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Integrated risk assessment of a hydroxyapatite–protein-composite for use in oral care products: A weight-of-evidence case study

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ABSTRACT

Risk assessment of cosmetic ingredients represents a regulatory standard requirement in Europe and other regions. An integrated approach was designed to assess the safety of *HPC*, a particulate composite of hydroxyapatite and protein (gelatin) for use in oral care products, employing a weight-of-evidence assessment and considering specific physico-chemical properties and exposure conditions. An initial evaluation of the constituents suggested that their chemical nature does not represent a particular health hazard *per se*. Hydroxyapatite is the main component of teeth and bones in mammals; gelatin is used in food and assumed to be safe once a BSE/TSE risk has been excluded. *In vitro* screening tests were chosen to further evaluate the biocompatibility: Hen's egg test-chorioallantoic membrane (HET-CAM) to assess irritating effects towards mucous membranes; MTT cytotoxicity test with 3T3 fibroblasts; human corneal epithelial models to investigate inflammatory mediators and cytotoxicity; macrophage assays to measure cytotoxicity, inflammatory mediators and oxidative stress. Together with results from clinical studies, exposure estimates and analyses of kinetic properties, the presented information provides sound evidence to support the safe use of *HPC*. This is an example of a risk assessment for cosmetic use of small particles without the need for additional animal studies.

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1. Introduction

Sensitive teeth still present an unsolved problem in practical dentistry. It is commonly accepted that exposure of dentine to the conditions in the oral cavity is the main reason for this phenomenon. It has been reported that approximately 36% of adults in the United States and Western Europe may suffer from sensitive teeth (Addy, 2002) and it is likely that the incidence will increase because of the demographic development. The hydroxyapatite–protein-composite *HPC* represents an active ingredient in toothpaste especially suited to protect sensitive teeth. *HPC* has been demonstrated to adsorb to the surface of exposed dentine, inducing the crystallization of calcium and phosphate from the saliva to grow a thin protective layer which covers the dentin tubuli that conduct unpleasant stimuli to the nerve, thereby preventing pain induction caused by external stimuli like hot/cold or sweet/sour food and drinks (Henkel, 2005).

The natural mineralization repair mechanism of the saliva thus is used in a self-organizing process which can be referred to as neomineralization. In general, engineered hydroxyapatite materials and their composites are intended for a variety of biomedical applications, often serving as bone substitute in intraosseous implantation or as implant coating materials, including small particle sizes down to the nano range (Arts et al., 2006; Huber et al., 2007, 2006c; Palmer et al., 2008; Webster and Ahn, 2007).

According to European Cosmetics legislation (EC, 1976), a cosmetic product is only allowed to be marketed if it has been proven to be safe for the consumer. Toothpaste, like any other cosmetic product, therefore requires a thorough risk assessment which is essentially based on the safety of its ingredients. Information on the properties of the formulation (e.g. clinical data) can be included in the assessment. Legal restrictions to testing of ingredients and formulations are provided in Directive 2003/15/EC (EC, 2003), where a phasing out of animal testing is laid down (animal testing ban).

Abbreviations: ANOVA, analysis of variance; ECETOC, European Centre for Ecotoxicology and Toxicology of Chemicals; ECVAM, European Center on the Validation of Alternative Methods; EPR, electron paramagnetic resonance; GCP, Good Clinical Practice; gd, glycerol/water dispersion; HA, hydroxyapatite; *HA-FN*, hydroxyapatite-fine/dull needles; *HA-NN*, hydroxyapatite-nano, needles; *HA-NP*, hydroxyapatite-nano, plate-like; *HA-NR*, hydroxyapatite-nano, rods; HET-CAM, hen's egg test-chorioallantoic membrane; GIT, gastrointestinal tract; HCE, human corneal epithelium; *HPC*, hydroxyapatite protein composite; ICP, inductively coupled plasma; ITT, intend-to-treat; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SCCP, Scientific Committee on Consumer Products (former name of SCCS); SCCS, Scientific Committee on Consumer Safety; SGF, simulated gastric fluid; TEM, transmission electron microscopy; WoE, Weight of Evidence; WST-1, water-soluble tetrazolium salt 1; XTT, sodium-3′-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate.

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The discussion about safety concerns associated with small particles is ongoing for many decades and is to a large extent related to potential risks following inhalative exposure, e.g. (Schmid et al., 2009). Especially ultrafine or nanoparticles are in the focus of the debate, e.g. (ECETOC, 2005; Holsapple et al., 2005), usually meant to have one or more size dimensions between 0.1 and 100 nm.

Due to the diversity of particulate materials and their applications, no fixed scheme is applicable to assess the potential risk of small particles. Consequently, a specific, taylor-made risk assessment and testing strategy needed to be designed for *HPC*, consisting of several building blocks. This strategy comprises a weight-of-evidence (WoE) analysis which is often used to assess the potential hazard, including multiple elements like physicochemical characterization, available information on constituents, *in vitro* screening tests and clinical studies.

The assessment of biocompatibility is a key request in this regard. When selecting suitable test systems for tooth paste ingredients, specific attention needs to be paid to the local compatibility towards mucous membranes as the primary site of contact.

Beyond that, it needs to be investigated whether a particulate material can penetrate into the body leading to systemic exposure. In case part of the material should become systemically available, the investigation of potential interactions with cells is of particular interest, mainly the responses of macrophages as the first line of defense of the body. Cytotoxicity, inflammation and oxidative stress through reactive oxygen species (ROS) formation are considered to be relevant to the potential development of several chronic diseases (Borm et al., 2006; Brown et al., 2001; Donaldson et al., 2005; Hirvonen et al., 1996; Lewinski et al., 2008; Unfried et al., 2007) and consequently need to be included in the analysis. To finally assess the relevance of effects, information on the biodegradability of the material under consideration is also of importance.

This study aims at describing the whole process of the risk assessment of *HPC*, comprising the initial description of scientific objectives, the identification of information requirements and data gaps, the selection of suitable non-animal methods to generate missing data and the subsequent integration of all relevant information into a comprehensive assessment.

2. Materials and methods

2.1. Integrated risk assessment and weight-of-evidence (WoE) analysis

"Integrated risk assessment" is not a standardized term and is used in a number of different contexts (Bridges and Bridges, 2004). In our study it describes a qualitative, expert judgment-based integration of data from different sources to assess the safety of consumers for the intended and foreseeable use of a cosmetic ingredient.

In particular for the hazard assessment part, the risk assessment is making use of a WoE analysis, a term which has also been used in many different ways in the public literature (Weed, 2005). We refer to WoE as a systematic, expert-judgment based approach to assess available information relevant for human safety and to identify potential data gaps, triggering a tiered testing approach. Testing results in turn were included in the overall WoE analysis, and exposure estimates were performed. The process was continued until a responsible safety decision could be made based on the current knowledge (with the general option for re-evaluation as soon as new relevant knowledge becomes available).

2.2. Strategy of literature search and review

In addition to retrieval of supplier information on the constituents, a literature search on the biocompatibility and toxicological profile of gelatin and hydroxyapatite or composites thereof was performed in PubMed and other databases or networks (TOXNET (US National Library of Medicine), Ariel™/ChemEXPERT, other internet sources, in-house databases). Hits were first screened for relevance to the issue by reading the abstracts. Additional references were identified through cross-references and additional searches on specific topics. About 80 references were selected in summary for detailed review with a particular focus on papers with well-documented materials and procedures which are applicable to this assessment.

2.3. Test samples

Synthesis and characterization of *HPC* was described previously (Albrecht et al., 2009). In brief, *HPC* is synthesized by a co-precipitation of calcium chloride dihydrate, collagen (gelatin with an average molecular weight of 140,000 Da with a molecular weight distribution between approx. 6000 and >400,000 Da) and ammonium phosphate. The calcium/phosphorus ratio was analyzed with inductively coupled plasma (ICP) spectroscopy and equals to 1.95 ± 0.28. The protein content is 36.2%. Particle morphology and size were analyzed by transmission electron microscopy (TEM), showing irregular shaped particles in the micrometer range with average sizes of 1200×2100 nm; pictures shown in (Albrecht et al., 2009). The specific surface is 67 m^2/g as analyzed by the Brunauer–Emmet–Teller (BET) method. For formulation into cosmetic products, *HPC* (9.5%) is provided in a preserved dispersion of glycerol (15%) and water (73%). In order to specifically investigate the properties of *HPC*, the material was used in cellular test systems without preservative (due to the known cytotoxic effects of preservatives) and also was isolated from the sediment. The final concentration of *HPC* in a marketed toothpaste is approx. 0.1%. Trade names for *HPC* are Nanit or Nanit®active (glycerol dispersion).

Depending on the specific test system, a number of controls and reference substances were applied along with *HPC*. An overview of test samples is provided in Table 1. Pure hydroxyapatite without gelatin was produced in an analogous precipitation process as *HPC* but without the presence of gelatin (*HA-NP*). This material was used either as sediment (macrophage tests) or as 5.3% glycerol dispersion (HCE and 3T3 experiments). Two other HA materials, *HA-NR* (hydroxyapatite – nano, rods) and *HA-FN* (hydroxyapatite – fine/dull needles), were prepared with different sizes and morphologies as previously described (Albrecht et al., 2009) and included in macrophage experiments. Ostim® (Heraeus Kulzer, Hanau, Germany; abbreviated as *HA-NN* (hydroxyapatite-nano/needle-shaped) in the following), a bone grafting material approved for clinical use, was used as a benchmark control in most *in vitro* tests. The calcium/phosphate ratio of this material is 1.67, the surface area/mass is 106 $m^2\ g^{-1}$ (Huber et al., 2007).

2.4. HET-CAM (Hen's egg test-chorioallantoic membrane) to assess mucosal membrane irritation

The HET-CAM was carried out as previously described (Steiling et al., 1999) using the reaction time method for transparent and the endpoint assessment for non-transparent test items. In brief, fertilized eggs were incubated for 9 days prior to use. Six eggs were used for each test item: *HPC* (*gd*) and two dilutions thereof with 1% and 5% *HPC*, tooth gel with 1% *HPC*, standard reference toothpaste (Sensodyne Fresh Mint). Of the dispersions 300 μl were applied to the CAM. The irritation potential is evaluated by occurrence of specific effects to the membranes and/or vessels (hemorrhage (H), lysis (L), coagulation (C)) which are interpreted in comparison to 5% sodium magnesium lauryl-myristyl-6-ethoxysulphate (Texapon ASV, Cognis, Germany). This internal reference compound is

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