Scientific Committee on Consumer Safety

SCCS

OPINION ON

Hydroxyapatite (nano)

The SCCS adopted this document
during the plenary meeting on 21-22 March 2023

ACKNOWLEDGMENTS

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This opinion was subject to a commenting period of seven weeks after its initial publication (from 11 January to 1 March 2023). There were no comments received, therefore the final opinion was not changed comparing to its preliminary version.

Keywords: SCCS, scientific opinion, Hydroxyapatite, HAP, nano, CAS/EC No.: 1306-06-5/215-145-7, Regulation 1223/2009

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on Hydroxyapatite (nano), preliminary version 4 January 2023, final version 21-22 March 2023, SCCS/1648/22

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 hydroxyapatite (nano) safe when used in oral cosmetic products according to the maximum concentrations and specifications as reported in the submission, taking into account reasonably foreseeable exposure conditions?

   Based on the data provided, the SCCS considers hydroxyapatite (nano) safe when used at concentrations up to 10% in toothpaste, and up to 0.465% in mouthwash.

   This safety evaluation only applies to the hydroxyapatite (nano) with the following characteristics:
   - composed of rod-shaped particles of which at least 95.8% (in particle number) have an aspect ratio less than 3, and the remaining 4.2% have an aspect ratio not exceeding 4.9;
   - the particles are not coated or surface modified.

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

   This Opinion is not applicable to hydroxyapatite (nano) composed of needle-shaped particles.

   Although the use of hydroxyapatite (nano) is indicated also for breath spray, no data were provided to allow assessment of consumer safety from inhalation exposure. Therefore, this Opinion is not applicable to sprayable products that might lead to exposure of the consumer's lungs to nanoparticles by inhalation.

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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.).

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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.

The nanomaterials definition covers materials in the nano-scale that are intentionally made and are insoluble/partially-soluble or biopersistent. It does not cover those that are soluble or degradable/non-persistent in biological systems. Article 16 of the Cosmetics Regulation requires cosmetic products containing nanomaterials other than colourants, 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, the Commission shall refer it to the Scientific Committee on Consumer Safety (SCCS) for a full risk assessment.

The Commission services received a number of 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. 215-145-7) in nano form. Hydroxyapatite 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.

In view of potential concerns to human safety, the Commission services mandated the SCCS on the safety of Hydroxyapatite (nano). In October 2015 and in December 2021, the SCCS, having considered the data submitted via the CPNP, additional data requested from the Responsible Persons and other relevant information available in scientific literature, could not conclude on the safety of the Hydroxyapatite (nano) composed of rod–shaped nanoparticles for use in oral cosmetic products at the maximum concentrations and specifications reported. Furthermore, the SCCS stressed that the available data/information was not sufficient to exclude concerns over the genotoxic potential of Hydroxyapatite (nano).

During 2022, industry submitted additional information to support the safety of Hydroxyapatite (nano) in oral products, specifically addressing the potential genotoxicity of Hydroxyapatite (nano).

Terms of reference

1. In view of the above, and taking into account the scientific data provided, does the SCCS consider Hydroxyapatite (nano) safe when used in oral cosmetic products according to the maximum concentrations and specifications as reported in the submission, taking into account reasonably foreseeable exposure conditions?

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

3. OPINION

Preamble

In its previous Opinion (SCCS/1624/20), the SCCS concluded that valid studies were not provided on mammalian gene mutation and/or chromosomal aberration/clastogenicity to address concerns over genotoxicity/mutagenicity of hydroxyapatite (nano) (HAP-nano). The results of the provided studies were not acceptable due to many limitations detailed in section 3.3.3 of that Opinion. Therefore, the SCCS could not exclude concerns over the genotoxic potential of HAP-nano.

HAP-nano is not likely to have any significant systemic exposure and therefore, systemic toxicity is not expected (SCCS/1624/20). As new data on genotoxicity have been provided, this opinion is focused on the toxicological evaluation regarding genotoxicity.

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)

Ref.: SCCS/1624/20 Final Opinion

3.1.1.2 Chemical names

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

Ref.: SCCS/1624/20 Final Opinion

3.1.1.3 Trade names and abbreviations

nanoXIM•CarePaste

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

Ref.: SCCS/1624/20 Final Opinion

3.1.1.5 Structural formula

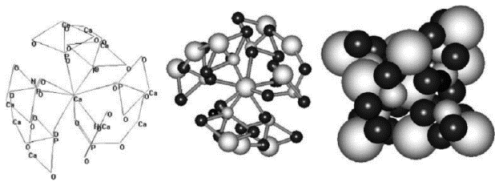

Figure 1: Spatial model of the hydroxyapatite molecule with calcium as the central atom (Ca, big white; O, small black; P, small white; H, smallest black).

Ref.: SCCS/1624/20 Final Opinion

3.1.1.6 Empirical formula

Formula: Ca10 (PO4)6 (OH)2

Ref.: SCCS/1624/20 Final Opinion

3.1.2 Physical form

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3.1.3 Molecular weight

Molecular weight: 1004.6 g/mol

Ref.: SCCS/1624/20 Final Opinion

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%

Ref.: SCCS/1624/20 Final Opinion

SCCS comment
Hydroxyapatite (nano) is fully synthetic and inorganic in nature.

3.1.5 Impurities / accompanying contaminants

From Ref.: SCCS/1624/20 Final Opinion

According to the information provided by the Notifier, 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

Ref.: SCCS/1624/20 Final Opinion

3.1.6 Solubility

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

Ref.: SCCS/1624/20 Final Opinion

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 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.

Ref.: SCCS/1624/20 Final Opinion

3.1.9 Particle size

Pristine HAP
The pristine material (i.e., nanoXIM•CarePaste dispersed in water) was analysed by transmission electron microscopy energy-dispersive X-ray spectroscopy (TEM-EDX) to characterise the hydroxyapatite (nano) primary particles and their aggregation /agglomeration state. The stability of suspension was assessed by DLS analysis.

Before determining particle size distribution, a sonication test was performed to obtain non-agglomerated/non-aggregated particles. No improvements with respect to the un-sonicated material were observed. Indeed, particles in the sonicated suspension were equally agglomerated but less distinguishable due to the matrix.

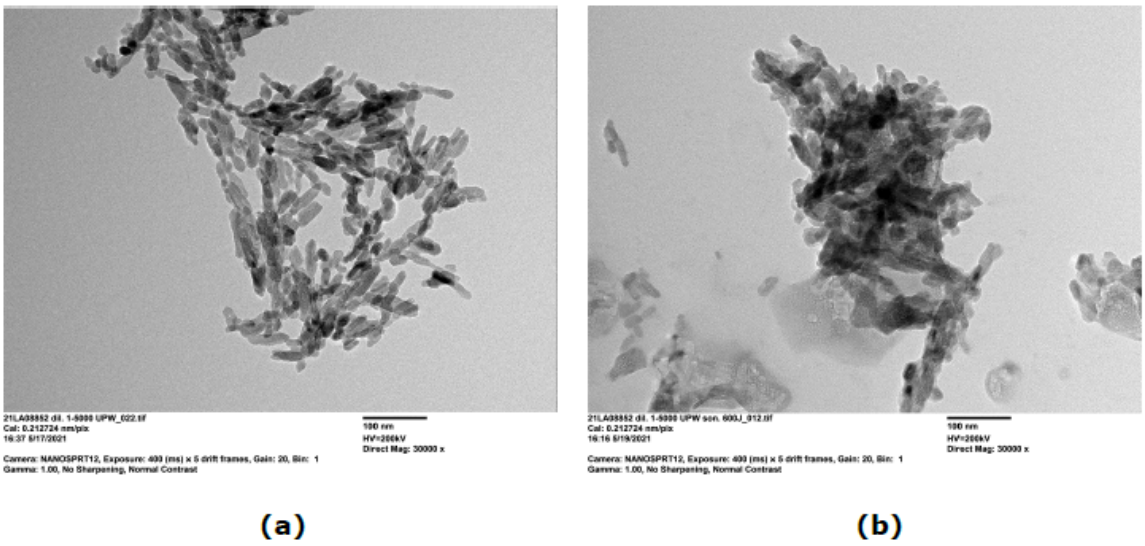


Figure 2: Comparison between TEM micrographs of (a) an un-sonicated and (b) a sonicated suspension of hydroxyapatite (nano) at 600 J/mL energy density (scale bar: 100nm).

Figure 3 presents some representative micrographs of nanoXIM•CarePaste (i.e., pristine product) diluted in ultrapure water to a final concentration of 31.4 ng/mL of hydroxyapatite (nano) (dilution factor: 5000x). Primary particles are rod-shaped with rounded edges and they form ellipsoidal/spheroidal aggregates/agglomerates made of tens to hundreds of particles. EDX spectrum in Figure 3d confirms the constitutive elements of hydroxyapatite, calcium (Ca), phosphorous (P) and oxygen (O). To define size distribution of primary particles, both length and width were independently measured.

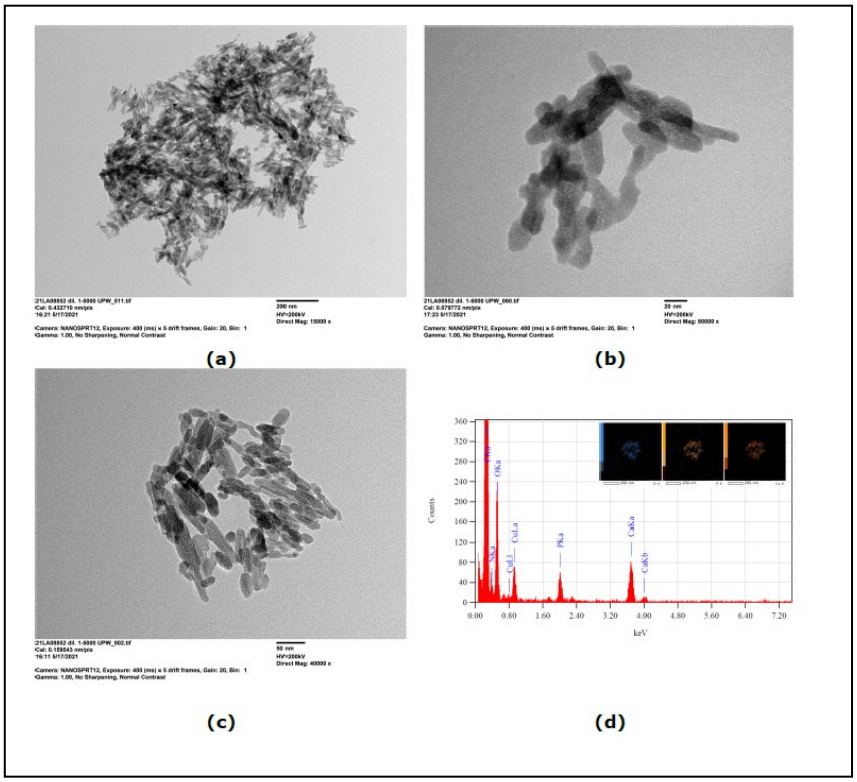
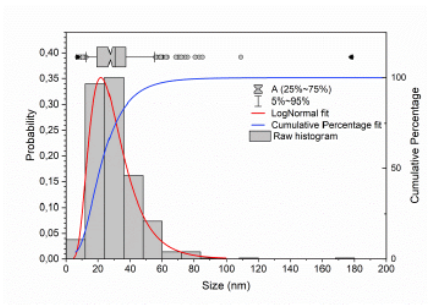


Figure 3 a-d: TEM number-based size distribution of hydroxyapatite (nano) primary particles in nanoXIM•CarePaste diluted 1:5000 in ultrapure water. TEM micrographs at magnitude (a) 15.000, (b) 80.000 and (c) 40.000. (d) EDX spectrum of particles in Figure (c).

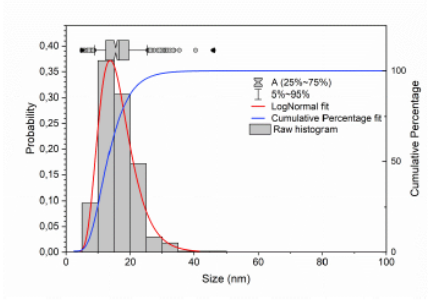
The two independent distribution curves are reported in Figure 3e and Figure 3g, while their descriptive parameters are reported in Figure 3f and Figure 3h, respectively. The median particle length and width obtained on a significant number of measured particles (≥ 500 particles) are 27.6 nm and 15.4 nm, respectively.



(e)

Dimensional Parameters - Length	Value
Analyzed particles	500
Minimum size (nm)	7.22 ± 0.89
First quartile (nm)	19.3 ± 2.1
Median (nm)	27.6 ± 3.1
MAD (nm)	8.82 ± 0.85
Average (nm)	30.3 ± 2.5
Standard deviation (nm)	15.6 ± 5.1
Third quartile (nm)	37.1 ± 3.4
Maximum size (nm)	178 ± 62
D10 (nm)	14.8 ± 1.9
D50 [median] (nm)	27.6 ± 3.1
D90 (nm)	48.9 ± 4.6

(f)



(g)

Dimensional Parameters - Width	Value
Analyzed particles	500
Minimum size (nm)	5.01 ± 0.62
First quartile (nm)	12.4 ± 1.9
Median (nm)	15.4 ± 2.8
MAD (nm)	3.66 ± 0.84
Average (nm)	16.3 ± 2.2
Standard deviation (nm)	5.6 ± 1.8
Third quartile (nm)	19.5. ± 3.1
Maximum size (nm)	46 ± 16
D10 (nm)	10.1 ± 1.3
D50 [median] (nm)	15.4 ± 2.8
D90 (nm)	23.2 ± 3.5

(h)

Figure 3 e-h: TEM number-based size distribution of hydroxyapatite (nano) primary particles in nanoXIM•CarePaste diluted 1:5000 in ultrapure water. (e) Number-based particle size distribution of particles length and (f) its descriptive dimensional parameters. (g) Number-based particle size distribution of particles width and (h) its descriptive dimensional parameters. Parameters are reported as value expanded uncertainty. The uncertainty is expressed with two significant figures and decimal places of the values are reported accordingly.

Ref.: Benetti, F. 2021. Report 21LA08852
Benetti, F. 2022a. Report 21LA08852/DLS_MN,
Benetti, F. 2022b, Report 21LA08852/MLA

According to ECHA guidance (ECHA, 2022), the aspect ratio (AR) of each individual particle was calculated as length to width ratio. Figure 4 reports an example of the segments used to determine length and width of particles.

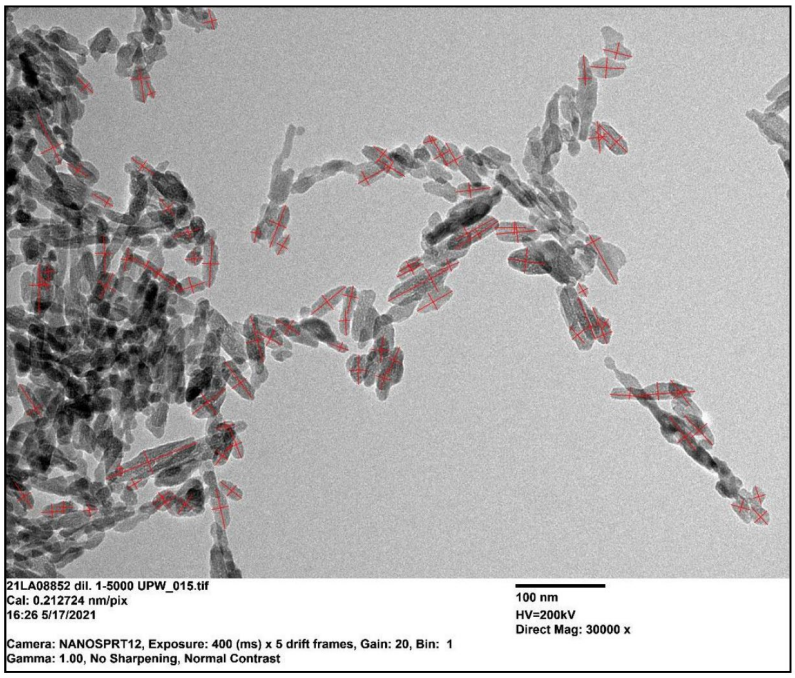
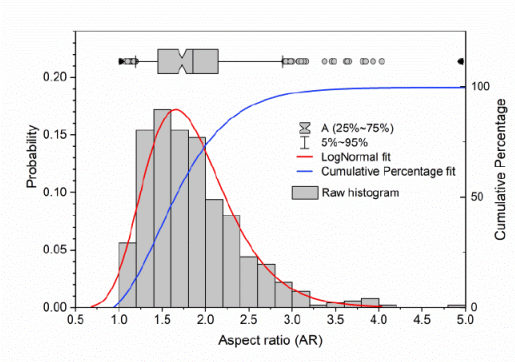


Figure 4: Example of manual size measurements on TEM-EDX micrographs. Red lines represent segments used to obtain length and width and calculate the Aspect Ratio (AR) of particles.

The descriptive parameters of particle AR distribution were produced and reported as minimum, first quartile, median, median absolute deviation (MAD), mean, standard deviation, third quartile, maximum and 10th, 50th and 90th percentiles. The 10^{th}, 50^{th} and 90^{th} percentile values correspond to the minimum threshold below which 10%, 50% and 90% of the particles in the distribution have an aspect ratio smaller than these values, respectively. Therefore the 50^{th} percentile corresponds to the median value of the aspect ratio

Figure 5 reports graphical representation and descriptive parameters of AR distribution calculated for individual particles of hydroxyapatite (nano). In the analysed particles, AR ranges from 1.0 (lower limit) to 4.9 (upper limit) with a median (i.e., 50^{th} percentile) ± MAD of 1.7 ± 0.3. The average ± standard deviation is 1.9 ± 0.6. AR values corresponding to the 10^{th} and 90^{th} percentile are 1.3 and 2.6 respectively, indicating that 10% and 90% of particles have AR smaller than these values. Only 4.2% of hydroxyapatite (nano) particles in the distribution have an AR equal to or higher than 3.0. Different fitting curves were applied to data (i.e., normal, lognormal and gamma), but according to the Kolmogorov-Smirnov statistical test, lognormal fit is the best model to analyse data. The descriptive parameters for the fit are also reported in Figure 5.



(a)

AR Descriptive parameters	
Number of particles	500
Minimum	1.0
First quartile	1.5
Median	1.7
MAD	0.3
Average	1.9
Standard deviation	0.6
Third quartile	2.1
Maximum	4.9
10^{th} percentile	1.3
50^{th} percentile	1.7
90^{th} percentile	2.6
% of particles with AR ≥ 3.0	4.2 %
Fit parameters	
R^2	0.98
μ	1.77
σ	0.49

(b)

Figure 5: (a) Graphical representation of the AR distribution for hydroxyapatite (nano) particles of nanoXIM•CarePaste. (b) Descriptive parameters of the AR distribution.

SCCS comments on pristine particles

The descriptive parameters provided for the pristine materials (median length and width, min and max length, min and max width; AR average, AR median, AR 3rd quartile, AR 90% percentile, AR max) are as follows:

- Length: 27.6 ± 3.1 nm (median), 7.22 ± 0.89 nm (min.), 178 ± 62 nm (max.)
- Width: 15.4 ± 2.8 nm (median), 5.01 ± 0.62 nm (min.), 46 ± 16 nm (max.)
- Aspect Ratio (AR): 1.9 ± 0.6 (average), 1.7 ± 0.3 (median), 2.1 (AR 3rd quartile), 2.6 (AR 90% percentile), 4.9 (AR max), with 4.2 % of particles exhibiting an AR larger or equal than 3.0.

Cell culture medium for micronucleus (MN) assay

nanoXIM•CarePaste is a product made of nanoparticles composed of Ca, P and O, consistent with hydroxyapatite (nano). Median length and width of particles are 28 nm and 15 nm, respectively. Particles in nanoXIM•CarePaste were in aggregated /agglomerated form and sonication did not lead to any improvement in particle dispersibility.

A preliminary characterisation by DLS and TEM-EDX of nanoXIM•CarePaste in cell culture media used for the MN assay was performed to find a stable concentration in terms of absence of visible precipitates and reproducibility of particle size measurement. Based on that, 1 mg/mL for condition without S9 and 0.250 mg/mL for condition with S9 resulted as the lowest dilutions that gave precipitates and the genotoxicity tests were performed starting from these concentrations as requested by the OECD TG 487 (2016).

Details regarding other concentrations and the adopted test conditions are reported in the genotoxicity test report (Ref. Cassata, F. 2022b. FINAL REPORT N. 21.513299.0002).

After establishing the concentrations to test in the MN assay, the dispersion state of nanoXIM•CarePaste in cell culture medium for each tested condition during the MN assay was evaluated by DLS and TEM-EDX. Analyses were performed before and after the MN assay with the aim to observe any changes in nanoXIM•CarePaste particle size and agglomeration/aggregation state.

According to the Notifier, based on TEM results (***Table 4 - Annex:*** *Micronucleus Assay – Particle size distribution determined by TEM observations)*, nanoXIM•CarePaste preserves its primary particles size distribution and agglomeration / aggregation phenomena did not get worse during exposure.

Ref.: Benetti, F. 2022a. Report 21LA08852/DLS_MN

Cell culture medium for the mouse lymphoma assay (MLA)

A preliminary characterisation by DLS and TEM-EDX of nanoXIM•CarePaste in cell culture media used for the MLA assay was performed to find a stable concentration in terms of absence of visible precipitates and reproducibility of particle size measurement.

In the presence of MLA cell culture medium without S9, 0.125 mg/mL of hydroxyapatite (nano), suspension resulted to be the last concentration with visible precipitates sufficiently stable in terms of particle size. In the presence of MLA cell culture medium with S9, the 0.250 mg/mL of hydroxyapatite (nano), concentration was the most diluted suspension with visible precipitates, but no useful DLS data were obtained to evaluate the stability of the suspension in terms of particle size.

Therefore, for the MLA assay, these concentrations (0.125 mg/mL for condition without S9 and 0.250 mg/mL for condition with S9) were tested as the lowest dilutions that give precipitates, as stated by the OECD TG 490 (2016). Details regarding other concentrations and the adopted test conditions are reported in the genotoxicity test report (Ref. Cassata, F. 2022a.FINAL REPORT N. 21.513299.0001). After establishing the concentrations to test in the genotoxicity study, the suspension state of nanoXIM•CarePaste in cell culture medium for each tested condition during the MLA assay was evaluated by DLS and TEM-EDX. Analyses were performed before and after the MLA assay with the aim to observe any changes in nanoXIM•CarePaste particle size and agglomeration/aggregation state.

Based on TEM results (***Table 1- Annex 1:*** *MLA assay – Particle size distribution determined by TEM observations)*, nanoXIM•CarePaste preserves its primary particle size distribution and no changes in agglomeration/aggregation phenomena were observed under any of the tested conditions.

Ref.: Benetti, F. 2022b, Report 21LA08852/MLA

SCCS comment on characterization of HAP-nano in MLA and MN cell culture media

The characterisation data for the test material used for MN and MLA tests indicate that the AR90% was equal or greater than 3, which is regarded as the threshold between rod shape and fibre shape:

Under the MN test conditions:

- AR90% = 3.0: MN-(C5 24h – S9)
- AR90% = 3.1: MN-(C3 – S9); MN-(C5 + S9; MN-(C5 4h + S9); MN-(C6 4h + S9)
- AR90% = 3.2: MN-(C2 4h – S9)
- AR90% = 3.4: MN-(C3 4h –S9)
- AR90% = 3.6: MN-(C4 –S9; MN-(C4 + S9)
- AR90% = 3.7: MN-(C2 – S9); MN-(C5 – S9)

Under the MLA test conditions:

- AR90% = 3.0: MLA-(C4 + S9)
- AR90% = 3.2: MLA-(C5 + S9)
- AR90% = 3.4: MLA-(C7 – S9)
- AR90% = 3.5: MLA (C6 + S9)

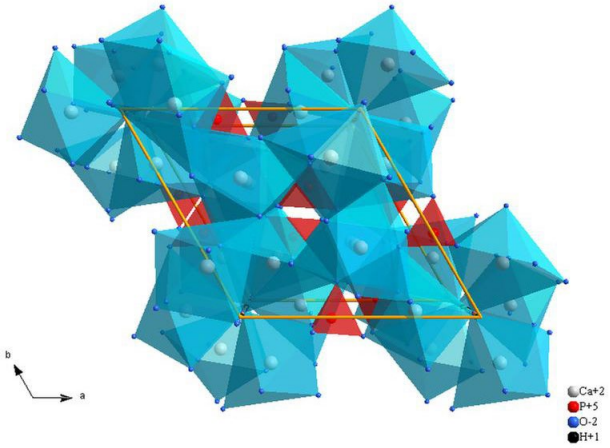
These data indicate that whilst most (90^{th} percentile) of the particles are rod shaped, a fraction of the particles can be considered to be at the borderline between rod and fibre shapes.

3.1.10 Microscopy

SCCS comment
TEM images have been provided for the test material at different concentrations (C2 – 1mg/mL, C3-0.5mg/mL, C4-0.250 mg/mL, C5 – 0.125mg/mL, C6-0.063 mg/mL, and C7 – 0.031 mg/mL), before the exposure, and after 4 and 24 hours for MN and MLA assays with or without S9.

3.1.11 Crystal structure

Structure – hexagonal, space group P63/m



Ref.: SCCS/1624/20 Final Opinion

3.1.12 UV absorption

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3.1.13 Surface characteristics

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3.1.14 Droplet size in formulations

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3.1.15 Homogeneity and stability

According to the information provided by the Notifier, 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 ensure 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.

Ref.: SCCS/1624/20 Final Opinion

3.1.16 Other parameters of characterisation – cellular uptake

Cellular uptake during Mouse Lymphoma Thymidine-Kinase (MLA) assay

Cellular uptake and internalisation of hydroxyapatite (nano) particles of nanoXIM•CarePaste during the MLA assay was investigated by TEM-EDX, analysing cells exposed to nanoXIM•CarePaste suspensions prepared in Fisher's medium with 10% heat inactivated Horse serum (F10 medium) at the concentrations reported in Analytical methods (paragraph 3), and used in the MLA assay (ref. Cassata, F. 2022a. FINAL REPORT N. 21.513299.0001). One hydroxyapatite (nano) concentration inducing visible precipitates (i.e., 0.125 mg/mL for condition without S9 and 0.250 mg/mL for condition with S9) and two lower concentrations (i.e., 0.063 mg/mL and 0.031 mg/mL for condition without S9 and 0.125 mg/mL and 0.063 mg/mL for condition with S9) were tested.
Exposed and controlled cells were detached from culture flasks by trypsinisation and washed several times. After the last washing step, samples were stained with 2% (vol/vol) uranyl acetate for 1 h and rinsed with sodium cacodylate buffer, dehydrated through an ascending acetone gradient from 30, 50, 70, 90, 100% (vol/vol), infiltrated with propylene oxide:resin mixtures and embedded in flat moulds. The resin was cured in a drying oven at 60 °C for 48 h. The samples were trimmed and absorbed onto 300-mesh copper grids. The grids were analysed with a JEOL JEM-2100 Plus TEM Microscope, working at 200 KeV, coupled with EDX for chemical identification of hydroxyapatite (nano) particles.

Hydroxyapatite (nano) particles were internalized only after 24 h exposure to a concentration of 0.063 mg/L hydroxyapatite (nano) in the absence of S9 (i.e. concentration number 6 (C6) 24 h – S9 corresponding to the first concentration without visible precipitates) and particles were localized in the cytoplasm. Representative TEM micrographs, EDX spectrum and number-based particle size distribution of the internalised particles are reported below. Even though the number of detected particles is limited (60 particles), their morphology and size are strongly comparable to primary particles in the pristine material.
For the (C6 24 h – S9) MLA assay condition, the median (Median ± MAD) and average (Average ± SD) aspect ratio values of individual internalised HAP particles detected in L5178Y/TK$^{+/-}$ cell culture medium are equal to 2.0 ± 0.4 and 2.0 ± 0.6, respectively.

Ref. Benetti, F. 2022d. Report 22LA12924/01

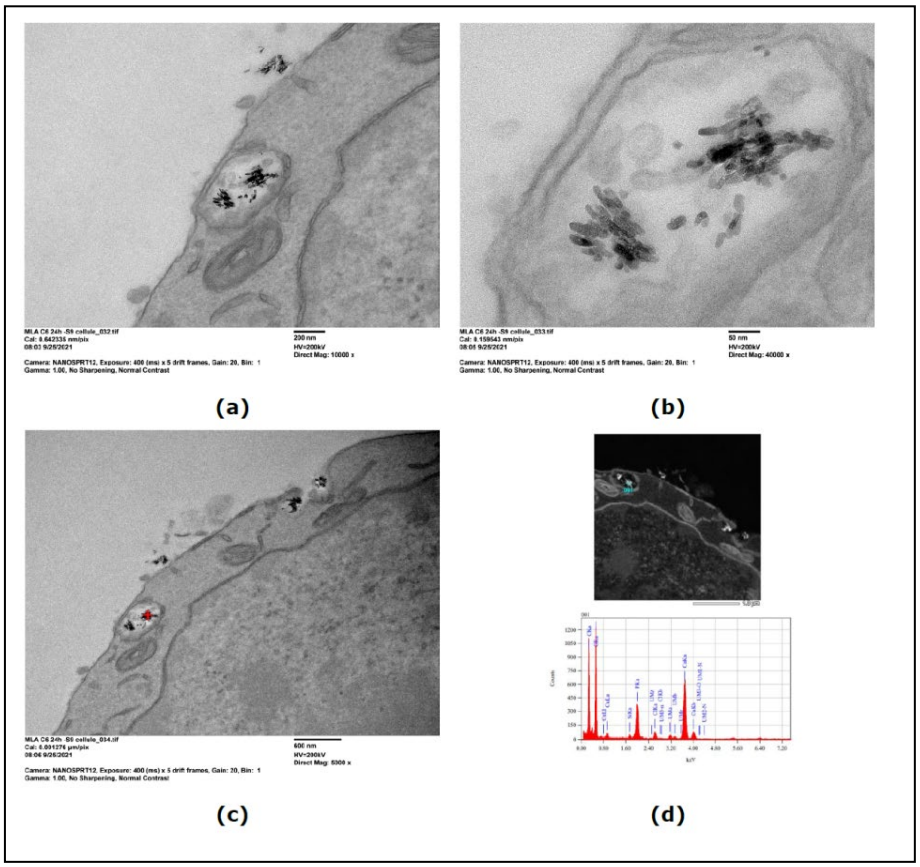
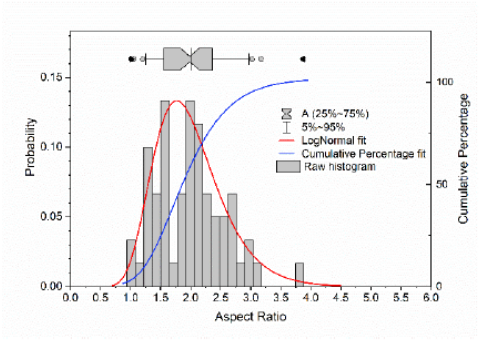


Figure 6: Representative TEM micrographs (a, b), EDX spectrum and analysed area (c, d) of hydroxyapatite (nano) particles localised in the cytoplasm. Red cross in panel (c) and light blue cross in panel (d) indicate the analysed point by EDX.

Ref.:
Benetti, F. 2022b**.** Report 21LA08852/MLA
Cassata F. 2022a. FINAL REPORT N. 21.513299.0001



(a)

AR Descriptive parameters	
Number of particles	60
Minimum	1.0
First quartile	1.6
Median	2.0
MAD	0.4
Average	2.0
Standard deviation	0.6
Third quartile	2.4
Maximum	3.9
10^{th} percentile	1.3
50^{th} percentile	2.0
90^{th} percentile	2.7
Fit parameters	
R^2	-0.16
μ	0.03
σ	0.00

(b)

Figure 7: (a) Graphical representation of the AR distribution of the internalised hydroxyapatite (nano) particles after exposure of L5178Y/TK$^{+/-}$ cells to a hydroxyapatite (nano) concentration that produces visible precipitates after 24 h exposure time without S9 (C6 24 h – S9). (b) Descriptive parameters of the AR distribution. Low values for R_2, μ and σ are due to the small number of size used to produce the distribution (due to limited number of particles detected in the cells) (from Benetti, F. 2022d**.** Report. 22LA12924/01).

The full set of data is shown in Table 2 – Annex 1.

SCCS comment

The procedure (trypsinisation) used for harvesting cells was unusual, as cells in suspension should not need trypsinisation. The SCCS has also noted that L5178Y TK$^{+/-}$ mouse lymphoma cells showed limited uptake of HAP-nano; cytoplasmic internalisation of HAP-nano was observed during the MLA assay but only under one condition – the highest concentration that did not cause precipitation (0.063 mg/mL) after 24 h exposure without S9 mix. The chemical composition of the internalised particles was determined by EDX-TEM analysis.

Cellular uptake during micronucleus (MN) assay

Cellular uptake of hydroxyapatite (nano) from nanoXIM•CarePaste was investigated by TEM and EDX-TEM. CHO K1 cells were exposed to nanoXIM•CarePaste suspensions prepared in HAM's medium enriched with 5% Foetal Bovine Serum (FBS) without or with (1%) S9 mix at the concentrations inducing visible precipitates (i.e., 1.0 mg/mL of hydroxyapatite (nano) under condition without S9 – namely C2 – S9 – and 0.25 mg/mL of hydroxyapatite (nano) with S9 - namely C4 + S9), and three lower concentrations that did not produce visible precipitates i.e., C3 – S9 and C5 + S9, corresponding to 0.5 mg/mL of hydroxyapatite (nano) under condition without S9 and 0.125 mg/mL of hydroxyapatite (nano) under condition with S9 respectively; C4 – S9 and C6 + S9, corresponding to 0.25 mg/mL of hydroxyapatite (nano) under condition without S9 and 0.063 mg/mL of hydroxyapatite (nano) under condition with S9 respectively; and C5 – S9, corresponding to 0.125 mg/mL of hydroxyapatite (nano) under condition without S9.

Exposed and control cells were detached from culture flasks by trypsinisation and washed several times. After the last washing step, samples were stained with 2% (vol/vol) uranyl acetate for 1 h and rinsed with sodium cacodylate buffer, dehydrated through an ascending acetone gradient from 30, 50, 70, 90, 100% (vol/vol), infiltrated with propylene oxide:resin

mixtures and embedded in flat moulds. The resin was cured in a drying oven at 60°C for 48 h. The samples were trimmed and absorbed onto 300-mesh copper grids. The grids were analysed with a JEOL JEM-2100 Plus TEM Microscope, working at 200 KeV, coupled with EDX for chemical identification of hydroxyapatite (nano) particles.

Regardless of the micronucleus assay conditions (i.e., 4 h – S9; 4 h + S9; 24 h – S9) and concentrations (i.e., C2, C3, C4, C5, C6 – namely 1.0 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.063 mg/mL of hydroxyapatite (nano)), cells exposed to nanoXIM•CarePaste consistently internalised hydroxyapatite (nano) particles.

Representative EDX spectra of internalized particles are reported in Figure 8. Representative TEM micrographs of CHO-K1 cells after 4 h exposure to the concentration of hydroxyapatite (nano) producing visible precipitates without S9 (C1 4 h – S9) and after 24 h exposure to the first concentration of hydroxyapatite (nano) not producing visible precipitates without S9 (C2 24 h – S9), and size distribution curves and their descriptive parameters (length, width) of the internalized hydroxyapatite (nano) are shown in Figures 9 and 10, respectively.

The full set of data related to the AR distribution of the internalised hydroxyapatite (nano) after CHO-K1 cells exposure as a function of concentration is shown in Table 5 – Annex 1.

Conclusion

In the MN assay, cytoplasmic internalisation of hydroxyapatite (nano) particles was observed in all test conditions, and the size of internalised particles was comparable with hydroxyapatite (nano) particles in pristine form. Median particle size of internalised nanoparticles ranged from 25.8 to 29.5 nm for length and from 14.1 to 15.9 nm for width.

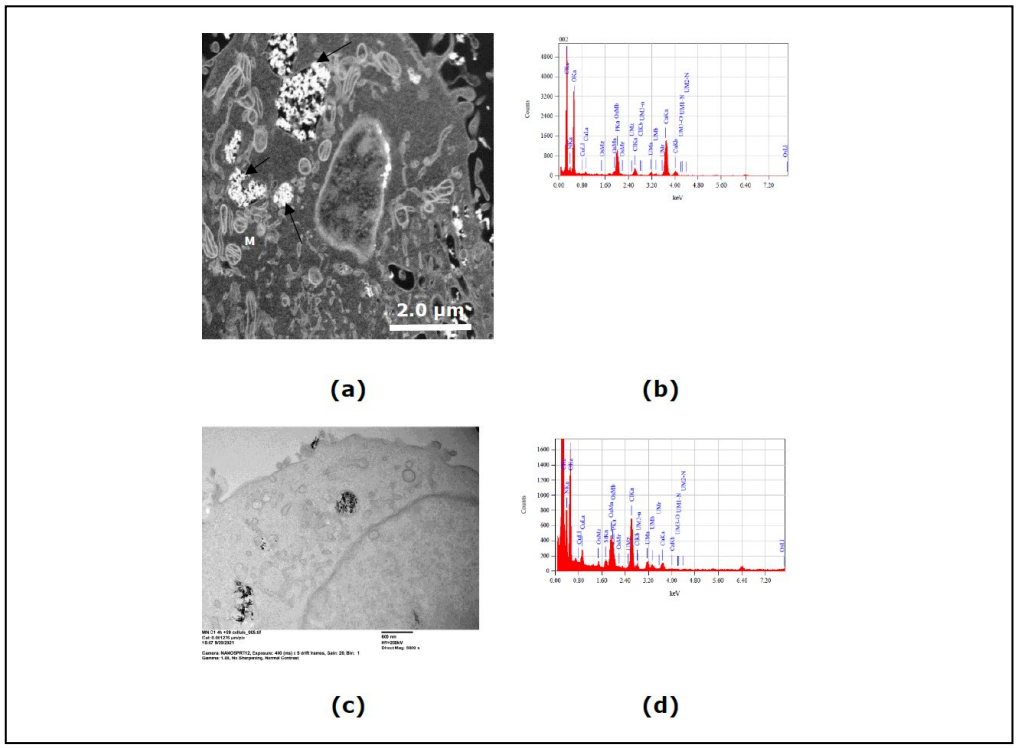
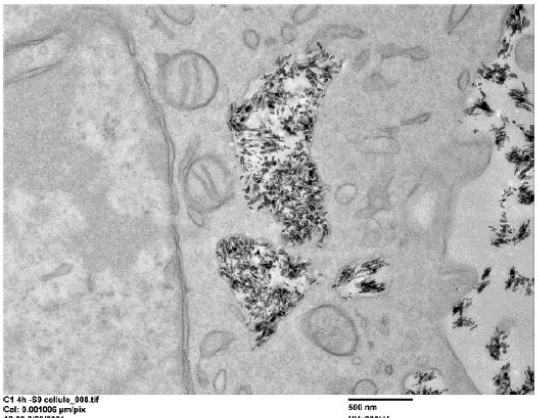
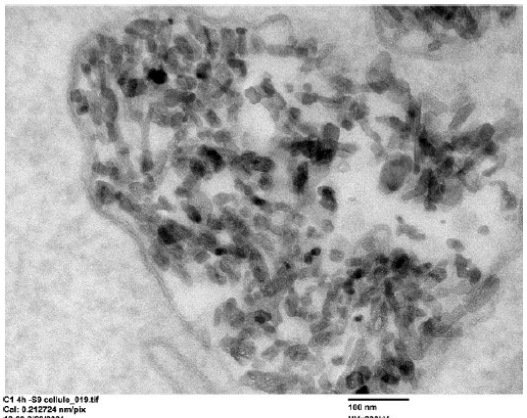


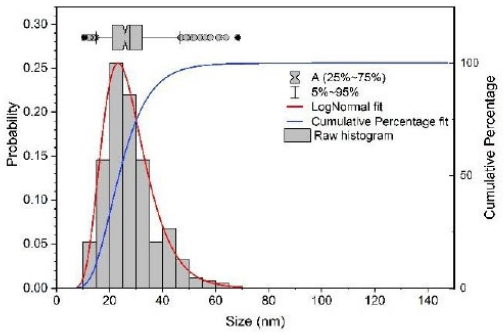
Figure 8: Representative EDX analysed areas and spectra of hydroxyapatite (nano) particles in CHO-K1 cells. Cells treated without S9: (a) Dark field shot at high contrast for better visualization of cytoplasmic localisation of particles (arrows point particles) and (b) EDX spectrum of hydroxyapatite (nano) in the cytoplasm. M = mitochondria. Cells treated with S9 mix (c) TEM micrograph with cytoplasmic internalisation of hydroxyapatite (nano) particles, and (d) EDX spectrum of hydroxyapatite (nano) in the cytoplasm.



(a)



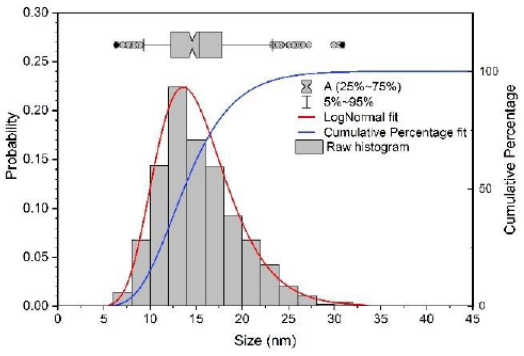
(b)



(c)

Dimensional Parameters - length	Value
Analyzed particles	500
Minimum size (nm)	10.4 ± 1.3
First quartile (nm)	20.1 ± 2.1
Median (nm)	25.9 ± 3.0
MAD (nm)	5.5 ± 0.84
Average (nm)	27.6 ± 2.5
Standard deviation (nm)	9.7 ± 3.2
Third quartile (nm)	32.2 ± 3.3
Maximum size (nm)	68 ± 24
D10 (nm)	16.9 ± 2.1
D50 [median] (nm)	25.9 ± 3.0
D90 (nm)	42.1 ± 4.3

(d)

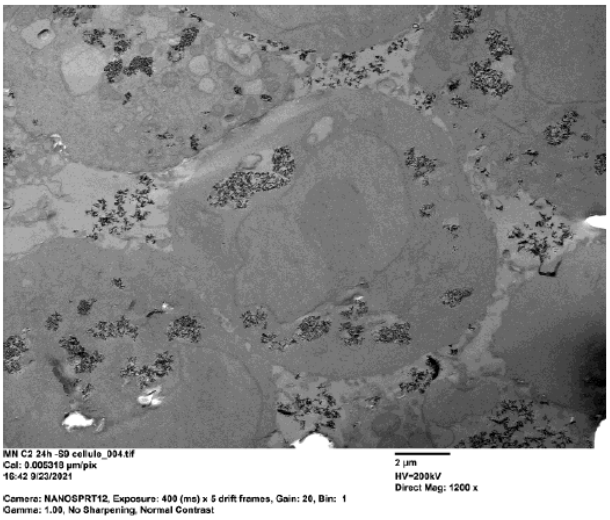


(e)

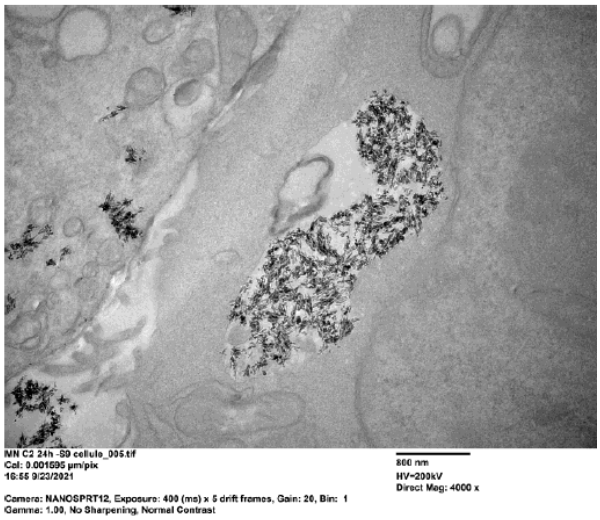
Dimensional Parameters - width	Value
Analyzed particles	500
Minimum size (nm)	6.40 ± 0.79
First quartile (nm)	12.3 ± 1.9
Median (nm)	14.6 ± 2.7
MAD (nm)	2.8 ± 0.84
Average (nm)	15.3 ± 2.2
Standard deviation (nm)	4.3 ± 1.4
Third quartile (nm)	17.8 ± 3.1
Maximum size (nm)	30.8 ± 10.8
D10 (nm)	10.5 ± 1.3
D50 [median] (nm)	14.6 ± 2.7
D90 (nm)	21.3 ± 3.4

(f)

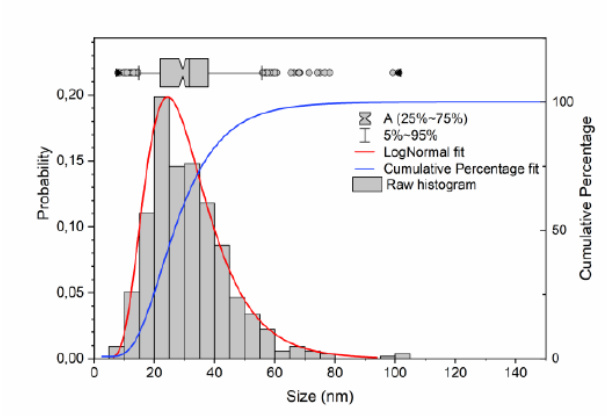
Figure 9: Representative TEM micrographs at magnification 6000x (a) and 30000x (b) of CHO-K1 cells after 4 h exposure to a concentration of hydroxyapatite (nano) producing visible precipitates without S9 (C1 4 h – S9). Size distribution curve and its descriptive parameters for length (c, d) and width (e, f) of the internalised nano-hydroxyapatite particles. Parameters are reported as value ± expanded uncertainty. The uncertainty is expressed with two significant figures and decimal places of the values are reported accordingly.



(a)



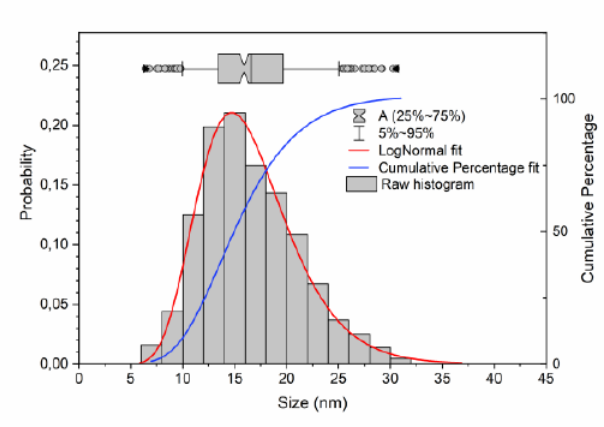
(b)



(c)

Dimensional Parameters - length	Value
Analyzed particles	534
Minimum size (nm)	7.98 ± 0.98
First quartile (nm)	21.9 ± 2.2
Median (nm)	29.4 ± 3.1
MAD (nm)	7.94 ± 0.85
Average (nm)	31.6 ± 2.6
Standard deviation (nm)	13.4 ± 4.4
Third quartile (nm)	37.9 ± 3.4
Maximum size (nm)	101 ± 35
D10 (nm)	17.1 ± 2.2
D50 [median] (nm)	29.4 ± 3.1
D90 (nm)	48.3 ± 4.6

(d)



(e)

Dimensional Parameters - width	Value
Analyzed particles	534
Minimum size (nm)	7.28 ± 0.90
First quartile (nm)	12.7 ± 1.9
Median (nm)	15.4 ± 2.8
MAD (nm)	3.06 ± 0.84
Average (nm)	16.2 ± 2.2
Standard deviation (nm)	4.8 ± 1.6
Third quartile (nm)	18.8 ± 3.1
Maximum size (nm)	40.6 ± 14.2
D10 (nm)	10.8 ± 1.4
D50 [median] (nm)	15.4 ± 2.8
D90 (nm)	22.6 ± 3.5

(f)

Figure 10: Representative TEM micrographs at magnification 1200x (a) and 4000x (b) of CHO-K1 cells after 24 h exposure to the first concentration of hydroxyapatite (nano) not producing visible precipitates without S9 (C2 24 h – S9). Size distribution curve and its descriptive parameters for length (c, d) and width (e, f) of the internalised hydroxyapatite (nano) particles. Parameters are reported as value ± expanded uncertainty. The uncertainty is expressed with two significant figures and decimal places of the values are reported accordingly.

Ref.: Benetti, F. 2022c. Report 21LA08852/MN

SCCS comment
Cytoplasmic internalisation of HAP-nano during the MN assay was studied for various concentrations after 4 h with and without S9; and after 24 h without S9. Cytoplasmic internalisations were observed at all concentrations tested including concentrations that caused visible precipitates. The chemical composition of the internalised particles was determined by EDX-TEM analysis.

Overall SCCS comment
The SCCS appreciates that a thorough study of the internalisation of HAP-nano was carried out. It was noted that there were large differences observed in uptake between lymphoblastoid (suspension) cells and adherent CHO-K1 cells.

3.2 FUNCTION AND USES

SCCS comment
From the previous Opinion (SCCS/1624/20 - Final Opinion)

The following information was provided by the Notifiers:
Hydroxyapatite as a cosmetic 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 products
- toothpaste at concentrations up to 10%
- mouthwash products at concentrations up to 0.465%

Skin products
- skin care products at concentrations up to 5%

The following information was provided in the notification files:

nanoXIM•CarePaste is intended to be incorporated in oral care products. The 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 ensure 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 Environmental Working Group's 'Skin Deep' cosmetic database, hydroxyapatite may be used as an abrasive, a bulking agent, as an oral care agent or as a stabilising emulsion.

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

3.3 SAFETY EVALUATION

From SCCS/1624/20 Final Opinion
The SCCS published an Opinion on hydroxyapatite (nano), which was adopted on 30-31 March 2021 at its plenary meeting (SCCS/1624/20 Final Opinion).

In that Opinion, the SCCS could not 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 that Opinion considering the data provided and other relevant information available in scientific literature. This was due to the fact that the available data/information was not sufficient to exclude concerns over the genotoxic potential of HAP-nano.

In that Opinion, the SCCS had concluded that needle-shaped HAP-nano should not be used in cosmetic products. For rod-shaped HAP-nano, the SCCS concluded that based on the available data, 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 had concerns about possible genotoxicity and, based on the provided data, could not exclude the genotoxicity potential of the HAP-nano.

Ref.: SCCS/1624/20 Final Opinion

As the genotoxicity potential was of concern, in this Opinion the SCCS evaluated genotoxicity of the HAP-nano based on further studies performed by the Notifier in order to address the SCCS' concerns.

3.4 TOXICOLOGICAL EVALUATION

As described above, HAP-nano is not likely to lead to any significant systemic exposure. Therefore, the toxicological evaluation is focused on genotoxicity.

The local toxicity was already addressed in the previous Opinion (see SCCS/1624/20).

3.4.1 Mutagenicity/genotoxicity

In its previous Opinion (SCCS/1624/20 Final Opinion), the SCCS concluded that valid studies were not provided on mammalian gene mutation and/or chromosomal aberration/clastogenicity to address concerns over genotoxicity/mutagenicity of HAP-nano. The results of the provided studies were not acceptable due to many limitations detailed in section 3.3.3 of that Opinion. Therefore, the SCCS could not exclude concerns over the genotoxicity potential of HAP-nano.

In the current Opinion, the SCCS evaluated genotoxicity of the HAP-nano on the basis of newly provided studies.

3.4.1.1 Mutagenicity/genotoxicity *in vitro*

Mammalian gene mutation assay (Mouse Lymphoma Assay)

Guideline:	OECD 490 (2016) plus requirements of SCCS opinion (SCCS/1624/2020) and SCCS Guidance (SCCS/1611/19)
Test system:	L5178Y $TK^{+/-}$ mouse lymphoma cells
Replicates:	Duplicates
Test substance:	nanoXIM•CarePaste
Batch (Purity):	F09-013 (HAP-nano: 15.7%; Potassium Chloride (KCl): 4.5%; water: 79.8%)
Vehicle:	F10 medium (Fisher's medium, 10% heat-inactivated Horse serum, 1 mM sodium pyruvate, 1% Pen./Strep., 0.1% Pluronic F-127)
Concentrations:	
Without S9:	0, 0.05, 0.10, 0.20, 0.399, 0.797 mg/mL (corresponding to HAP-nano: 0, 0.008, 0.016, 0.031, 0.063, 0.125 mg/mL)
With S9:	0, 0.10, 0.20, 0.399, 0.797, 1.593 mg/mL (corresponding to HAP-nano: 0, 0.016, 0.031, 0.063, 0.125, 0.250 mg/mL)
Treatment:	4 h ±S9, 24 h -S9
Expression period:	2 days
Colony counting:	After 12 days incubation in medium containing Trifluorothymidine (TFT)
Positive controls:	-S9: Methylmethanesulphonate (MMS): 4.0, 7.5 µg/mL +S9: Cyclophosphamide monohydrate (CP): 2.5, 5 µg/mL
Negative control:	Vehicle
Statistics:	One-way ANOVA and non-parametric Post Test
GLP:	Yes
Study period:	21.06.2021 – 21.02.2022

The test substance "nanoXIM•CarePaste, batch n° F09-013" was subjected to the MLA assay according to OECD 490 (2016) "In vitro Mammalian Cell Gene Mutation Tests using the Thymidine Kinase Gene" and to SCCS Guidance document (SCCS/1611/19).
The test was carried out by using L5178Y/$TK^{+/-}$ cells. Cells were exposed to five different concentrations of the test substance at three different exposure conditions: 4 hours in

presence and in absence of metabolic activation (4h ± S9) and 24 hours in absence of metabolic activation (24h - S9). At the end of the expression period, the mutagenic potential of the test substance was assessed by colony counting to determine the Mutant Frequency increase.

Stability of dispersion in culture medium by DLS was measured in each tested concentration. Uptake of nanoXIM•CarePaste by L5178Y cells was assessed by TEM.

Study design and methodology were compliant with OECD testing and reporting requirements as well as with the SCCS Notes of Guidance. No obvious deviations were evident. Osmolality and pH were monitored and were acceptable. Suspension growth (SG) and relative suspension growth (RSG%) as growth measures passed the requirements for an acceptable test. A preliminary cytotoxicity range-finding experiment to assess the cytotoxic potential of the test substance was carried out. The cloning efficiency (CE) and relative total growth (RTG%) as measures for viability and cytotoxicity passed the acceptability criteria. In principle and although acceptable, the lowest viability was observed for the positive controls irrespectively of treatment duration or presence/absence of metabolic activation. Sufficient numbers of historical control data were provided and indicated that the negative and positive controls were within the acceptable historical range of the laboratory.

Quality of cell culture (mycoplasma test, karyotype, cell viability and doubling time) was checked prior to the experiment. L5178Y $TK^{+/-}$ cells were checked for background $TK^{-/-}$ mutants in order to define the background or basal Mutant Frequency (MF), as well as for appropriate doubling time.

The nanoXIM•CarePaste product batch used contained 15.7% hydroxyapatite (nano) as the nanomaterial tested. So, to prepare a 20 mg/ml concentration of hydroxyapatite (nano), 127.4 mg of nanoXIM•CarePaste was weighted per ml of F10 medium. A stock solution of test substance was prepared by diluting 4179.1 mg in 32.8 ml of F10 medium in order to obtain the first working solution C1 20 mg/ml of hydroxyapatite (nano). Then, 8 dilutions (labelled from C2 to C9) were prepared. The highest concentration analysed was the lowest concentration producing a visible precipitate (for experiments without S9mix, 0.125mg/mL and for experiments with S9mix, 0.250mg/mL).

In each test condition, the highest test concentrations chosen led to precipitation, as requested by OECD 490 criteria. No cytotoxicity was evident at any concentration after 4 h exposure with/without S9 or 24 h without S9.

Table 4. Test concentrations in MLA assay (X = tested concentrations)

Test concentration in MLA assay						
Acronym	Initial Concentration (mg/ml)		Final Concentration (mg/ml)		Treatment without S9 mix	Treatment with S9 mix
	nanoXIM•CarePaste	nano-hydroxyapatite	nanoXIM•CarePaste	nano-hydroxyapatite		
C1	127,4	20,000	12,74	2,000		
C2	63,700	10,000	6,370	1,000		
C3	31,850	5,000	3,185	0,500		
C4	15,930	2,500	1,593	0,250		X
C5	7,970	1,250	0,797	0,125	X	X
C6	3,990	0,625	0,399	0,063	X	X
C7	2,000	0,313	0,200	0,031	X	X
C8	1,000	0,156	0,100	0,016	X	X
C9	0,500	0,078	0,050	0,008	X	

After establishing the test concentrations of the MLA, the dispersion state of nanoXIM•CarePaste in cell culture medium for each tested condition during the MLA assay was evaluated by DLS and TEM-EDX.

Cellular uptake of HAP-nano in the form of cytoplasmic internalisation was observed at 0.063 mg/mL HAP-nano after 24 h in the absence of S9.

It was demonstrated that the test item did not lead to increases in the mutant frequency at either concentration, neither in the form of incidences for small and large colonies or combined, when compared to concurrent or historical controls after 4 h ±S9 or 24 h -S9 exposures. The negative and positive controls led to the expected results and further indicated the sensitivity and validity of the test system.

Table 5. Summary of MLA assay results for treatment 4h-S9

4 h - S9									
Treatment	Mean SG	Mean RSG %	Mean $CE_{VIABILITY}$	Mean RTG %	RTG% ≥ 10%	Mean MF/10^6 cells	Mean SMF/10^6 cells	Mean LMF/10^6 cells	$MF>MF_{NEG}+126$
NEGATIVE	20,114	100,000	97,532	100,000	-	90,085	79,354	10,732	no
MMS 7,5 µg/ml	17,115	85,088	43,719	36,747	PASS	705,376	585,381	71,998	yes
MMS 4 µg/ml	20,243	100,642	54,438	56,551	PASS	423,647	359,681	36,965	yes
0,125 mg/ml (*)	25,961	129,067	47,094	62,648	PASS	107,088	85,826	10,243	no
0,063 mg/ml	27,104	134,752	65,344	90,374	PASS	91,861	91,861	15,310	no
0,031 mg/ml	26,712	132,801	44,657	60,713	PASS	134,754	112,355	22,399	no
0,016 mg/ml	25,470	126,629	63,063	81,986	PASS	71,256	55,395	15,861	no
0,008 mg/ml	29,441	146,368	49,438	74,226	PASS	70,562	50,318	20,245	no

Under the conditions of reliable test conditions, the test item was not able to induce gene mutations in L5178Y $TK^{+/-}$ mouse lymphoma cells, when tested up to precipitation concentrations, including a non-precipitating concentration leading to cellular uptake. Overall, nanoXIM•CarePaste, including its relevant constituent HAP-nano, was clearly negative and revealed no genotoxic potential *in vitro* in the MLA.

Table 6. Summary of MLA assay results for treatment 4h+S9

4 h + S9									
Treatment	Mean SG	Mean RSG %	Mean $CE_{VIABILITY}$	Mean RTG %	RTG% ≥ 10%	Mean MF/10^6 cells	Mean SMF/10^6 cells	Mean LMF/10^6 cells	$MF>MF_{NEG}+126$
NEGATIVE	18,499	100,000	73,969	100,000	-	143,606	122,867	13,565	no
CF 5 µg/ml	8,319	44,970	29,125	17,716	PASS	1578,631	1406,945	68,675	yes
CF 2,5 µg/ml	7,850	42,435	45,438	26,525	PASS	693,820	589,883	68,003	yes
0,250 mg/ml (*)	15,737	85,067	57,219	65,581	PASS	164,063	154,793	9,270	no
0,125 mg/ml	22,788	123,183	41,719	69,493	PASS	179,688	155,716	23,972	no
0,063 mg/ml	17,648	95,400	57,001	71,534	PASS	137,027	118,961	18,066	no
0,031 mg/ml	23,161	125,202	55,657	94,335	PASS	136,121	118,058	18,062	no
0,016 mg/ml	15,105	81,654	60,407	66,596	PASS	167,943	151,149	16,794	no

Table 7. Summary of MLA assay results for treatment 24h-S9

24 h - S9									
Treatment	Mean SG	Mean RSG %	Mean $CE_{VIABILITY}$	Mean RTG %	RTG% ≥ 10%	Mean MF/10^6 cells	Mean SMF/10^6 cells	Mean LMF/10^6 cells	$MF>MF_{NEG}+126$
NEGATIVE	68,262	100,000	66,844	100,000	-	67,762	52,775	14,988	no
MMS 7,5 µg/ml	27,307	40,003	18,657	11,214	PASS	1687,411	1422,110	132,651	yes
MMS 4 µg/ml	37,979	55,638	28,563	24,076	PASS	1097,643	916,618	123,431	yes
0,125 mg/ml (*)	82,959	121,530	65,282	119,776	PASS	96,949	81,216	15,733	no
0,063 mg/ml	91,326	133,788	51,282	100,005	PASS	65,097	44,945	20,152	no
0,031 mg/ml	98,734	144,640	55,751	119,261	PASS	74,277	66,123	18,118	no
0,016 mg/ml	93,718	137,292	41,407	85,147	PASS	109,157	72,522	36,635	no
0,008 mg/ml	110,293	161,573	44,344	107,286	PASS	102,322	90,778	22,564	no

Ref.: Cassata, F. 2022a, FINAL REPORT N. 21.513299.0001

SCCS comment
HAP-nano was assessed by the mammalian gene mutation test under GLP according to OECD TG 490 and following the SCCS Guidance on the safety assessment of nanomaterials in cosmetics (SCCS/1611/19). Stability of the dispersion in medium prior to and after the experiment was tested. Uptake of HAP-nano after 24 h treatment of L5178Y $TK^{+/-}$ cells was only confirmed for one concentration. The SCCS notes that very small increases in mutant frequency were observed in a few exposed samples. This was not concentration-dependent and did not reach the GEF (Global Evaluation Factor 126 x 10^{-6}) and was, therefore, considered of no biological relevance. The SCCS has therefore concluded that the test is valid and agrees with the Notifier that nanoXIM•CarePaste does not induce gene mutations in the MLA assay under the experimental conditions used.

In vitro Mammalian Cell Micronucleus Test (MNT) complex

Guideline: OECD 487 (2016) plus requirements of SCCS Opinion (SCCS/1624/2020) and SCCS Guidance (SCCS/1611/19)
Test system: Chinese hamster CHO-K1 cells (ECACC 85051005)
Replicates: Triplicates
Test substance: nanoXIM•CarePaste
Batch (Purity): F09-013 (HAP-nano: 15.7%; Potassium Chloride (KCl): 4.5%; water: 79.8%; specified by CoA)
Vehicle: F-12 HAM (HAM'S) medium enriched with 5% Foetal Bovine Serum (FBS)
Concentrations:
Without S9: 0, 0.797, 1.593, 3.185, 6.370 mg/mL (corresponding to HAP-nano: 0, 0.125, 0.250, 0.500, 1.000 mg/mL)
With S9: 0, 0.399, 0.797, 1.593 mg/mL (corresponding to HAP-nano: 0, 0.063, 0.125, 0.250 mg/mL)
Treatment: 4 h ±S9, 24 h -S9
After treatment exposure: 24 h: Cytochalasin B (cytoB): 3 µg/mL
Positive controls: -S9: Mitomycin C (MMC): 4.7 µg/mL (4 h), 0.02 µg/mL (24 h)
Colchicine: 0.5 µg/mL (4 h), 0.06 µg/mL (24 h)
+S9: Cyclophosphamide (CP) 1mg/mL (4h)

Negative control: Vehicle
Statistics: ANOVA
GLP: Yes
Study period: 16.06.2021 – 21.02.2022

NanoXIM•CarePaste (batch: F09-013, consisting of 15.7% HAP-nano, 4.5% KCl, 79.8% water) was tested for its possible potential to induce chromosomal damage *in vitro* in the mammalian cell Micronucleus test in CHO-K1 cells according to OECD 487 under GLP conditions and in line with SCCS recommendations and guidance for testing Nanomaterials (SCCS/1611/19).
All quality and acceptability criteria were met. Study design and methodology were compliant to OECD testing and reporting requirements as well as the recommended SCCS guidance. Osmolality and pH were monitored and were acceptable. The cell cultures were free of mycoplasms and more than 50% of the cells showed the karyotype with 20 chromosomes and passed the requirements for an acceptable test.

Cellular uptake of HAP-nano in the form of cytoplasmic internalisation was observed at all test concentrations.

The cytokinesis block proliferation index (CBPI) and the Cytostasis % as measures for a sufficient number of cell cycles and cytotoxicity passed the acceptability criteria.

Table 8: Test substance concentrations

	Treatment without S9 mix		Treatment with S9 mix	
	nanoXIM·CarePaste (mg/ml)	nano-hydroxyapatite (mg/ml)	nanoXIM·CarePaste (mg/ml)	nano-hydroxyapatite (mg/ml)
C1*	6.370	1.000	1.593	0.250
C2	3.185	0.500	0.797	0.125
C3	1.593	0.250	0.399	0.063
C4	0.797	0.125	0.200	0.031
C5	0.399	0.063	0.100	0.016

NOTE (*): Concentration producing precipitate.

In the preliminary cytotoxicity assay, CHO-K1 cells were exposed to 5 different concentrations of test substance, separated by a spacing factor of 1:2 (Table 8). Treatment was for 4 hours in presence (4h+S9) and absence of S9 (4h-S9) and for 24 hours in absence of S9 (24h-S9), in triplicates, respectively. At the end of the exposure, cells were treated with cytochalasin B (3 µg/ml final concentration) for 24 hours (2 cell cycles). To determine CBPI and the Cytostasis %, a total of 500 cells for each plate was observed under the microscope (40x) and the number of mononucleate, binucleate and multinucleate cells was recorded. Only cells with intact cell membrane and intact cytoplasm were evaluated.
In general, no cytotoxicity was observed, neither with any concentration of the test item nor with the positive controls. A sufficient number of historical control data were provided and indicated that the negative and positive controls were within the acceptable historical range of the laboratory. The selection of investigated test concentrations for the main MNT was based on the results of a preliminary cytotoxicity test, which itself used concentrations based on results of Dynamic light scattering (DLS and TEM). In each case, the highest test concentrations led to precipitation. No cytotoxicity was evident at any concentration after 4 h exposure with/without S9 or 24 h without S9.

The presence of micronuclei was evaluated only in binucleated cells that showed intact cell membrane and intact cytoplasm. At least 1000 cells/plate, for a total of 3000 cells per concentration tested, were scored for the evaluation of the micronuclei.

It was clearly demonstrated that the test item did not lead to increases in the incidence of micronuclei as indication for clastogenicity, when compared to concurrent or historical controls after 4 h ±S9 or 24 h -S9 exposures (Tables 9-11).
The negative and positive controls led to the expected results and further indicated the sensitivity and validity of the test system.

Table 9: Results of micronuclei counting for 4h treatment without S9 mix

Micronuclei Results 4 hours - S9									
Treatment		**Mean Cytostasis %**	**MN replica A**	**MN replica B**	**MN replica C**	**Mean MN**	**% MN**	**IF**	**Statistical evaluation**
Negative control	NEG_{medium}	/	14	14	16	14.7	1.5	-	/
Positive control	Colchicine (0.5 µg/ml)	-11.1	45	52	50	49.0	4.9	3.3	Statistically significant differences
	Mitomycin (4.7 µg/ml)	22.2	95	81	73	83.0	8.3	5.5	Statistically significant differences
Test substance nano-hydroxyapatite	1 mg/ml	18.5	20	13	18	17.0	1.7	1.1	IF > 1 No statistically significant differences (Anova test)
	0.500 mg/ml	11.1	17	18	15	16.7	1.7	1.1	IF > 1 No statistically significant differences (Anova test)
	0.250 mg/ml	7.4	11	10	15	12.0	1.2	0.8	Not performed, IF < 1
	0.125 mg/ml	0.0	12	13	15	13.3	1.3	0.9	Not performed, IF < 1

Table 10: Results of micronuclei counting for 4h treatment with S9 mix

Micronuclei Results 4 hours + S9									
Treatment		**Mean Cytostasis %**	**MN replica A**	**MN replica B**	**MN replica C**	**Mean MN**	**% MN**	**IF**	**Statistical evaluation**
Negative control	$NEG_{medium+S9}$	/	16	13	17	15.3	1.5	/	/
Positive control	Cyclophosphamide (50 µg/ml)	16.7	55	69	73	65.7	6.6	4.4	Statistically significant differences
Test substance nano-hydroxyapatite	0.250 mg/ml	12.5	14	15	12	13.7	1.4	0.9	Not performed, IF < 1
	0.125 mg/ml	4.2	12	19	14	15.0	1.5	1.0	IF = 1 No statistically significant differences (Anova test)
	0.063 mg/ml	0.0	15	15	13	14.3	1.4	0.9	Not performed, IF < 1

Table 11: Results of micronuclei counting for 24h treatment without S9 mix

Micronuclei Results (Phase A) 24 hours - S9									
Treatment		Mean Cytostasis %	MN replica A	MN replica B	MN replica C	Mean MN	% MN	IF	Statistical evaluation
Negative control	NEG_{medium}	/	13	12	16	13.7	1.4	/	/
Positive control	Colchicine (0.06 µg/ml)	0.0	47	39	42	42.7	4.3	3.1	Statistically significant differences
	Mitomycin (0.2 µg/ml)	22.2	150	136	141	142.3	14.2	10.1	Statistically significant differences
Test substance nano-hydroxyapatite	1 mg/ml	18.5	8	17	12	12.3	1.2	0.9	Not performed, IF < 1
	0.500 mg/ml	18.5	10	15	14	13.0	1.3	0.9	Not performed, IF < 1
	0.250 mg/ml	7.4	13	17	18	16.0	1.6	1.1	IF > 1 No statistically significant differences (Anova test)
	0.125 mg/ml	3.7	14	15	11	13.3	1.3	0.9	Not performed, IF < 1

Conclusion:
Under the conditions of the study, the test item was not able to induce chromosomal breaks and/or gain or loss in CHO-K1 cells, when tested up to precipitation concentrations and including a non-precipitating concentration, all of them leading to cellular uptake. All acceptance criteria were met, and the negative and positive controls led to the expected results, indicating the validity of the study. Overall, nanoXIM•CarePaste, including its relevant constituent HAP-nano, was clearly negative and revealed no clastogenic potential *in vitro* in the mammalian cell Micronucleus test.

Ref.: Cassata, F. 2022b. FINAL REPORT N. 21.513299.0002

SCCS comment
HAP-nano was assessed by the micronucleus assay under GLP, according to OECD TG 487 (2016) and following the SCCS Guidance on the safety assessment of nanomaterials in cosmetics (SCCS/1611/19). Stability of the dispersion in medium prior to and after the experiment was determined. Uptake of nanoXIM•CarePaste by CHO-K1 cells was confirmed at all concentrations tested. The SCCS considers the test valid and agrees with the Notifier's conclusion that nanoXIM•CarePaste does not induce chromosome breaks and/or gain or loss under the experimental conditions used.

Overall SCCS comment on mutagenicity/genotoxicity
The Notifier used appropriate methodologies to test genotoxicity of HAP-nano according to OECD TGs, SCCS Guidance on the safety assessment of nanomaterials in cosmetics (SCCS/1611/19) and the current state-of-knowledge. The genotoxicity studies were performed under GLP and were performed along with characterisation of the test nanomaterial in cell culture medium. The uptake of HAP-nano by CHO-K1 cells was demonstrated at all tested concentrations.

Although the uptake of HAP-nano by L5178Y $TK^{+/-}$ mouse lymphoma cells was only observed at one concentration after 24 h treatment, the SCCS considers both genotoxicity studies valid.

Both the mammalian gene mutation test and the micronucleus assay were negative in the experimental conditions tested, and the SCCS is of the opinion that HAP-nano did not induce gene mutations in mammalian cells nor structural or numerical chromosomal damage when tested up to precipitation concentrations.

3.4.2 Carcinogenicity

/

3.4.3 Reproductive toxicity

/

3.4.4 Photo-induced toxicity

/

3.4.5 Human data

/

3.5 SAFETY EVALUATION (INCLUDING CALCULATION OF THE MOS)

In its previous Opinion (SCCS/1624/20) the SCCS concluded that based on the available data, 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.

As the genotoxicity potential was of concern, the SCCS evaluation in this Opinion focused on the genotoxicity of the HAP-nano. Based on the new data provided at the SCCS request, the conclusion drawn by the SCCS is that HAP-nano as specified in this Opinion does not have genotoxicity potential.

Also, as the systemic exposure could be considered only negligible, the MoS calculation was not considered relevant for this evaluation.

3.6 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 descriptive parameters of the pristine materials (median length and width, min and max length, min and max width; Aspect Ratio (AR) average, AR median, AR 3rd quartile, AR 90% percentile, AR max) are as follows:

- Length: 27.6 ± 3.1 nm (median), 7.22 ± 0.89 nm (min.), 178 ± 62 nm (max.),

- Width: 15.4 ± 2.8 nm (median), 5.01 ± 0.62 nm (min.), 46 ± 16 nm (max.)
- Aspect Ratio (AR): 1.9 ± 0.6 (average), 1.7 ± 0.3 (median), 2.1 (AR 3rd quartile), 2.6 (AR 90% percentile), 4.9 (AR max), with 4.2 % of particles exhibiting an AR larger or equal than 3.0.

The large majority of the particles are rod shape, while based on the aspect ratio (larger than 3), a minor fraction of the particles can be considered to be of fibre shape.

Function and uses

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

Oral products

- toothpaste at concentrations up to 10%
- mouthwash products at concentrations up to 0.465%

Skin products

- skin care products at concentrations up to 5%

The approach followed in this Opinion to assess the safety of HAP-nano is based on the SCCS Note of Guidance (11th revision, 2021, SCCS/1628/21) and the Guidance on the Safety Assessment of Nanomaterials in Cosmetics. In the previous Opinion (SCCS/1624/20), systemic exposure of the HAP-nano and local toxicity were explored and were both excluded. As the SCCS Opinion further concluded that the available data were not sufficient to exclude concerns over the genotoxic potential of HAP-nano, in the current Opinion the SCCS focused on the characterisation of HAP-nano in culture medium, cellular uptake and genotoxicity, based on the newly provided studies. Since no information was provided on the use of HAP-nano in breath spray, this evaluation did not include any use in a sprayable product.

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 the oral route has been considered. After entering the mouth, part of the cosmetic formulation comes in contact with the buccal mucosa and part of it may be ingested. Therefore, systemic exposure to the HAP-nano may occur either via uptake by mucosal cells or by getting into the intestinal tract. Both routes have been assessed by the Notifiers.

<u>Penetration into buccal mucosal cells (from SCCS/1624/20)</u>

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 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

<u>Absorption by gastric compartment (from SCCS/1624/20)</u>

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 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 (from SCCS/1624/20)
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-keratinising model that was taken as the worst-case scenario. As no toxicity was revealed using this model, one should not expect any toxic effects in a keratinised model that has additional protective layers of stratum corneum. Also, the data showed that 3.1% HAP-nano after an incubation period of 48 hr was not cytotoxic to the mucosal cells.

Cellular internalisation of HAP-nano
Uptake of HAP-nano by CHO-K1 and L5178Y $TK^{+/-}$ mouse lymphoma cells was tested in all experimental conditions used for the mammalian gene mutation test and the micronucleus assay. The uptake by CHO-K1 cells was demonstrated at all tested concentrations. However, the uptake of HAP-nano by L5178Y $TK^{+/-}$ mouse lymphoma cells was marginal and only observed at one concentration, the highest one tested, after 24 h treatment.

Mutagenicity / genotoxicity
The genotoxicity of HAP-nano was investigated in three endpoints of genotoxicity: gene mutations (by the mammalian gene mutation test), structural chromosome aberrations and aneuploidy (by the micronucleus assay). The genotoxicity studies were performed along with characterisation in culture media and uptake of HAP-nano by cells. Stability of the dispersion of the test nanomaterial in cell culture medium prior to and after the experiment was provided. The Notifier used appropriate methodologies according to OECD TGs, the SCCS Guidance on the safety assessment of nanomaterials in cosmetics (SCCS/1611/19) guidance and the current state-of-knowledge.

The results showed that HAP-nano did not induce gene mutation on the mammalian gene mutation test using the thymidine kinase gene in L5178Y/$TK^{+/-}$ cell line, and did not induce structural or numerical chromosomal damage in CHO-K1 cells when tested up to precipitation concentrations. Based on these valid *in vitro* study results on gene mutations and micronucleus tests, the SCCS is of the opinion that HAP-nano does not pose a genotoxicity hazard.

4. CONCLUSION

3. In view of the above, and taking into account the scientific data provided and reasonably foreseeable exposure conditions, does the SCCS consider hydroxyapatite (nano) safe when used in oral cosmetic products according to the maximum concentrations and specifications as reported in the submission?

   Based on the data provided, the SCCS considers hydroxyapatite (nano) safe when used at concentrations up to 10% in toothpaste, and up to 0.465% in mouthwash.

   This safety evaluation only applies to the hydroxyapatite (nano) with the following characteristics:
   - composed of rod-shaped particles of which at least 95.8% (in particle number) have an aspect ratio of less than 3, and the remaining 4.2% have an aspect ratio not exceeding 4.9;
   - the particles are not coated or surface modified.

4. Does the SCCS have any further scientific concerns with regard to the use of hydroxyapatite (nano) in oral cosmetic products?

   This Opinion is not applicable to hydroxyapatite (nano) composed of needle-shaped particles.

   Although the use of hydroxyapatite (nano) is indicated also for breath spray, no data were provided to allow assessment of consumer safety from inhalation exposure. Therefore, this Opinion is not applicable to sprayable products that might lead to exposure of the consumer's lungs to nanoparticles by inhalation.

5. MINORITY OPINION

None.

6. REFERENCES

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Annex 1: Size distribution determined by TEM observations

Table 1: MLA cell culture medium Assay – Particle size distribution determined by TEM observations: descriptive parameters (median length and width, min and max length, min and max width); Aspect Ratio* (AR) average, AR median, AR 3rd quartile, AR 90% percentile) as a function of concentration (C4-0.250 mg/mL, C5-0.125 mg/mL, C6 – 0.063 mg/mL and C7 – 0.031 mg/mL), with or without S9, before exposure, after 4 and 24 hours

Material / Condition	Length (median – nm)	Width (median - nm)	Length (min. - nm) (max. - nm)	Width (min. - nm) (max. - nm)	AR average	AR Median	AR max	AR 3rd Quartile	AR 90% percentile
Pristine	27.6 ± 3.1	15.4 ± 2.8	7.22 ± 0.89 178 ± 62	5.01 ± 0.62 46 ± 16	1.9 ± 0.6	1.7 ± 0.3	4.9	2.1	2.6 4.2% AR ≥ 3.0
MLA-(C4 + S9) MLA-C4 before exposure time with S9	27.5 ± 3.1	15.0 ± 2.7	8.2 ± 1.0 160 ± 56	4.74 ± 0.58 62 ± 22	2.0 ± 0.8	1.7 ± 0.4	7.7	2.2	3.0
MLA-(C4 4 h + S9) MLA-C4 after 4 h exposure with S9	25.2 ± 3.0	15.1 ± 2.7	7.40 ± 0.91 210 ± 73	4.57 ± 0.56 84 ± 29	1.8 ± 0.7	1.5 ± 0.3	7.3	2.0	2.7
MLA – (C5-S9) MLA-C5 before exposure time without S9	25.9 ± 3.0	13.9 ± 2.7	6.02 ± 0.74 150 ± 52	4.73 ± 0.58 70 ± 24	1.9 ± 0.8	1.8 ± 0.4	8.1	2.2	2.8
MLA (C5 4h – S9) MLA-C5 after 4 h exposure without S9	25.0 ± 3.0	13.4 ± 2.7	9.2 ± 1.1 75 ± 26	6.48 ± 0.80 32 ± 12	1.9 ± 0.6	1.8 ± 0.4	5.1	2.2	2.8
MLA-(C5 24h – S9) MLA-C5 after 24 h exposure without S9	25.8 ± 3.0	13.9 ± 2.7	4.87 ± 0.60 59 ± 20	3.88 ± 0.48 37 ± 13	1.9 ± 0.6	1.8 ± 0.4	4.0	2.2	2.6
MLA-(C5 + S9) MLA-C5 before exposure with S9	28.2 ± 3.1	15.0 ± 2.7	9.5 ± 1.2 98 ± 34	6.53 ± 0.80 62 ± 22	2.0 ± 0.8	1.9 ± 0.5	6.0	2.4	3.2
MLA-(C5 4h + S9) MLA-C5 after 4h	24.4 ± 3.0	14.6 ± 2.7	7.40 ± 0.91 131 ± 46	4.6 ± 0.56 78 ± 27	1.8 ± 0.7	1.6 ± 0.3	7.3	2.0	2.6

exposure with S9									
MLA-(C6 – S9) MLA-C6 before exposure without S9	24.2 ± 3.0	13.9 ± 2.7	6.90 ± 0.85 150 ± 52	5.71 ± 0.70 70 ± 24	1.8 ± 0.6	1.7 ± 0.4	4.8	2.2	2.6
MLA-(C6 4h – S9) MLA-C6 after 4 h exposure without S9	25.0 ± 3.0	14.1 ± 2.7	9.2 ± 1.1 75 ± 26	6.82 ± 0.84 42 ± 15	1.9 ± 0.6	1.7 ± 0.3	4.8	2.1	2.7
MLA-(C6 24h – S9) MLA-C6 after 24h exposure without S9	23.4 ± 3.0	13.0 ± 2.7	4.87 ± 0.60 94 ± 33	4.64 ± 0.57 46 ± 16	1.9 ± 0.8	1.7 ± 0.4	7.3	2.2	2.8
MLA (C6 + S9) MLA-C6 before exposure exposure with S9	29.8 ± 3.1	14.8 ± 2.7	9.2 ± 1.1 114 ± 40	4.94 ± 0.61 50 ± 17	2.2 ± 1.0	1.9 ± 0.5	9.3	2.7	3.5
MLA-(C6 4h + S9) MLA-C6 after 4 h exposure exposure with S9	25.2 ± 3.0	14.8 ± 2.7	7.70 ± 0.95 118 ± 41	5.57 ± 0.69 56 ± 20	1.8 ± 0.7	1.6 ± 0.3	6.4	2.0	2.8
MLA-(C7 – S9) MLA-C7 before exposure without S9	31.2 ± 3.1	14.4 ± 2.7	8.1 ± 1.0 140 ± 49	6.33 ± 0.78 50 ± 17	2.2 ± 0.9	2.1 ± 0.6	5.0	2.8	3.4
MLA-(C7 4h – S9) MLA-C7 after 4 h exposure exposure without S9	26.3 ± 3.0	13.7 ± 2.7	9.6 ± 1.2 106 ± 37	6.66 ± 0.82 36 ± 13	2.0 ± 0.7	1.9 ± 0.4	6.1	2.4	2.9
MLA-(C7 24h –S9) MLA-C7 after 24 h exposure exposure without S9	27.4 ± 3.1	14.6 ± 2.7	11.7 ± 1.4 103 ± 36	6.86 ± 0.84 46 ± 16	1.9 ± 0.6	1.8 ± 0.3	5.3	2.2	2.8

(*) The aspect ratio is calculated from the size distribution of particles for which both length and width were measured on the same individual particle.

Ref.
Benetti, F. 2022b. Report 21LA08852/MLA,
Benetti, F. 2022d. Report 22LA12924/01,
Benetti, F. 2022e. Report 22LA12924

Table 2: MLA assay - Cellular uptake and internalization of hydroxyapatite (nano) particles of nanoXIM•CarePaste during MLA assay - Particle Size distribution investigated by TEM-EDX analyzing cells exposed to nanoXIM•CarePaste: Descriptive parameters (median length and width, min and max length, min and max width); Aspect Ratio* (AR) average, AR median, AR 3rd quartile, AR 90% percentile) as a function of MLA assay conditions, i.e. C6 (0.063 mg/L) after 24h without S9

Material / Condition	Length (median – nm)	Width (median - nm)	Length (min. - nm) (max. - nm)	Width (min. - nm) (max. - nm)	AR average	AR Median	AR max	AR 3rd Quartile	AR 90% percentile
Pristine	27.6 ± 3.1	15.4 ± 2.8	7.22 ± 0.89 178 ± 62	5.01 ± 0.62 46 ± 16	1.9 ± 0.6	1.7 ± 0.3	4.9	2.1	2.6 4.2% AR ≥ 3.0
MLA-(C6 24h – S9) MLA-C6 after 24h exposure without S9	23.4 ± 3.0	13.0 ± 2.7	4.87 ± 0.60 94 ± 33	4.64 ± 0.57 46 ± 16	1.9 ± 0.8	1.7 ± 0.4	7.3	2.2	2.8
MLA-IP-(C6 24h –S9) Internalized Particles MLA-C6 after 24h exposure without S9	24.5 ± 3.0	12.7 ± 2.7	11.3 ± 1.4 70 ± 24	7.01 ± 0.86 20.4 ± 7.1	2.0 ± 0.6	2.0 ± 0.4	3.9	2.4	2.7

(*) The aspect ratio is calculated from the size distribution of particles for which both length and width were measured on the same individual particle.

Ref.:
Benetti, F. 2022b. Report 21LA08852/MLA,
Benetti, F. 2022d. Report 22LA12924/01,
Benetti, F. 2022e. Report 22LA12924

Table 3: From Notifier's Table 3. Summary of the median aspect ratio (± MAD) and the average (± SD) aspect ratio for individual hydroxyapatite (nano) particles detected in the L5178Y/TK$^{+/-}$ cell culture medium in all tested conditions for MLA assay. Results are comparable to those obtained for the internalized particles and to the median AR and average AR in the pristine material

Concentration	Condition	Aspect Ratio Median ± MAD	Aspect Ratio Average ± SD
Concentration that produces visible precipitates	C5 - S9	1.8 ± 0.4	1.9 ± 0.8
	C5 4 h – S9	1.8 ± 0.4	1.9 ± 0.6
	C5 24 h – S9	1.8 ± 0.4	1.9 ± 0.6
	C4 + S9	1.7 ± 0.4	2.0 ± 0.8
	C4 4 h + S9	1.5 ± 0.3	1.8 ± 0.7
First concentration that does not produce visible precipitate	C6 – S9	1.7 ± 0.4	1.8 ± 0.6
	C6 4 h – S9	1.7 ± 0.3	1.9 ± 0.6
	C6 24 h – S9	1.7 ± 0.4	1.9 ± 0.8
	C5 + S9	1.9 ± 0.5	2.0 ± 0.8
	C5 4 h + S9	1.6 ± 0.3	1.8 ± 0.7
Second concentration that does not produce visible precipitates	C7 – S9	2.1 ± 0.6	2.2 ± 0.9
	C7 4 h – S9	1.9 ± 0.4	2.0 ± 0.7
	C7 24 h – S9	1.8 ± 0.3	1.9 ± 0.6
	C6 + S9	1.9 ± 0.5	2.2 ± 1.0
	C6 4 h + S9	1.6 ± 0.3	1.8 ± 0.7
Internalized particles	C6 24 h – S9	2.0 ± 0.4	2.0 ± 0.6
Pristine material		1.7 ± 0.3	1.9 ± 0.6

(*) The aspect ratio is calculated from the size distribution of particles for which both length and width were measured on the same individual particle.

Ref.: Benetti, F. 2022d. Report 22LA12924/01

Table 4: Micronucleus Assay – Particle size distribution determined by TEM observations: Descriptive parameters size Distribution (median length and width, min and max length, min and max width); Aspect Ratio* (AR): AR average, AR median, AR 3rd quartile, AR 90% percentile) as a function of concentrations (C2 – 1mg/mL, C3-0.5mg/mL, C4-0.250 mg/mL, C5 – 0.125mg/mL, C6-0.063 mg/mL), with or without S9, before exposure, after 4 and 24 hours

Material / Condition	Length (median – nm)	Width (median - nm)	Length (min. - nm) (max. - nm)	Width (min. - nm) (max. - nm)	AR Median	AR average	AR max	AR 3rd Quartile	AR 90% percentile
Pristine	27.6 ± 3.1	15.4 ± 2.8	7.22 ± 0.89 178 ± 62	5.01 ± 0.62 46 ± 16	1.7 ± 0.3	1.9 ± 0.6	4.9	2.1	2.6 4.2% AR ≥ 3.0
MN-(C2 – S9) MN - C2- before exposure without S9	29.2 ± 3.1	13.8 ± 2.7	10.4 ± 1.3 114 ± 40	7.34 ± 0.90 30 ± 10	2.1 ± 0.5	2.4 ± 1.0	7.8	2.8	3.7
MN-(C2 4h – S9) MN - C2-4h exposure without S9	29.9 ± 3.1	14.6 ± 2.7	11.3 ± 1.4 99 ± 35	6.45 ± 0.80 37.4 ± 13.1	1.9 ± 0.4	2.2 ± 0.9	7.3	2.5	3.2
MN-(C2 24h – S9) MN-C2-24h exposure without S9	25.4 ± 3.0	11.7 ± 1.4	14.5 ± 2.7 82.8 ± 28.9	5.86 ± 0.72 46 ± 16	1.7 ± 0.3	1.8 ± 0.6	6.9	2.0	2.5
MN-(C3 – S9) MN-C3 before exposure without S9	27.7 ± 3.1	14.5 ± 2.7	8.3 ± 1.0 90 ± 31	5.6 ± 0.7 34 ± 12	1.9 ± 0.5	2.1 ± 0.7	5.3	2.5	3.1
MN-(C3 4h – S9) MN-C3 after 4 h exposure without S9	33.5 ± 3.2	16.2 ± 2.8	11.6 ± 1.4 144 ± 50	7.66 ± 0.94 52.2 ± 18.2	2.0 ± 0.5	2.3 ± 0.9	7.0	2.7	3.4
MN-(C3 24h –S9) MN-C3 after 24 h exposure without S9	23.8 ± 3.0	13.1 ± 2.7	9.4 ± 1.2 83 ± 29	4.47 ± 0.55 36 ± 13	1.7 ± 0.3	1.9 ± 0.6	6.9	2.2	2.7
MN-C4-without S9 MN cell culture medium at 0.250 mg/L without S9	24.9 ± 3.0	15.0 ± 2.7	6.47 ± 0.80 177 ± 62	5.18 ± 0.64 75 ± 26					
MN-(C4 –S9) MN-C4 before exposure without S9	29.6 ± 3.1	14.1 ± 2.7	8.9 ± 1.1 114 ± 40	6.43 ± 0.79 28.0 ± 9.8	2.1 ± 0.5	2.3 ± 1.0	7.8	2.8	3.6

MN-(C4 4h – S9) MN-C4 after 4 h exposure without S9	26.3 ± 3.0	14.5 ± 2.7	11.8 ± 1.5 106 ± 37	6.79 ± 0.84 40.7 ± 14.2	1.9 ± 0.4	2.0 ± 0.7	6.0	2.3	2.8
MN-(C4 24h –S9) MN-C4-after 24 h exposure without S9	25.5 ± 3.0	13.9 ± 2.7	7.61 ± 0.94 66.2 ± 23.1	4.47 ± 0.55 29 ± 10	1.8 ± 0.3	1.9 ± 0.6	4.6	2.1	2.6
MN-(C4 + S9) MN-C4-before exposure with S9	30.3 ± 3.1	15.3 ± 2.8	9.5 ± 1.2 119 ± 42	5.64 ± 0.70 41 ± 14	2.1 ± 0.6	2.4 ± 1.0	7.0	2.9	3.6
MN-(C4 4h + S9) MN-C4-4h exposure with S9	25.7 ± 3.0	15.4 ± 2.8	10.7 ± 1.3 70 ± 24	5.57 ± 0.69 42 ± 15	1.7 ± 0.3	1.8 ± 0.6	5.5	2.0	2.6
MN-(C5 – S9) MN-C5-before exposure without S9	30.2 ± 3.1	14.6 ± 2.7	11.9 ± 1.5 98 ± 34	7.34 ± 0.90 36 ± 13	2.1 ± 0.6	2.4 ± 1.0	9.3	2.8	3.7
MN-(C5 4h – S9) MN-C5-after 4 h exposure without S9	30.6 ± 3.1	15.8 ± 2.8	11.7 ± 1.4 144 ± 50	6.79 ± 0.84 64 ± 22	1.9 ± 0.4	2.1 ± 0.9	7.0	2.4	3.1
MN-(C5 24h – S9) MN-C5-after 24 h exposure without S9	27.1 ± 3.0	13.2 ± 2.7	7.61 ± 0.94 69 ± 24	4.47 ± 0.55 28.4 ± 9.9	1.9 ± 0.4	2.1 ± 0.7	4.6	2.4	3.0
MN-(C5 + S9) MN-C5-before exposure with S9	27.0 ± 3.0	15.2 ± 2.8	10.7 ± 1.3 119 ± 42	5.64 ± 0.70 41.3 ± 14.4	1.7 ± 0.4	2.0 ± 0.9	5.8	2.4	3.1
MN-(C5 4h + S9) MN-C5-after 4 h exposure with S9	26.8 ± 3.0	15.5 ± 2.8	11.2 ± 1.4 119 ± 42	5.57 ± 0.69 42 ± 14	1.8 ± 0.4	2.0 ± 0.8	5.6	2.4	3.1
MN-(C6 + S9) MN-C6-belfore exposure with S9	25.5 ± 3.0	15.7 ± 2.8	7.61 ± 0.94 66 ± 23	5.64 ± 0.70 41 ± 14	1.7 ± 0.4	1.8 ± 0.7	4.9	2.1	2.7
MN-(C6 4h + S9) MN-C6-after 4h exposure with S9	25.7 ± 3.0	15.4 ± 2.8	10.7 ± 1.3 70 ± 24	5.57 ± 0.69 42 ± 15	1.8 ± 0.4	2.0 ± 0.7	5.5	2.3	3.1

(*) The aspect ratio is calculated from the size distribution of particles for which both length and width were measured on the same individual particle.

Ref.: Benetti, F. 2022c. Report 21LA08852/MN,
Benetti, F. 2022d. Report 22LA12924/01,
Benetti F. 2022e. Report 22LA12924

Table 5: Cellular uptake and internalization of hydroxyapatite (nano) particles of nanoXIM•CarePaste during micronucleus assay (MN) - Particle size distribution determined by TEM observations: descriptive parameters (median length and width, min and max length, min and max width); Aspect Ratio* (AR) : AR average, AR median, AR 3rd quartile, AR 90% percentile) as a function of micronucleus assay conditions (i.e., 4 h – S9; 4 h + S9; 24 h – S9) and concentration (i.e., C2, C3, C4, C5, C6 – namely 1.0 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.063 mg/mL of hydroxyapatite (nano)).

Material / Condition	Length (median – nm)	Width (median - nm)	Length (min. - nm) (max. - nm)	Width (min. - nm) (max. - nm)	AR Median	AR average	AR max	AR 3rd Quartile	AR 90% percentile
Pristine	27.6 ± 3.1	15.4 ± 2.8	7.22 ± 0.89 178 ± 62	5.01 ± 0.62 46 ± 16	1.7 ± 0.3	1.9 ± 0.6	4.9	2.1	2.6 4.2% AR ≥ 3.0
MN-IP-(C2 4h –S9) Internalized Particles C2 4h – S9	25.9 ± 3.0	14.6	10.4 ± 1.3 68 ± 24	6.40 ± 0.79 30.8 ± 10.8	1.5 ± 0.2	1.6 ± 0.5	5.1	1.8	2.2
MN-IP-(C2 24h –S9) Internalized Particles C2 24h – S9	27.8 ± 3.1	15.9 ± 2.8	9.8 ± 1.2 91.0 ± 31.7	6.40 ± 0.79 30.6 ± 10.7	1.7 ± 0.3	1.8 ± 0.6	4.6	2.1	2.6
MN-IP-(C3 4h –S9) Internalized Particles C3 4h - S9	27.6 ± 3.1	15.0 ± 2.7	9.2 ± 1.1 79 ± 27	6.48 ± 0.80 35.1 ± 12.3	1.8 ± 0.4	1.9 ± 0.7	5.9	2.2	2.8
MN-IP-(C3 24h –S9) Internalized Particles C3 24h - S9	29.4 ± 3.1	15.4 ± 2.8	7.98 ± 0.98 101 ± 35	7.28 ± 0.90 40.6 ± 14.2	1.9 ± 0.4	2.0 ± 0.7	5.6	2.3	2.8
MN-IP-(C4 4h +S9) Internalized Particles C4 4h + S9	25.8 ± 3.0	15.2 ± 2.8	9.1 ± 1.1 85.7 ± 29.9	6.23 ± 0.77 38.9 ± 13.6	1.6 ± 0.3	1.8 ± 0.5	5.0	2.0	2.3
MN-IP-(C4 4h –S9) Internalized Particles C4 4h - S9	27.6 ± 3.1	15.0 ± 2.7	9.2 ± 1.1 79 ± 27	12.3 ± 1.9 35.1 ± 12.3	1.6 ± 0.3	1.8 ±0.6	5.1	2.0	2.6
MN-IP-(C4 24h –S9) Internalized Particles C4 24h - S9	29.5 ± 3.1	15.8 ± 2.8	7.98 ± 0.98 101 ± 35	5.71 ± 0.70 38 ± 13	1.8 ± 0.4	1.9 ±0.7	5.6	2.3	2.8

MN-IP-(C5 4h – S9) Internalized Particles C5 4h - S9	25.8 ± 3.0	14.3 ± 2.7	7.27 ± 0.90 87 ± 30	5.45 ± 0.67 30 ± 10	1.8 ± 0.4	1.9 ± 0.7	6.4	2.3	3.0
MN-IP-(C5 24h –S9) Internalized Particles C5 24h - S9	25.8 ± 3.0	14.5 ± 2.7	6.90 ± 0.85 90 ± 32	5.38 ± 0.66 31 ± 11	1.7 ± 0.3	1.9 ± 0.7	4.6	2.2	2.8
MN-IP-(C5 4h + S9) Internalized Particles C5 4h + S9	25.9 ± 3.1	14.1 ± 2.7	10.4 ± 1.3 100 ± 35	5.72 ± 0.70 35 ± 12	1.8 ± 0.4	1.9 ± 0.7	6.4	2.3	2.8
MN-IP-(C6 4H + S9) Internalized Particles C6 4h + S9	25.9 ± 3.1	14.6 ± 2.7	10.4 ± 1.3 68 ± 24	6.40 ± 0.79 31 11	1.7 ± 0.3	9 ± 0.6	4.9	2.1	2.7

(*) The aspect ratio is calculated from the size distribution of particles for which both length and width were measured on the same individual particle.

Ref.:
Benetti, F. 2022c. Report 21LA08852/MN,
Benetti, F. 2022d. Report 22LA12924/01,
Benetti, F. 2022e. Report 22LA12924

Table 6: From Notifier's Table 3. Summary of median and average aspect ratio* of individual hydroxyapatite (nano) particles detected in CHO-K1 cell culture medium. Table also reports median and average AR of individual hydroxyapatite (nano) particles in the pristine material

Concentration	Condition	Aspect ratio Median ± MAD	Aspect ratio Average ± SD
Concentration that	MN-C2- S9	2.1 ± 0.5	2.4 ± 1.0
produces visible	MN-C2 4 h – S9	1.9 ± 0.4	2.2 ± 0.9
precipitates	MN-C2 24 h – S9	1.7 ± 0.3	1.8 ± 0.6
	MN-C4 + S9	2.1 ± 0.6	2.4 ± 1.0
	MN-C4 4 h + S9	1.7 ± 0.3	1.8 ± 0.6
First concentration	C3 – S9	1.9 ± 0.5	2.1 ± 0.7
that does not produce visible precipitates	C3 4 h – S9	2.0 ± 0.5	2.3 ± 0.9
	C3 24 h – S9	1.7 ± 0.3	1.9 ± 0.6
	C5 + S9	1.7 ± 0.4	2.0 ± 0.9
	C5 4 h + S9	1.8 ± 0.4	2.0 ± 0.8
Second concentration that does not produce	C4 – S9	2.1 ± 0.5	2.3 ± 1.0
	C4 4 h – S9	1.9 ± 0.4	2.0 ± 0.7
visible precipitates	C4 24 h – S9	1.8 ± 0.3	1.9 ± 0.6
	C6 + S9	1.7 ± 0.4	1.8 ± 0.7
	C6 4 h + S9	1.8 ± 0.4	2.0 ± 0.7
Third concentration that does not produce visible precipitates	C5 – S9	2.1 ± 0.6	2.4 ± 1.0
	C5 4 h – S9	1.9 ± 0.4	2.1 ± 0.9
	C5 24 h – S9	1.9 ± 0.4	2.1 ± 0.7
Pristine material		1.7 ± 0.3	1.9 ± 0.6
(4.2% AR ≥ 3.0)			

(*) The aspect ratio is calculated from the size distribution of particles for which both length and width were measured on the same individual particle.

Ref:
Benetti, F. 2022d. Report 22LA12924/01,
Benetti, F. 2022e. Report 22LA12924

Table 7: From Notifier's Table 4. Summary of median and average aspect ratio of individual hydroxyapatite (nano) particles internalized and detected in CHO-K1 cells after exposure. Table also reports median and average AR of individual hydroxyapatite (nano) particles in the pristine material.

Concentration	Condition	Aspect ratio Median ± MAD	Aspect ratio Average ± SD
Concentration that produces visible precipitates	C2 4 h – S9 C2 24 h – S9 C4 4 h + S9	1.5 ± 0.2 1.7 ± 0.3 1.6 ± 0.3	1.6 ± 0.5 1.8 ± 0.6 1.8 ± 0.5
First concentration that does not produce visible precipitates	C3 4 h – S9 C3 24 h – S9 C5 4 h + S9	1.8 ± 0.4 1.9 ± 0.4 1.8 ± 0.4	1.9 ± 0.7 2.0 ± 0.7 1.9 ± 0.7
Second concentration that does not produce visible precipitates	C4 4 h – S9 C4 24 h – S9 C6 4 h + S9	1.6 ± 0.3 1.8 ± 0.4 1.7 ± 0.3	1.8 ± 0.6 1.9 ± 0.7 1.9 ± 0.6
Third concentration that does not produce visible precipitates	C5 4 h – S9 C5 24 h – S9	1.7 ± 0.4 1.7 ± 0.3	1.9 ± 0.8 1.9 ± 0.7
Pristine material	//	1.7 ± 0.3	1.9 ± 0.6

(*) The aspect ratio is calculated from the size distribution of particles for which both length and width were measured on the same individual particle.

Ref.:
Benetti, F. 2022d. Report 22LA12924/01,
Benetti, F. 2022e. Report 22LA12924