notes that the Notifiers actually confirmed that they did not use DLS as the nanoparticles present in nanoXIM product naturally form agglomerates. However, data on TEM are only in stock dispersion and not in medium.

The Notifiers admitted that DLS results showed that the nanoparticles present in nanoXIM product naturally form agglomerates. The purpose of DLS is to assess satisfactory stability of NPs suspensions before preforming any *in vitro* cell exposures. If other means of assuring good dispersion (i.e. preventing excessive agglomeration) cannot be used, at least serial dilutions of the suspensions should be measured by DLS to try to select the optimal NPs concentrations for cell exposures. In the studies the Notifiers did not perform such assessment, and in contrary, increased the already used high concentration of 500 ug/mL to 1000 and 2000 ug/mL, without providing any justification for this or stability assessment.

Moreover, TEM analysis was most probably performed on stock suspension of the HAP NPs and not in culture medium, therefore it not clear what was the agglomeration status of the NPs during cell exposures.

Concerning the characterization by means of transmission electron microscopy (TEM):

The Notifiers performed characterisation using TEM to show the particle shape and size but at that time the size distribution was not performed. They present the particle size distribution that was measured by image analysis using TEM figures. Three different batches of nanoXIM hydroxyapatite were analysed and over 100 nanoparticles were measured for each batch. The results are illustrated, with the histogram for each sample and the corresponding Gaussian curves. All particles are below 100 nm, particularly below 70 nm in length. The mean particle size for each sample was calculated using GraphPad software with a Gaussian non-linear fitting and a confidence level of 95 %. Those results are presented in a table.

SCCS comments concerning the characterization by means of transmission electron microscopy (TEM)
SCCS asked data on size distribution but the particle size distribution was assessed by image analysis using TEM figures. SCCS consider that it is arbitrary and it was shown only in stock dispersion and not in medium. Thus, SCCS does not know state of agglomerations/aggregation in the time of the treatment.

SCCS overall comment on genotoxicity/mutagenicity
In spite of clear requests, valid studies on genotoxicity/mutagenicity were not provided. The results of the provided studies are not acceptable due to many limitations listed above. Therefore, the SCCS cannot exclude concerns over the genotoxic potential of HAP-nano.

3.3.4 Assessment of the systemic toxicity of the non nanoform of HAP

HAP is a naturally occurring mineral represented by the formula $Ca_{10}(PO_4)_6(OH)_2$, it accounts for 97% of tooth enamel and 70% of dentin. Enamel is formed of prisms comprising rod-like HAP-nano in parallel arrangement. In a healthy oral environment with normal saliva flow, enamel density is relatively stable, with demineralization and remineralisation occurring continuously at the tooth surface.

If some calcium phosphate particles are ingested, they enter the stomach where a complete dissolution occurs at a pH of 1–2 (see above). There is no chance that dispersed calcium phosphate nanoparticles will survive these highly acidic conditions. Thus, their nanoparticulate identity is completely lost, and they are only present as calcium and hydrogen phosphate ions.

Calcium phosphate is a common mineral on earth and the most common calcium phosphate mineral is hydroxyapatite. Calcium phosphates have been generally recognized as safe (GRAS) in food by the FDA in 1975. Calcium phosphate is highly biocompatible in contact with hard tissue because the body is well accustomed to this mineral. Therefore, it has found wide application in biomedicine, especially for the treatment of bone defects and the coating of metallic implants in bone contact (like total hip endoprostheses or tooth implants).

There are no concerns regarding the application of calcium phosphate cements, except for occasional slight local inflammation, as a number of *in vivo* studies have confirmed, This indicates that there is no negative side effect of calcium phosphate (nano)particles migrating from the cemented site.

The question whether the presence of calcium phosphate nanoparticles in the blood enhances the risk of atherosclerosis may also be raised. This is difficult to answer because

atherosclerosis is a long-term process that cannot be reproduced by short-term experiments as in cell-culture or imaging studies. The calcium concentration in blood is highly regulated to about 100 mg L-1, corresponding to 500 mg in 5 L blood for an adult human. To match this amount of calcium, one would have to disperse 1250 mg calcium phosphate nanoparticles in the blood. This is clearly much more than could reasonably be expected from any practical medical or cosmetic application. Thus, unless the applied dose is very high (which should not be the case in any conceivable application), a negative side effect is unlikely (Epple, 2018).

3.3.5 Special investigations

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3.4 SAFETY EVALUATION (INCLUDING CALCULATION OF THE MOS)

Based on the available data, the SCCS concludes that HAP-nano under the conditions of uses in cosmetic products would not have:

- any significant systemic exposure *via* the oral mucosa
- any significant systemic exposure *via* ingestion (due to solubility in gastric fluid)
- any cytotoxicity at the level of the oral epithelium after 48h exposure

However, the SCCS still has concerns about possible genotoxicity and, based on the provided data, cannot exclude the genotoxicity potential of the HAP-nano. For this reason, the MOS could not be calculated.

3.5 DISCUSSION

HAP is a naturally occurring, water-insoluble mineral of a molecular weight of 502.31 g/mol. HAP is of hexagonal crystal structure comprising different crystal phases. The OH- ions in HAP can be replaced by different counter anions to form other members of the apatite group. HAP-nano materials added to oral cosmetic products are listed either as powder or suspension.

Physicochemical properties

HAP-nano is characterised by a specific surface area of 80 m^2/g and a Zeta potential of + 30 ± 1 mV. The material is reported to be in the form of nanorod shape only forming agglomerates with particle size > 100 nm; however according to TEM nanorod shaped entities with size below 100 nm are observable. Needle-shape HAP-nano is not supported by the Notifiers. Therefore, this Opinion will only assess the safety of rod-shaped HAP-nano.

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).

Reported shelf lives of HAP-nano is > 18 months.

Function and uses

Hydroxyapatite as an ingredient is reported in the CosIng database without any reference to the nano form with the function of abrasive, bulking and emulsion stabilising.
HAP-nano is intended to be used in the following categories of cosmetic products:

- Oral hygiene products > Tooth care products >Toothpaste at concentrations up to 10%
- Skin products > Skin care products > Other skin care at concentrations up to 5%
- Oral hygiene products > Mouth wash / breath spray >Mouth wash at concentrations up to 0.465%

The approach followed in this Opinion to assess the safety of HAP-nano is based on the SCCS Note of Guidance (10th edition, 2018) and the Guidance on the Safety Assessment of Nanomaterials in Cosmetics. As a first step, systemic exposure of the HAP-nano has been explored and as significant systemic exposure to the HAP-nano could be excluded, only local toxicity and the genotoxicity of the nanoforms have been assessed. This assessment is described below.

Exposure

As the nanoXIM® ingredient is only intended to be used in oral cosmetic products (toothpastes, mouthwashes…), only exposure *via* oral route has to be considered. After entering into the mouth, part of the cosmetic formulation will enter into contact with the buccal mucosa and part may be ingested. Therefore systemic exposure to the HAP-nano may either occur either *via* uptake by mucosal cells or by crossing the intestinal tract. Both routes have been assessed by the Notifiers.

Penetration into buccal mucosal cells

As a preliminary step to investigate whether HAP-nano can enter systemic tissues through the oral epithelium, it was histologically studied to what extent HAP-nano could penetrate the stratified layers in two types of three-dimensional (3-D) reconstituted human oral epithelial models, one with and one without a *stratum corneum*. The results showed that the NPs did not penetrate the *stratum corneum* in SkinEthic HGE samples and penetrated only the outermost layer of cells in SkinEthic HOE samples without *stratum corneum*, and no permeation into the deeper layers of the epithelium in either tissue model was observed.

Absorption by gastric compartment

The stability of nanoXIM.CarePaste HAP-nano was assessed in a stability study in simulated gastric fluid (SGF) by determination of calcium content at different time points (7.5, 15 and 30 mins). The results have confirmed that the material would solubilise in the gastric fluid if ingested. Therefore, there should not be any issue of nano-related concerns over its safety following ingestion.

As it was concluded that systemic exposure to HAP-nano following cosmetic use in oral care products was not significant, only local toxicity and genotoxicity have to be assessed.

Toxicological Evaluation

Local toxicity

To determine the biocompatibility / oral irritation test on human oral epithelium of HAP-nano, an in vitro model of reconstructed human oral epithelium was used after exposure to nanoXIM nanoparticles (SkinEthic reconstructed Human Oral Epithelium). Most probably, it was a non-keratinizing model that was taken as the worst case scenario, as no toxicity was revealed using this model, one should not assume any toxic effects in a keratinized model, which has an additional protective layers of stratum corneum. After an incubation time for 48 hours, it can therefore be concluded that 3.1 % HAP- nano was not cytotoxic for the tissues after an incubation period of 48 hr.

Mutagenicity / genotoxicity

Despite SCCS requests, valid studies were not provided on mammalian gene mutation or chromosomal aberration/clastogenicity to address concerns over genotoxicity/mutagenicity of HAP-nano. The results of the provided studies are not acceptable due to reasons detailed in section 3.3.3. Therefore, the SCCS cannot exclude concerns over the genotoxic potential of HAP-nano.

4. CONCLUSION

1. *In view of the above, and taking into account the scientific data provided, does the SCCS consider the nanomaterial Hydroxyapatite safe when used in leave-on and rinse-off dermal and oral cosmetic products according to the maximum concentrations and specifications reported in the attached list, taking into account reasonably foreseeable exposure conditions?*

Having considered the data provided, and other relevant information available in scientific literature, the SCCS cannot conclude on the safety of the hydroxyapatite composed of rod–shaped nanoparticles for use in oral-care cosmetic products at the maximum concentrations and specifications given in this Opinion. This is because the available data/information is not sufficient to exclude concerns over the genotoxic potential of HAP-nano.

2. *Does the SCCS have any further scientific concerns with regard to the use of Hydroxyapatite in nano form in cosmetic products?*

Although the particle shape in the HAP-nano considered in this Opinion is reported as rod-shaped, the SCCS is aware that, depending on the manufacturing process, needle-shaped HAP nanoparticles may also be produced. The available information indicates that HAP-nano in needle-shaped form is of concern in relation to potential toxicity. Therefore, needle-shaped HAP-nano should not be used in cosmetic products.

As detailed in Annex I, the SCCS has concluded that there is a basis for concern that the use of HAP-nano in cosmetic products can pose a risk to the consumer. The SCCS will be ready to assess any evidence provided to support safe use of the materials in cosmetic products.

5. MINORITY OPINION

None.

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ANNEX I

Safety concerns for Hydroxyapatite used as cosmetic ingredient based on public information

Physicochemical properties (see section 3.1 of the Opinion)

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 properties (e.g. see www.astm.org/Standards/F1185.htm).

According to the information provided by the Applicants, 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. SCCS in the previous opinion on HAP-nano has raised concerns regarding the use of needle –shape HAP in cosmetic products. Therefore, its use in cosmetic products is not supported anymore. In the last opinion, the SCCS has only assessed the evidence for safety of rod-shaped HAP-nano.

The HAP-nano intended for use in oral care products is characterised by a specific surface area of 80 m^2/g and a Zeta potential of +30 ± 1 mV. The material is reported to be in the form of nanorod shape only in the form of 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).

The reported shelf life of HAP-nano is reported as >18 months.

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. HAP-nano is obtained continuously by wet chemical precipitation, resulting in a diluted slurry that is then concentrated to its final value of 15.5 %wt. The product is not obtained by diluting nano-powder in water. It is in the form of a slurry from the beginning of reaction to the final product concentration. All the reaction and manufacturing process is done close to room temperature and below 60 ºC. The process was, therefore, designed to produce only particles with rod shape. No needle-like nanoparticles are reported in the product that was produced in this manner.

The TEM analysis shown in reports and papers provided by the Applicant were done with

samples being prepared just by placing the HAP-nano suspension on the TEM grid and letting it dry. No calcination step of the materials was applied. More details about the NETmix technology are available at https://www.fluidinova.com/index.php/company-nano-hydroxyapatite-manufacturer-and-supplier/#technology .

Besides these, several papers by other researchers have been published which state that the HAP-nano produced following the above-described manufacturing process is characterized as nano rod-like particles. From the additional information and clarification provided, the SCCS acknowledged that the HAP-nano for which notification was sent and which is intended for these cosmetic uses are rod shaped.

Exposure (see section 3.3.1 of the Opinion)

HAP-nano is intended to be used in the following categories of cosmetic products:

- Oral hygiene products (Tooth care products, in toothpaste and in mouthwash)
- Skin products

As the nanoXIM® ingredient is only intended to be used in oral cosmetic products (toothpastes, mouthwashes…), only exposure *via* oral route has to be considered. After entering into the mouth, part of the cosmetic formulation will be in contact with the buccal mucosa and part may be ingested. Therefore systemic exposure to HAP-nano may either occur either *via* uptake by mucosal cells or by crossing the intestinal tract. Both routes have been assessed by the Applicants.

Penetration into buccal mucosal cells (see section 3.3.1.1 of the Opinion)
As a preliminary step to investigate whether HAP-nano can enter systemic tissues through the oral epithelium, it was histologically studied to what extent HAP-nano could penetrate the stratified layers in two types of three-dimensional (3-D) reconstituted human oral epithelial models, one with and one without a *stratum corneum*. The results showed that the NPs did not penetrate the *stratum corneum* in SkinEthic HGE samples and penetrated only the outermost layer of cells in SkinEthic HOE samples without *stratum corneum*, and no permeation into the deeper layers of the epithelium in either tissue model was observed.

Absorption by gastric compartment (see section 3.3.1.2 of the Opinion)
The stability of nanoXIM.CarePaste HAP-nano was assessed in a stability study in simulated gastric fluid (SGF) by determination of calcium content at different time points (7.5, 15 and 30 mins). The results have confirmed that the material would solubilise in the gastric fluid if ingested. Therefore, there should not be any nano-related concerns over its safety following ingestion.

HAP-nano is also used as a drug delivery material and as a bone defect filling material.

Hazard

Hardly any of the toxicological studies provided were 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 by SCCS 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 HAP-nano 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 HAP-nano would manifest in adverse effects as indicated in the scientific literature (e.g. expressed in Fox et al., 2012).

Acute toxicity

The acute toxicity of HAP-nano 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.

Sensitisation

No guideline-compliant study for skin sensitisation was provided. 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.

Local Toxicity (see section 3.3.2 of the Opinion)

In general, the cytotoxicity study, although with some limitations, has shown negative results. TEM analysis of the HAP-nano is only partially acceptable. Internalisation by the cells is documented by TEM but only in the outer layers. These findings suggest that HAP-nano at the concentration used are unlikely to enter systemic tissues *via* intact oral epithelium.

Genotoxicity (see section 3.3.3 of the Opinion)

For a genotoxic substance, adverse effects may occur even at very low doses. Such effects may also occur at local level, which is especially important for the oral and oesophageal mucosa considering the intended use of the HAP-nano in oral-care products.

Therefore, even if a significant systemic exposure is not reported, evidence from properly conducted studies on genotoxicity is required to exclude genotoxic potential of HAP-nano.

Although only rod-shaped (not needle-shaped) HAP-nano are intended for use in the current submission, the SCCS could still not conclude on the genotoxicity of the nanoXIM•CarePaste from the data provided.
Indeed, despite SCCS requests, valid studies were not provided on mammalian gene mutation or chromosomal aberration/clastogenicity to address concerns over genotoxicity/mutagenicity of HAP-nano. The results of the provided studies are not acceptable due to reasons detailed in section 3.3.3. of the Opinion. Therefore, the SCCS cannot exclude concerns over the genotoxic potential of HAP-nano.

Repeated 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 HAP-nano 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.

A 7-day repeated dose intravenous study using two types of rod-shaped HAP-nano 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.

Carcinogenicity

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

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

Overall concern on hazardous properties

Based on the available data, the SCCS concludes that HAP-nano under the conditions of uses in cosmetic products would not have:

- any significant systemic exposure *via* the oral mucosa
- any significant systemic exposure *via* ingestion (due to solubility in gastric fluid)
- any cytotoxicity at the level of the oral epithelium after 48h exposure

However, the SCCS still has concerns about possible genotoxicity and, based on the provided data, cannot exclude the genotoxicity potential of the HAP-nano. More data according to the SCCS Guidance on the Safety Assessment of Nanomaterials (SCCS/1611/19) are therefore required to conclusively exclude the genotoxicity potential of HAP-nano. The SCCS will be ready to assess any evidence provided in this regard to support the safe use of HAP-nano in cosmetic products.