cosmetics

MDPI

Review

Safety Assessment of Nanomaterials in Cosmetics: Focus on Dermal and Hair Dyes Products

Sara Cabanas Coimbra [1], Inês Sousa-Oliveira [1], Inês Ferreira-Faria [1], Diana Peixoto [1,2], Miguel Pereira-Silva [1,2], Ankita Mathur [3], Kiran D. Pawar [4], Faisal Raza [5], Priscila Gava Mazzola [6], Filipa Mascarenhas-Melo [1,2,*], Francisco Veiga [1,2] and Ana Cláudia Paiva-Santos [1,2,*]

1 Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra, 3000-548 Coimbra, Portugal
2 REQUIMTE/LAQV, Group of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra, 3000-548 Coimbra, Portugal
3 Research and Development Department, Abode Biotech India Pvt. Limited, MLA Colony, Banjara Hills, Hyderabad 500033, India
4 School of Nanoscience and Biotechnology, Shivaji University, Kolhapur 416004, India
5 School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, China
6 Faculty of Pharmaceutical Sciences, University of Campinas, Campinas 13083-871, Brazil
* Correspondence: filipamelo@ff.uc.pt (F.M.-M.); acsantos@ff.uc.pt (A.C.P.-S.); Tel.: +351-239-488-400 (F.M.-M. & A.C.P.-S.)

Abstract: Nanomaterials use in cosmetics is markedly enhancing, so their exposure and toxicity are important parameters to consider for their risk assessment. This review article provides an overview of the active cosmetic ingredients used for cosmetic application, including dermal cosmetics and also hair dye cosmetics, as well as their safety assessment, enriched with a compilation of the safety assessment tests available to evaluate the different types of toxicity. In fact, despite the increase in research and the number of papers published in the field of nanotechnology, the related safety assessment is still insufficient. To elucidate the possible effects that nanosized particles can have on living systems, more studies reproducing similar conditions to what happens in vivo should be conducted, particularly considering the complex interactions of the biological systems and active cosmetic ingredients to achieve newer, safer, and more efficient nanomaterials. Toward this end, ecological issues and the toxicological pattern should also be a study target.

Keywords: nanomaterial; safety assessment test; toxicology; genotoxicity; hair dye cosmetic

Citation: Coimbra, S.C.; Sousa-Oliveira, I.; Ferreira-Faria, I.; Peixoto, D.; Pereira-Silva, M.; Mathur, A.; Pawar, K.D.; Raza, F.; Mazzola, P.G.; Mascarenhas-Melo, F.; et al. Safety Assessment of Nanomaterials in Cosmetics: Focus on Dermal and Hair Dyes Products. *Cosmetics* **2022**, *9*, 83. https://doi.org/10.3390/cosmetics9040083

Academic Editor: Enzo Berardesca

Received: 26 June 2022
Accepted: 2 August 2022
Published: 8 August 2022

1. Introduction

Topical delivery of active cosmetic ingredients (ACIs) can be efficiently made by nanoparticles (NPs), including the nanoencapsulated hair dyes, which are able to penetrate hair follicles to a much greater extent [1]. Due to their small size, NPs can be successful carriers of hair and skin ACIs. By searching for articles published in the field of nanotechnology it directs us to a huge number of publications already done, attesting to the importance of this topic and its excellent upcoming prospects. Nevertheless, the number of papers considering its toxicological assessment, and depicting hair dyes, is much smaller. Further elucidation is needed about the possible impact that nanoscience and its specific nanomaterials (NMs) can have on living systems. Studies that could mimic the in vivo conditions should be carried out to clarify possible toxicological outcomes of a certain cosmetic formulation containing nanostructures. Several studies can be considered and analyzed regarding the wide range of possible interactions of the nanostructures with living systems, considering the broader range of NPs developed and conceived for different applications [2–5].

New approaches to the NMs use in cosmetic products enhanced safety requirements for a cosmetic product safety report preparation. Though the total replacement of animal testing with non-animal methods recurring to cell cultures and tissues is the final ideal. The 3R strategy is already an advancement and notable progress in the refinement, reduction, and replacement concepts related to laboratory animal testing. The "safety evaluation" of an ACI can be addressed by the process of risk assessment and its principles based on four main parts. The first part is hazard identification, in which the toxicological profile of the ACI of interest is undertaken via several tests (in vivo, in vitro), clinical and epidemiological studies, as well as case reports, that provide useful results and data, dose-response assessment, and some parameters can be measured, such as no observed adverse effect level (NOAEL) and no observed effect level (NOEL), in order to study the exposure—toxic response binomial, exposure assessment and, finally, risk characterization, where a margin of safety (MoS) can be calculated. Another important and inescapable point is the exposure assessment and the calculation of important parameters, such as the systemic exposure dosage (SED)—dermal exposure. Moreover, oral and inhalation exposures if needed, and the toxicological assessment, with similar parameters, when compared with the more conventional cosmetics formulations, have particular specifications considering nano-aspects as the absence of validation of conventional testing methods of toxicological endpoints for NM's purpose and the possibility of existing surface interactions motivated by the possible high surface energy. Therefore, an accurate characterization of the NM is needed, along with its probable interaction with the surrounding environment. Toxicological measurement's metric can be different once we are dealing with nanosized structures, the absorption, distribution, metabolism, and excretion (ADME) evaluation and bioavailability (BA)/toxicokinetic may assume even special importance, once their small properties and peculiar physicochemical characteristics that may increase their ability to cross membranes and attain different regions of the body that otherwise would remain loosely accessible, which can constitute both an advantage and a disadvantage [6,7]. Agglomeration/aggregation behavior should also be thoroughly examined, and dispersibility and solubility should be tested too. The risk of an NM is evaluated on the risk assessment and is calculated by dividing NO(A)EL (or LO(A)EL) by SED, yielding the margin of safety (MoS) of the NM on a final cosmetic product [8,9].

Erythema, edema, burning, and itching sensations are common manifestations of skin irritation and may arise from the use of certain ACIs, resulting from a localized inflammatory response. Triggering of a non-immunological response to a certain ACI in a formulation is generally characterized as a skin irritation with a specific signal, ranging from edema, and itching, to burning and erythema, although it is important to state that different types of irritant dermatitis may occur, such as acute or long irritant dermatitis, irritant reaction, that is frequent among hairdressers, and cumulative irritant dermatitis. Therefore, it is important to have models to predict the possible irritation and reactions to the exposition of new ACIs of interest in cosmetic formulations, like hair dyes. According to the 9th revision of the "Notes of Guidance for Testing of ACIs and Their Safety Evaluation by the SCCS" [10], since 2009 animal testing of ACIs were prohibited by the European Cosmetic Legislation, the animal's ban legislation, and at the present the principles assent on the development of new alternative methods that can replace the animal's use. Other changes were made, such as the introduction of the concept of a "responsible person", as the one that ensures that all legislative obligations are accomplished, needed for the market placement of the cosmetic product.

2. Hair Formulations and Their Impact on Living Systems

The emerging formulations and marketing of hair dyes brought some concerns regarding the possibility of certain ACIs contained in these formulations causing deleterious health outcomes.

Of note is the case of the permanent dyes 2-nitro-p-phenylenediamine and 4-amino-2-nytrophenol, among others, that were appointed as responsible for inducing carcinogenic

activity on tested laboratory animals. They manifested the capability of being able to permeate the dermal barrier on humans, thereafter, culminating in the removal of these molecules from the definitive formulation of hair colorants by the Food and Drug Administration (FDA) and their substitution by other chemically similar molecules with safer properties [11]. p-phenylenediamine (PPD) containing cosmetic formulations have raised special concerns once it is considered to be a strong sensitizer and could initiate an allergic response, particularly by some specific oxidation products, such as p-benzoquinone diamine (BQDI), a product resulting from an oxidative transformation process of the initial PPD [12]. The complexity of the mixtures of different ACIs, with special regard not only to the more "problematic" aromatic amines but also to the huge quantity of other ACIs, leads to the necessity of further studies to fill the lack of information in this field of research. A recent study showed that the inflammatory mediator IL-1 alpha had its release augmented, suggesting skin irritation, after application of a mixture consisting of resorcinol, a coupler, PPD, an intermediate, and hydrogen peroxide, all of them commonly present on hair dye formulations. Moreover, it was verified a decrease in cell viability as well as an increase in skin cells apoptosis, epidermal damage, and impairment of the normal skin functions and properties, such as its pivotal natural barrier [13]. Studies in a dendritic cell model showed that the skin sensitizer PPD decreased membrane expression of certain receptors, more precisely chemokine receptors CCR6 and CXCR4. This way is able to impact the immune system, and dendritic cell migration and plays a key role in allergic contact dermatitis [14]. PPD allergy can also be acquired by cross-sensitization, in which other exposures for example latex products, local anesthetics such as benzocaine and procaine, other drugs such as sulfones and sulphonamides or p-aminobenzoic acid (PABA) used for example in sunscreens, are responsible for the installment of allergic symptomatology [15].

Toxicology assessment is one of the most relevant processes for the determination of deleterious health outcomes that can occur with hair dyes. Several studies are needed in order to conduct a toxicology assessment, as the exposure of the ACI to the skin could promote a possible percutaneous absorption and guide a systemic delivery and distribution of the ACI of interest, permeability, and exposition to the ACI, the period of time in which it will be contacted with the surface of the skin because the longer the time of contact, the greater could be the effects and the implications that could be triggered by the chemical, and all the formula ACIs, such as permanent dyeing ACIs long-term exposure [16]. Therefore, it is important to establish and test the ACIs by doing a safety assessment, due to their potential to interact and modify the activity and equilibrium of living systems, with some tests such as acute and sub-chronic systemic toxicity, local effects, sensitizing potential, mutagenicity, tumorigenicity, and teratogenicity. It is necessary to establish possible routes of entry and some relevant ACI's characteristics, as a screening phase, that could provide important and useful data for the following studies.

3. Physicochemical Characteristics of NPs and Their Safety Testing

As far as the safety of NMs in cosmetic products is concerned, the first note that should be taken resides in the nanoscale issue. In fact, the NM's incredibly small size can have an impact on numerous properties, when compared to its micro or normal size correspondent. A nanoscale material may not only have the physicochemical properties different, but also the biological ones, thus potentially affecting several domains such as quality, safety, effects, and activities. This way, it is comprehensible the possible lack of applicability and adequacy of the traditional testing assays normally used to test an ACI's safety [17].

NP's safety assessment is of utmost importance and despite the need for more studies and guidance to establish a definitive and robust safety assessment, the first major topic should be the assessment of its characteristics. Morphology and solubility can play a key role in immune cells' uptake, as in the case of nanofibers, and dermal permeability respectively [18,19]. Both FDA and the Scientific Committee on Consumer Safety (SCCS) have provided useful information on the specific requirements and relevant testing methodologies for safety evaluation. Physicochemical characterization is perhaps the first to consider,

allowing a thorough description of several useful information on the NMs, including its structural formula, purity degree, presence or absence of impurities, particle size measurement, aggregation, or agglomeration phenomena. Surface chemistry studies, such as zeta potential assessment and morphology parameters elucidation as shape and surface area are relevant, as well as solubility and stability. Once the chemical and physical properties have been properly evaluated, toxicological assessment is extremely important, concerning the routes of exposure, possible uptake and absorption, using a variety of in vitro or in vivo tests that best fit each case. Thereafter, FDA recommends at least, acute, repeated dose, and subcronic toxicity testing, skin and eye irritation testing, as well as genotoxicity or mutagenicity, skin sensitization, and dermal photoirritation testing [17]. Similarly, the SCCS, in the "Guidance on the safety assessment of NMs in cosmetics", emphasizes the importance of characterization to identify and entail its particular properties and to detail its toxicological profile and safety issues, building upon previously studied physicochemical peculiarities. Physicochemical characterization can be summarized, according to the previously mentioned guidance, in the following Figure 1 representation [8].

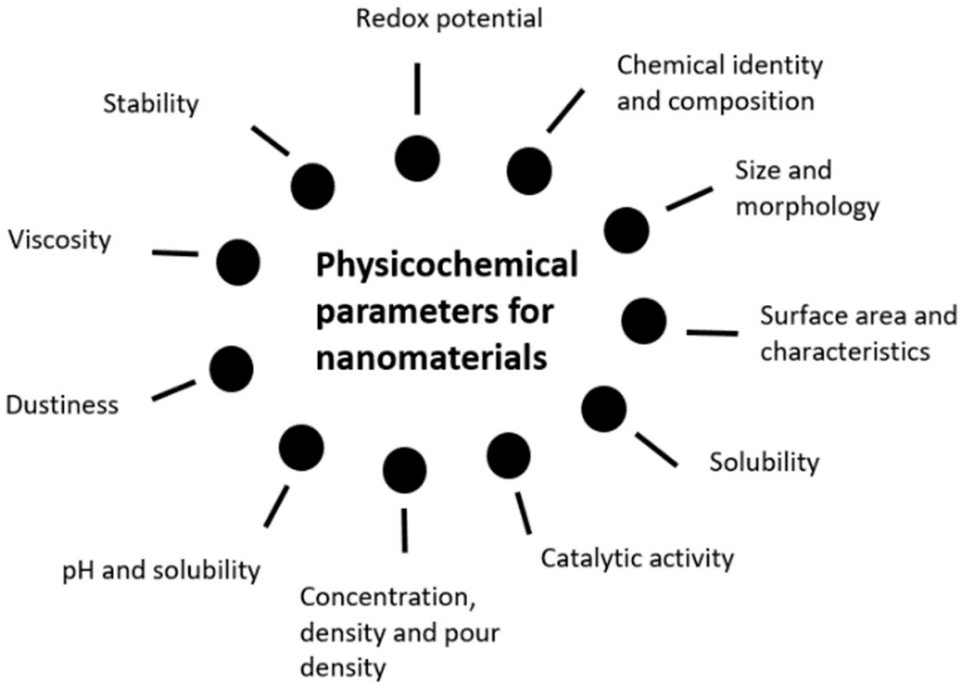

Figure 1. Physicochemical characterization for nanomaterials (NMs).

Taking on the SCCS's checklists for applicants submitting dossiers on ACIs to be evaluated by the SCCS, a final section is dedicated to addressing NMs principal assessments. Divided into three main "checklist" sections, offering a guide for the information previously mentioned and described characteristics, parameters, and general information regarding the NMs for cosmetic use, including in a cosmetic formulation, as seen summarily in Figure 2—checklist for NM's characterization, hazard assessment as toxicological data and the checklist for information on exposure, exposure assessment that is adapted from the document, noting the importance of addressing a description regarding the raw material (the produced pristine NPs); the NPs in the finished cosmetic formulation and the NPs present under toxicological investigations and exposure assessment [20].

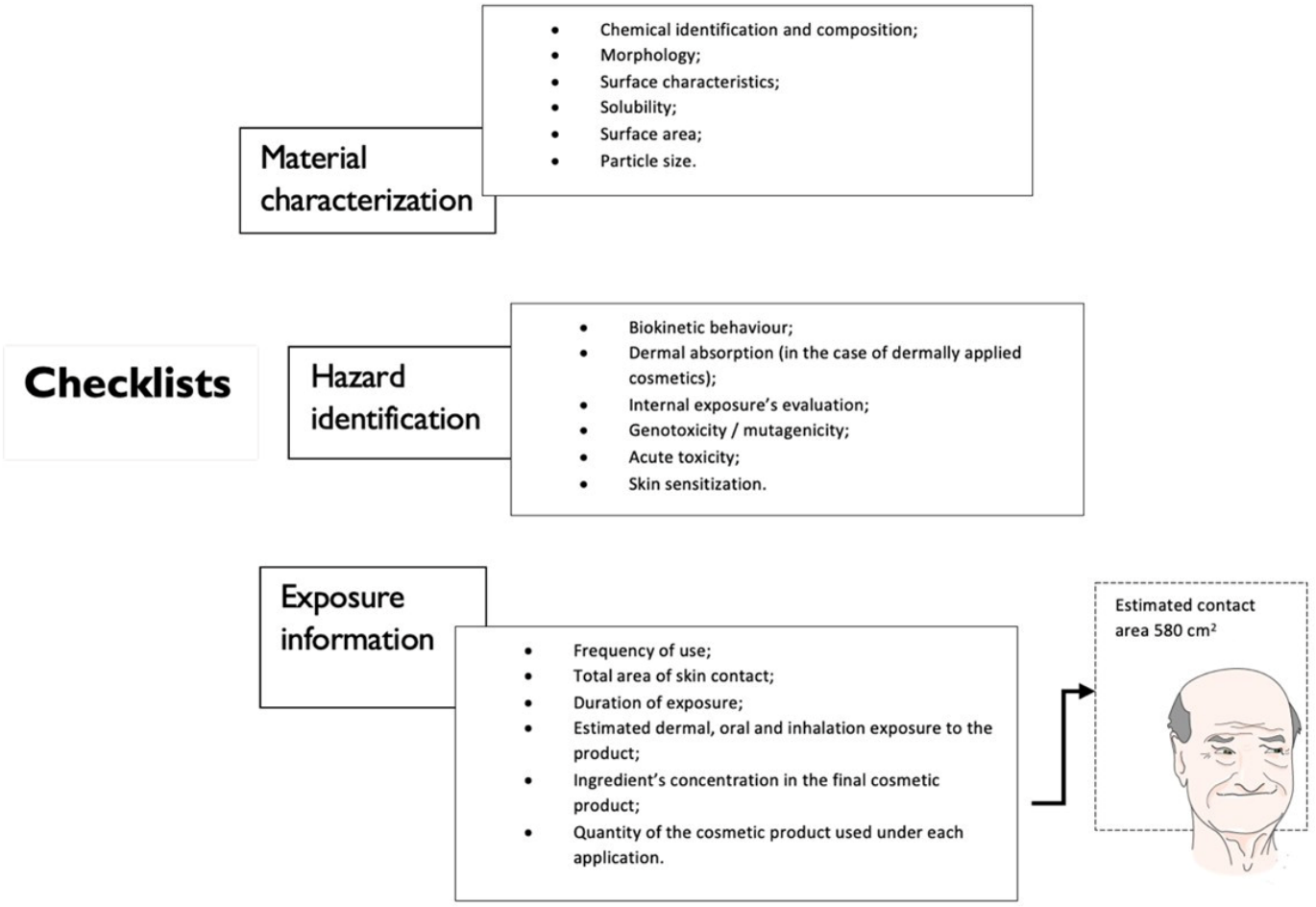

Figure 2. Schematic summary of SCCS's checklists for applicants submitting dossiers on ACIs. SCCS stands for: Scientific Committee on Consumer Safety. ACIs stands for: active cosmetic ingredients.

General studies for the safety of NMs, for example, a random search in vitro micronucleus assay [21], comet assay, and other tests are widely used to assess the toxicological profile of the NMs. Especially genotoxicity and cytotoxicity assays, several studies regarding zinc nanoparticles (ZnO), cosmetics, drugs, sensors [22–24], AgNPs [25], and gold nanoparticles (AuNP) [26], remark on the importance of the particle size and zeta potential on the overall toxicological contribution. Carbon NMs, including nanotubes and graphene [27,28] are also in deep focus, as well as silica nanoparticles [29], dendrimers [30], among others.

3.1. Exposure Assessment

NMs are widely used in products, such as cosmetics, so exposure and toxicity are important parameters to consider for their risk assessment [31]. It is required by the EU chemical legislation, acknowledged as Regulation on Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH), to do a consumer exposure assessment when there is a substance with a high hazard risk [32,33].

Nowadays, there is still no agreement regarding the methods to expect the consumer exposure assessment as mentioned above [34]. Predicting the consumer exposure assessment is difficult once it is required to know the nature, amount, exposure routes, and the intended use of the products, which makes it hard to monitor after the product is sold. For example, the application of hair dyes requires the use of gloves, but there is no way to monitor this after selling the product. This last example leads us to the dermal exposure which is the amount of ACIs that contact the exposed dermal surface [33]. The consumer's exposure is expectable to be higher with NPs in a free form. There have been developed

modeling tools to estimate the potential exposure [35]. Exposure assessments can be predicted using the NanoRisk framework, which was supported by two organizations, and consists of six steps, which are considered the physicochemical characterization of the NP, the defined life cycle, the assessment risks, and the assessment of risk management [36].

3.2. Safety Testing

Different studies can be encountered with regards to safety testing of metal-oxide NMs [37], carbon NMs [38,39], and gold NMs [40]. Delayed-type hypersensitivity is one of the reasons why ACIs are retired from the market. Testing methods to predict delayed-type hypersensivity were performed in a recent study, where several methods and strategies for testing engineered NMs were described and proposed, such as the human cell line activation test (hCLAT) and the myeloid U937 skin sensitization test (MUSST or U-SENS). Regarding the hCLAT and MUSST tests, the in vitro expression of surface markers CD54 and CD86 in THP-1 and U937 cell lines, respectively, was evaluated. The in vitro tests consist in calculating the expression of the surface marker by flow cytometry. The fixed minimum stimulation index was 3, so values above 3 are considered as having a positive stimulation. Generally, the MUSST test is more sensitive than the hCLAT [41].

The main definition of NM stated by the Cosmetics Regulation No. 1223/2009 considers it as a material that satisfies some requirements, such as its insolubility or bio persistence, made intentionally and "with one or more external dimensions, or an internal structure, on the scale of 1 to 100 nm" [42]. An NP is defined according to ISO/TS 80004:2015 as a nano-object "with all external dimensions in the nanoscale, where the lengths of the longest and the shortest axes of the nano-object do not differ significantly". Nanofibers and nanoplates can be separated from this definition, once they have only two and one external dimension in the nanoscale, respectively [43]. There are several nanocarriers for nanocosmetic delivery, allowing the successful improvement of not only the cosmetic formulation's properties at a completely different level but also toward a set of key-concepts such as toxicity, permeability, controlled-release, and efficacy. Therefore, the relevance of both the variety of the vehicles on display and the clear advantages of the more conventional normal sized forms, confers an attractiveness to the field of cosmetics, allowing the fusion of nanotechnology and cosmetology into one unique and enthralling concept.

Regarding the physicochemical characteristics of NMs, as described above, and the lack of safety testing evidence about these nanosized particles, it is important to perform more studies that also consider their long-term impact on safety. The following Table 1 presents a compilation with examples of models and testing performed on NPs for safety testing.

Table 1. Compilation of models and tests performed on NPs for safety testing.

NPs	Study	Model	Test	Ref.
Iron Oxide and Ionic Iron NPs	Genotoxicity (in vivo)	Earthworm Coelomocytes	Comet Assay Micronucleus test	[44]
Cerium Dioxide NPs	Genotoxicity (in vitro)	Human peripheral blood lymphocytes	Comet assay Micronucleus test Gamma H2AX	[45]
Titanium Dioxide NPs	Genotoxicity	Human bronchial epithelial BEAS-2B cells	Mini-gel comet assay and micronucleus test	[46]
AgNPs	Toxicity	Caenorhabditis elegans	Population-based observations Gene expression analysis	[47]
Multiwalled Carbon Nanotubes				
Dendrimers				

Table 1. *Cont.*

NPs	Study	Model	Test	Ref.
Micro-nano zinc oxide NPs	Cytotoxicity Genotoxicity Phototoxicity	Human skin keratinocyte cells	Comet assay (geno) UV radiation (photo) NRU assay MTT assay Intracellular ROS determination JC-1 staining	[48]
AgNPs	Toxicity	Zebrafish embryos	NA	[49]
AuNPs	Genotoxicity (in vitro/in vivo)	Drosophylla (in vivo)	Comet assay SMART assay	[50]
Fullerene	Genotoxicity (in vivo)	Rat lung cells	Comet assay	[51]
Graphene	Toxicology	Caenorhabditis elegans	Transcriptomics Network-based pathway analysis	[52]
Indium-based quantum dot NPs	Biodistribution (in vivo) Toxicology	Rat model	NA	[53]
Chitosan NPs	Toxicology (embryonic)	Zebrafish	NA	[54]

AgNP—silver nanoparticle; AuNP—gold nanoparticle; MTT—3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide; NA—not applicable; NP—nanoparticle; NRU—neutral red uptake; QSAR—quantitative structure—activity relationship; ROS—reactive oxygen species; SMART—single molecule analysis of resection tracks; UV—ultraviolet.

4. Safety Assessment Testing to Evaluate Different Types of Toxicity

NMs are widely used in many fields, such as cosmetics, urging the understanding of their potential toxicity. The increase of new NPs is unfortunately not accompanied by their safety assessment [55,56]. NMs unique properties, such as size, surface area, zeta potential, and aggregation, have influence over the biological reaction and can be a risk for the consumer, thus it is important to increase the knowledge regarding the potential toxicology of NPs supported by in vivo or in vitro studies [34,57].

In vivo studies are the ideal ones, once they can simulate biological mechanisms and chronic toxicology which is not possible by in vitro methods [56]. NPs toxicology is related to the route of administration [57]. The potential exposure routes must be identified and in vivo or in vitro studies should be conducted [34].

4.1. General Requirements for the Assessment of Toxicological Data

The toxicological profile is extremely important, some in vitro methods have been validated and, this way, contribute to the replacement of experimental animal use, despite the lack of in vitro methods in certain toxicological subcategories. The general requirements for the assessment of toxicological data of ACIs are described below and listed in Table 2 [10].

Table 2. General requirements for the assessment of toxicological data of the ACIs.

Irritation and corrosivity (skin and eye) (1)
Skin sensitization (2)
Dermal/percutaneous absorption (3)
Repeated dose toxicity (4)
Mutagenicity/genotoxicity (5)
Carcinogenicity (6)
Reproductive toxicology (7)
Photo-induced toxicity (8)
Human data (9)

4.1.1. Sensitization, Irritation, and Corrosivity of Skin and Eyes

Skin sensitization is often tested by the broadly used local lymph node assay (LLNA), an in vivo test method measuring the degree of stimulation of lymph nodes' lymphocytes proliferation after the test substance is applied and is present within this secondary lymphoid organ. In vitro skin sensitization ARE-Nrf2 luciferase test, a reporter gene assay using the KeratinoSensTM test method (reporter cell line), measuring cell culture keratinocytes activation through activation of the Keap1-Nrf2-ARE pathway (Kelch-like ECH-associated protein 1)-Nrf2 (nuclear factor-erythroid 2-related factor 2)-ARE) as a response for oxidative/electrophilic stress, an important cellular defense mechanism, can be useful for predicting its potential skin sensitization properties [58,59]. Further studies show its potential application to photosafety testing assessments [60]. The h-CLAT, this time studying and measuring dendritic cell activation, is another useful method [61–63]. Direct peptide reactivity assay is considered an "*in chemico*" assay for skin sensitization testing, measuring the phenomenon of haptenation, a process whereby small molecules turn into immunogenic after binding to larger ones, a consequence of the reaction of the ACI with certain proteins (aminoacids residues) [64]. In order to improve sensibility and selectivity for accurately identifying the sensitization potential of some ACIs, another study upgraded the analytical domains of the assay by developing and validating a high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC/MS-MS)-based direct peptide reaction (DPR) assay [65].

A considerable amount of methods and also models with predictive capability regarding skin irritation can be found in the literature. The use of cell cultures consisting of a culture of multi-layered epidermal cells and stratum corneum is probably the most cited method, in which different assemblages of skin cultures can be utilized to detect potential in vitro irritation by specific ACIs present in a determined formulation; newer strategies comprise the development of a structure of deepidermized dermis and epiderm keratinocytes, thus showing effective in the evaluation of specific ACIs so far, as for now, cell culture of keratinocytes remains the simplest method to study this specific topic. 3-(4,5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay, used to determine cell viability, once only viable cells are capable of reducing the tetrazolium salt that names the method to the purple chromogenic product, the formazan crystals, through an enzymatic process, is depicted in Figure 3 [66].

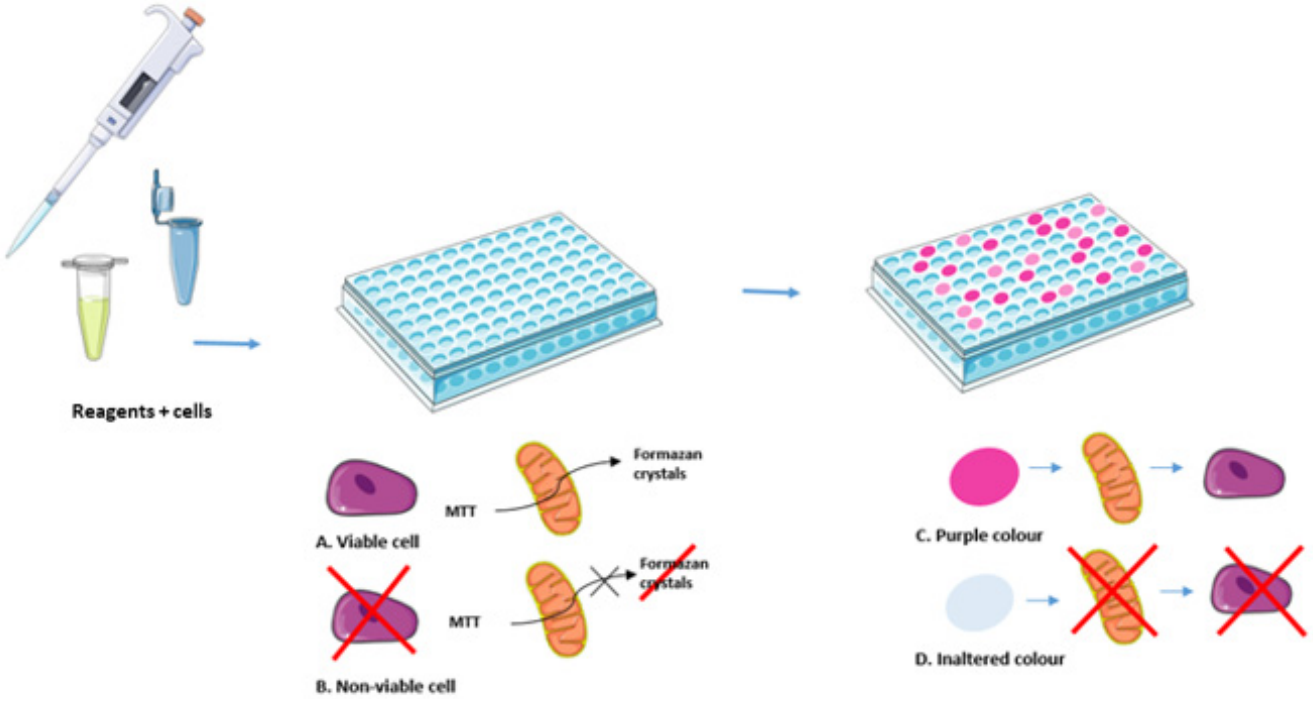

Figure 3. 3-(4,5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay.

Besides the methods described above to predict skin sensitization, a recent review article highlighted different non-animal methods as testing strategies, to elucidate several ACIs' safety: haptenization potential of a certain ACI to proteins in vivo by measuring the reaction of the test ACI toward certain peptides, using the method DPR assay, activation of an important regulator of protective cellular responses to cellular stress (oxidative and electrophilic stressors) [67], Keap1-Nrf2-ARE-pathway, by the ACI using a human keratinocyte cell line (HaCaT)-derived (KeratinoSensTM); other methods consist on measuring differential expression of co-stimulatory molecules (CD86 and CD54) in THP-1 cell lines (consisting of an acute monocytic leukemia-derived immortalized cell line [68]), h-CLAT method; skin sensitization of the incubated test ACIs can be evaluated by measuring CD 86 expression in a specific lymphoma cell line (U937)—U-SENSTM. Besides these, a stipulated increase in certain genes' expression of reconstituted epidermal cells can be used to measure and estimate skin sensitization properties, SENS-IS [69].

Beyond the skin sensibilization, the skin corrosion and irritation are also a concern regarding cosmetic products, such as hair dyes, and they are two different concepts: while the first consists of irreversible skin damage, the second one is responsible for reversible damage, with different physiological reactions, being less severe. Both can be studied by the "reconstructed human epidermis (RhE) test method", an in vitro-validated strategy that replaces in vivo skin irritation, using a model of human skin, with further application of the MTT assay (more difficult in the case of hair dyes, once the visualization of the presence or absence of formazan crystals' color) and, if needed, the interleukin-1x assay [10].

Considering eye damage and the testing of potential eye irritation can be assessed by different in vitro studies, three different categories are considered. The first category comprises organotypic test methods such as the bovine cornea opacity permeability (BCOP) test and the isolated chicken eye (ICE) test [70]. The second one englobes cytotoxicity/cell function methods as the fluorescein leakage (FL) test that studies the increase in permeability of sodium fluorescein and evaluates the toxic response in specific kidney cells and the short time exposure (STE) test that resorts to a corneal cell line from rabbit to evaluate the cytotoxic response [71]. The third category includes the reconstructed human tissue (RhT)-based test method as the reconstructed human cornea like epithelium (RhCE) test, similar to the RhE, followed by MTT assay [10].

These methods can be very helpful in replacing the Draize Rabbit eye testing method, following the animal ban law for cosmetic purposes [72,73]. New efforts are being made to further develop new in vitro tests regarding eye irritation [74,75].

4.1.2. Dermal/Percutaneous Absorption

Dermal/percutaneous absorption is included in the toxicokinetics domain and is of obvious importance since the main application of cosmetics is skin-related and hence the majority of the human exposure [76]. Percutaneous absorption of ACIs is still a challenge due to the skin structure. The different characteristics of NPs as physicochemical properties can influence skin permeation and toxicity [77,78]. Skin hydration, once corneocytes are polar on the inside, compatibility with the skin lipids, disruption of stratum corneum, the zeta potential, NP size, flexibility, and elasticity are the main parameters to consider for having a successful percutaneous absorption [79].

The stratum corneum, a layer of 10 to 20 µm, limits the absorption of ACIs, and it is known that the concentration of ACI in the stratum corneum is directly compared to the ACI concentration in the epidermis [79,80]. The ACIs are transported through the stratum corneum by passive diffusion, in three possible ways, intercellular, transcellular, and appendageal [79]. The tape stripping method allows measuring the ACI deposition in the stratum corneum. This method consists of quantifying the ACI by weighing the tape strips taken from the stratum corneum and then analyzed by high-performance liquid chromatography (HPLC), optical spectroscopy, protein assays in corneocytes, or radio labeling [80]. Studies have shown that metal and metal oxides NPs, may progress the understanding of NPs dermal absorption [78].

Penetration, permeation, and resorption are the three steps of dermal/percutaneous absorption; the possible ways in vitro testing of skin absorption should mimic the real human exposure by a realistic approach. Therefore, for solids, 1–5 mg/cm^2 is the indicated amount to be applied, in contrast to oxidative hair dyes, having special values of 20 mg/cm^2 and an application time of around 30–45 min. In vitro tests using cell diffusion techniques and in vivo indirect radioactivity measurements are two tests referred on the literature. The range of dermal absorption should be used to evaluate the potential risks associated with the systemic reaching of the ACIs [81].

4.1.3. Carcinogenicity

Despite rising awareness of the possibility of personal use of hair dyes being related to carcinogenicity patterns, the evidence remains inconclusive once the epidemiological studies conducted to the time of the release of the special report, about the carcinogenicity of specific aromatic amines, organic dyes and the associated exposures, point to a lack of adequacy of the results analyzed by the working group at IARC [82].

Further studies should be conducted to elucidate the impact of hair dyes on living systems and its possible relation with the arousal of new cancer cases on personal users. A considerable amount of studies and reports are conducted to understand if there is a link between hair dye use and the development of several types of cancer. Despite the need for further work to explore this specific and concerning question, it was verified that, based on different findings, there was evidence that suggested that there was a relation between the personal use of hair colorants and an increased risk of developing breast cancer on a review of the literature of epidemiological studies (8 case-control) [83]; association between hair dyes use and cancer incidence was also shown in a 2015 case-control study [84]. Following studies addressing showing an increase in bladder cancer among professionals (hairdressers and barbers), others were conducted on the terms of personal use and remain whether inconclusive or/and open to discussion [85–87]. Other possible cancer relations were also investigated in hair dye use [88].

Besides a recent case-control epidemiology evaluation indicated a correlation between hair dye use and bladder cancer, additional reports, as prospective surveys on large populations, detected no negative correlations for bladder or additional type of cancers. Even

though in vivo topical carcinogenicity assays hair dye ACIs or commercial formulations showed no indication for systemic toxicity or carcinogenicity, oral carcinogenicity investigations on hair dye ACIs at oral doses up to the maximum tolerated dose (MTD) proposed some ACIs have carcinogenic effects in rodents. Human systemic exposure to several 14C-labelled oxidative hair dyes was lower than 1.0% of the amount administrated under conditions of use. Conservative risk studies indicated no insignificant cancer risk, also considering the ACIs that were shown to be positive in the oral carcinogenicity studies.

One promising in vitro test allowing the study of carcinogenicity (both genotoxic and non-genotoxic—when the ACI does not promote the initiation of tumors by a direct impact on deoxyribonucleic acid (DNA) structure carcinogenicity) includes the cell transformation assay (CTA), although it does not entirely reproduce the in vivo neoplastic process, the CTA allows the detection of ACI with carcinogenic potential and their mode of action [10,89,90].

4.1.4. Reproductive Toxicology

As already known the physicochemical properties of NMs as size, shape, zeta potential, and aggregation, may influence the interaction with the target cells and lead to toxic effects. Reproductive toxicity assessment can be rather difficult once animal testing is not allowed. Therefore, there are alternative methods comprising embryotoxicity as the whole embryo culture (WEC) test and the embryonic stem cell test (EST) [91,92].

Two possible ways lead to reproductive toxicology, one is when NPs interfere with the outcome of the pregnancy and the other is affecting fertility. Regarding the reported studies for NMs affecting fertility, they have uncertain findings due to the doses applied and the routes of exposure. The studies made to predict the influence of NMs on the outcome of the pregnancy, suggest that almost all NMs may have an adverse effect [35].

The results of reproductive toxicity studies and epidemiological investigations suggested that hair dyes and their ACIs pose no risk of adverse reproductive effects [93].

4.1.5. Photo-Induced Toxicology

Phototoxic potential should also be addressed, especially when the cosmetic substance has a high degree of sunlight exposure, such as hair dyes, hence the importance of the photo-induced toxicity studies, which can be divided into four different concepts: photo-irritation, photo-sensitization, photo-mutagenicity, and photo-clastogenicity studies [10]. To evaluate the phototoxicity of sunscreens [94] and hair dyes' ACIs [95], perhaps the most remarkable test is the 3T3 Neutral Red Uptake Photo-Toxicity (3T3 NRY PT) test, a validated in vitro method in which cytotoxicity of a test substance, tested in the presence and also in the absence of non-cytotoxic light (ultraviolet (UV) or visible), is compared and this way the cytotoxicity assessment can provide useful data by analyzing light-exposed and light-private responses testing, evaluating the phototoxic potential of ACIs [96–98].

In regard to photo-mutagenicity and photo-genotoxicity, there are some methods described, such as the photo-Ames test, where a specific ACI can be tested regarding the photo-genotoxicity potential, recurring to Salmonella typhimurium strains, typical of a regular Ames test. It was conducted, for example, for the assessment of in vitro genotoxicity/mutagenicity of a red dye, an aromatic amine heterocycle named 3-amino-7-dimethylamino-2-methyl phenazine, a common dye used as an indicator in several biological systems, and a known photo-sensitizer, tested under different experimental conditions: with aromatic amine activation—metabolical activation; without metabolical activation; and under photoactivation [99], also for the polycyclic aromatic molecule named azulene [100], the photo hypoxanthine phosphorybosyl transferase (HPRT)/photo-mouse lymphoma assay and the photo-micronucleus assay. In addition, it is of substantial importance the information generated by "Human data", once there are several possible effects, not desirable, that can appear as response to the contact of the surface (skin, hair, nails) with the cosmetic substance, with different degrees of severity, such as allergic contact dermatitis, irritation, among others [10]. Hence, case reports, epidemiological and clinical studies

are of extreme importance due to their relevance in the actual cosmetic safety assessment paradigm [101].

4.1.6. Mutagenicity Assessment

NMs mutagenicity can't be assessed by the realization of the Ames Test, once the difficult bacterial incorporation of NMs constitutes a limit for testing [10]. Thereafter, the Scientific Committee on Consumer Products (SCCP) suggests three tests for NP genotoxicity assessment, including chromosome aberrations/clastogenicity testing in mammalian cells (for example by micronucleus test) and gene mutation in vitro testing, also in mammalian cell lines (for example hypoxanthine phosphorybosyl transferase (HPRT) test).

With the specific regards of oxidative hair dyes testing, the SCCP recommended a wide range of in vitro mutagenicity/genotoxicity tests [102]: a bacterial reverse mutation test is required, described in OECD guideline 471 [103] consisting of the detection of mutation points, that are a result of the addition, elimination or substitution of one or more DNA base pairs, by using strains of *Salmonella typhimurium* and *Escherichia coli*, despite it cannot be a stand-alone procedure to determine carcinogenic potential; an in vitro gene mutation testing assay using a mammalian cell line, described in OECD 476 [104], with special relevance the mouse lymphoma thymidine kinase assay, providing the detection of both gene mutations and changes at the chromosomal domain, and consisting of measuring by seeding an identified number of cells in the media, one with the selective agent that detects the mutated cells and other media without the selective agent in order to conclude the cloning efficiency; the third one required can be the in vitro micronucleus test or an in vitro chromosome aberration test in a mammalian cell line, as described in OECD 473 [105]. Further studies, although not obligatory required, can be conducted for providing more information and confirmations on the prior-mentioned studies, such as the in vitro Syrian hamster embryo (SHE) cell transformation assay, described on OECD 495, standing now alone in this additional section once the Unscheduled DNA Synthesis (UDS) in mammalian cells in vitro—OECD 482—a test focusing on tritium-labeled thymidine (3 H-TdR) incorporation into DNA of the lineage of a mammalian cell which is not in the DNA replication phase of the cell cycle was deleted on April the 2nd 2014 [106]. Additional assessments on dermal absorption and metabolic activation are of special relevance, concerning the carcinogenicity potential of these specific oxidative dyes and thus are important topics to be addressed, mainly for precursors and couplers, where systemic exposure to the molecules must not be excluded. Therefore, for precursors and couplers, the two main components of the oxidative hair dye formulation, along with hydrogen peroxide, are the molecules applied initially on the hair, then undergoing a series of chemical reactions described previously. The color intermediates formed by the oxidative reaction of the dye precursors and hydrogen peroxide with the formation of p-benzoquinone imines, react with the relative hydrogen peroxide-stable couplers, yielding the final color molecules responsible for the dyeing of the hair, in a rapid reaction forming larger molecules, with more than one aromatic rings, trapped inside the hair's structure [102]. A recent study suggested five physicochemical properties to be taken into account as an indicator of very low dermal absorption of a certain ACI: molecular weight higher than 500 Da; Logarithm of the partition coefficient n-octanol/water lower than -1 or higher than 4; melting point over 200 °C; high extent of ionization; topological polar surface area over 120 Å [107].

An ACI must not be genotoxic and cause mutagenicity. This way, it is recommended to carry out the bacterial reverse mutation (BRM) test (to identify gene mutations) and the in vitro micronucleous test, the latter allowing the study of chromosome aberrations (structural—clastogenicity; and numerical-aneugenicity), detecting genotoxic damage in a specific cell cycle moment (interphase). The BRM assay, also known as the Ames Test, can be found in different articles in the literature [108,109]. The in vitro micronucleous test allows for assessing the genotoxic potential of an active ingredient and is depicted in Figure 4, used for many substances [99,110]. Some hair dye testing in vivo can be encountered in a quick search, regarding the micronucleus assay principles [111–113].

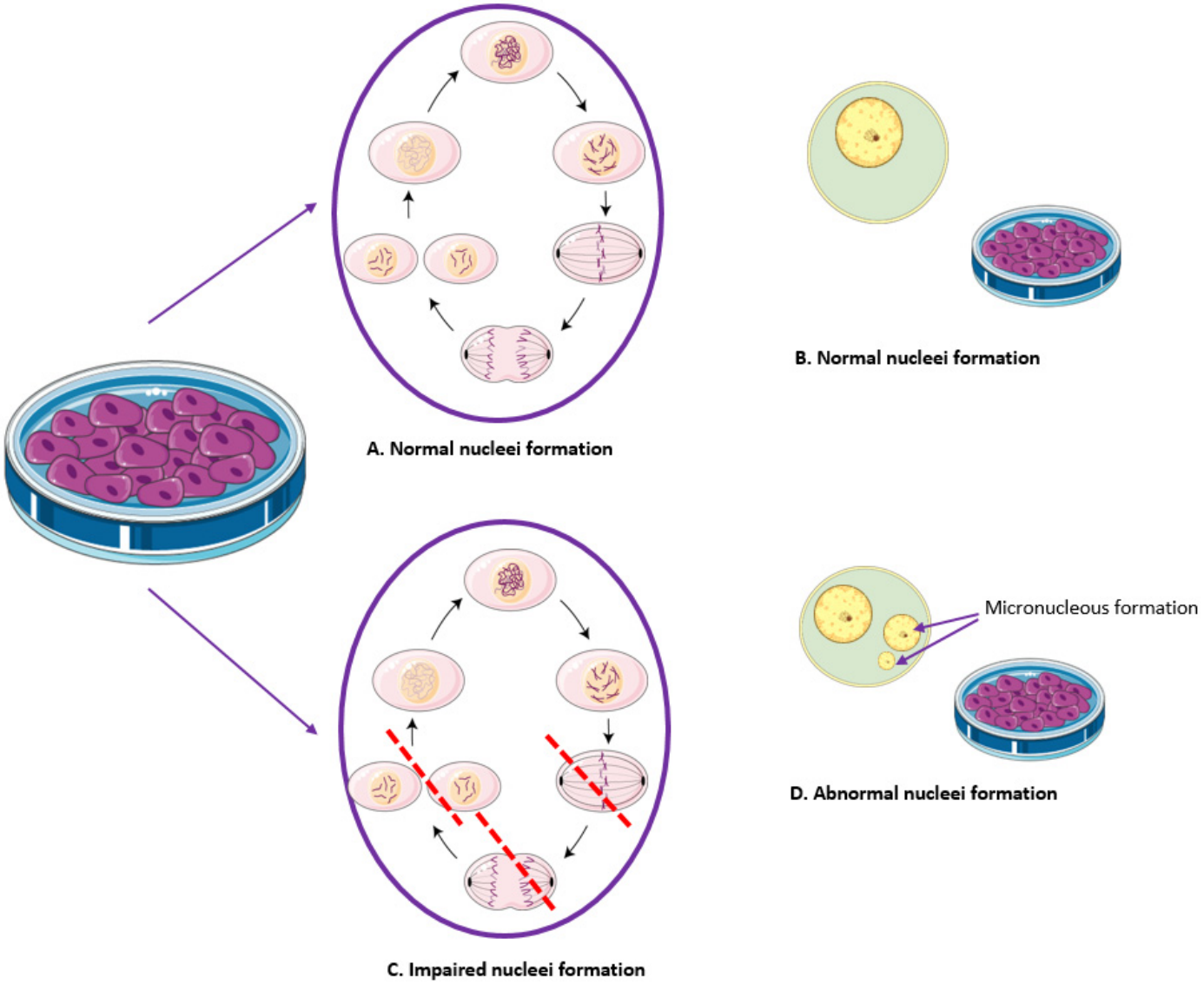

Figure 4. In vitro micronucleous test, used to assess the genotoxic potential of an ACI.

In the case of NPs, HPRT (hypoxanthine phosphorybosyl transferase) gene mutation test should be performed (mammalian cell lines) replacing the BRM test. In this test, a toxic HPRT analog (6- thioguanine) allows cell selection: mutated cells survive whereas the non-mutated cells die because HPRT is an important enzyme in DNA synthesis. This was, if a mutation alters the gene that codifies to the expression of that specific enzyme, it can be identified by 6-TG, by positive selection [114]. This important housekeeper gene can function, therefore, as a mutational biomarker, and is widely used to test the potential carcinogens [114,115], as shown in Figure 5.

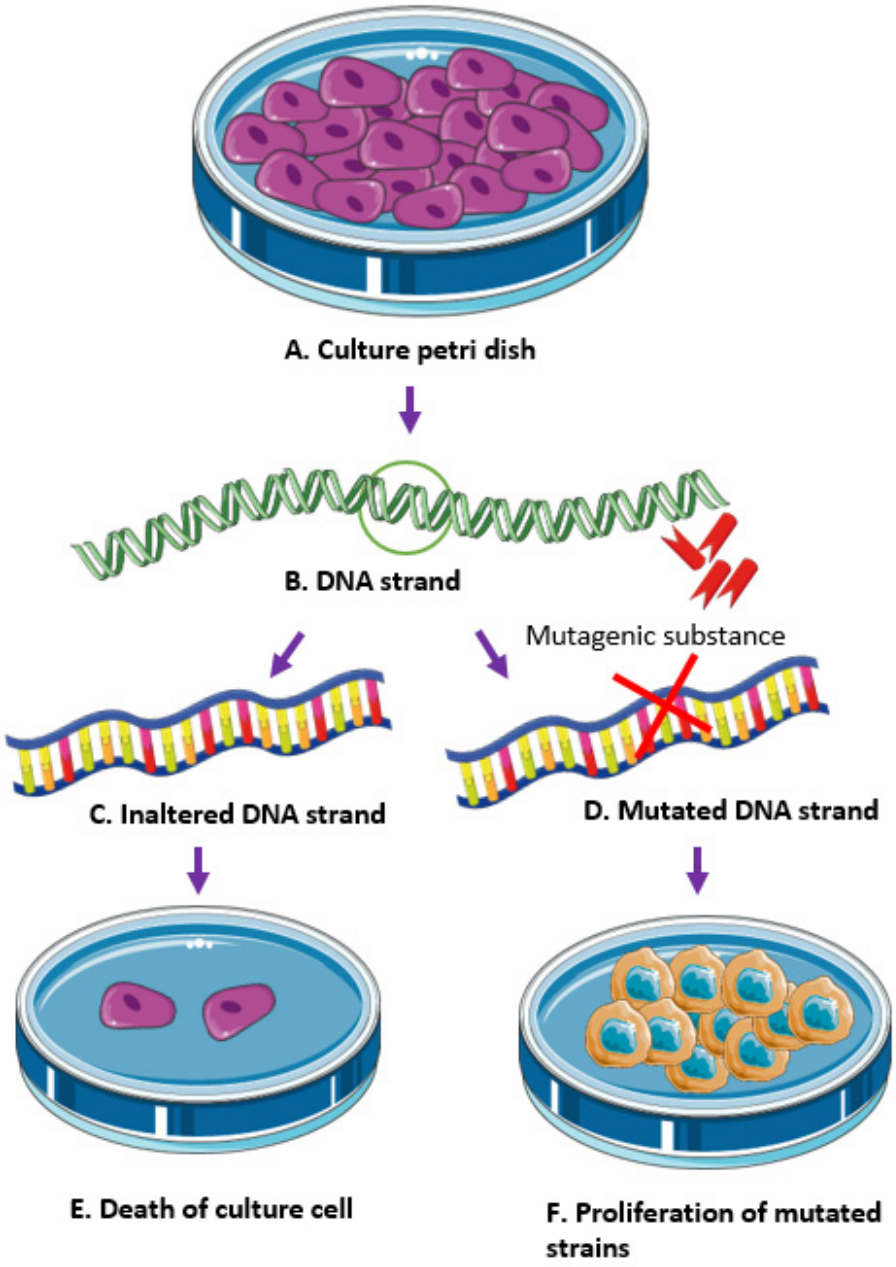

Figure 5. In vitro hypoxanthine phosphorybosyl transferase (HPRT), a gene mutation test. DNA: deoxyribonucleic acid.

4.1.7. Genotoxicity of Hair Dyes Molecules

Regarding hair dyes and cosmetics in general, besides the potential exposure paths of NPs and the in vivo and in vitro toxicological studies, more genotoxicity studies should be conducted to assure their safety [34,116]. Some NPs, and also related to their chronic exposure, can lead to genotoxicity by oxidative stress [57,117]. Extensively used for in vitro and in vivo genotoxicity tests, the COMET is a gel electrophoresis-based assay, where the DNA single-strand break is measured in single eukaryotic cells [56]. Moreover, a 3D microtissue model was developed. In vitro genotoxicity assays on hair dyes with ACIs usually show positive outcomes, besides their association with in vivo carcinogenicity for aromatic amines (i.e., the oxidative hair dyes ACIs' chemical class) is unclear. Moreover, positive in vivo genotoxicity outcomes regarding hair dyes are scarce. Investigations in humans did not detect genotoxic effects for hair dyes and/or for the respective ACIs. On

the basis of mechanistic studies, in vivo positive hair dye ACIs (including p-aminophenol, Lawsone) represent no negligible risk to human health.

4.1.8. Cytotoxicity

Mitochondrial enzyme succinate dehydrogenase catalyzes this reaction, turning the yellow tetrazolium salt into a purple formazan dye if cells are metabolically viable. Trypan blue assay is used to count the number of viable cells and therefore can be used to perform cell viability tests and to evaluate the cytotoxicity of a certain ACI of interest, once trypan blue molecule cannot be absorbed by the live-cell in normal conditions (viable cell), due to the cell's strict membrane selectivity and its intact membrane properties. Once the cell is dead or severely damaged, these dye molecules can penetrate the cell, transversing the membrane and giving the dead (nonviable) cells a blue color, when visualized under a microscope [114]. Cell uptake of an ACI interest is also an important step to characterize the absorption and internalization of the ACI, or an NP loaded with an ACI of interest. For that matter, a qualitative study can be conducted, using specific cell lines incubated with the NP, followed by the washing of the cell culture medium after a couple of hours and staining with a specific molecule that allows ulterior visualization, such as phalloidin (a mushroom toxin that binds and stabilizes the filamentous actin of the cell, called "F-actin") fluorescent derivatives, used in the halloysite cellular uptake study [114]. Enzyme-release test can also be used for this endeavor; lactate dehydrogenase and aspartate aminotransferase are the key enzymes that are measured; also, the interleukin 1alfa-assay test can be used, in which is released the pro-inflammatory interleukin from irritated cells.

The study of permanent hair dye incorporation into two NPs composed of hyaluronic acid and poly-y-glutamic acid/glycol chitosan was the base for the following Table 3. Allowing to trace a strategy for hair dye testing, specifically, cytotoxicity and apoptosis testing, using different techniques and using HaCaT cells. The studies provided an interesting conclusion once the incorporation into NMs—NPs in these two particular examples showed a reduction both in cytotoxicity and apoptosis in the cell line in question, emerging as interesting vehicles for hair dye-targeting and providing stimulation for more studies and new prospects in what hair dyes are concerned.

Table 3. Cytotoxicity and apoptosis testing of permanent hair dye incorporation into two NPs composed of hyaluronic acid and poly-y-glutamic acid/glycol chitosan.

Cell Culture	HaCaT Cells			Ref.
Variable studied	Cytotoxicity	Apoptosis and Necrosis (Reagents: PI and FITC-annexin V)	Apoptosis	[118]
Technique	MTT assay	Flow Cytometry Analysis	TUNEL assay of HaCaT cells	[118]
NP	PDA-2 (50:10:05) [a] Reduction PDA cytotoxicity by nanoparticle formation	PDA-2 (50:10:05) [a] Reduction PDA cytotoxicity by nanoparticle formation	NA	[119]
	Form. 9/1 (45/5) [b] Reduced intrinsic toxicity	Form. 9/1 (45/5) [b] Reduced intrinsic toxicity	Form. 9/1 (45/5) [b] Reduced intrinsic toxicity	[118]

[a]—Weight ratio of PGA/PDA/GC; [b]—weight ratio of HA/PDA (mg/mg); FITC—fluorescein isothiocyanate; HaCaT—human keratinocyte cell line; NA—not applicable; NP—nanoparticle; PDA—p-phenylenediamine; PI—propidium iodide.

4.1.9. Human Data

Comprising the formation of reaction products (RPs) of oxidative hair dye ACIs in hair dyeing processes, the SCCS published an important opinion building on previously published documents on this area and pushing further the study of several scientific domains regarding hair dyeing, its chemical perspective, reaction kinetics, RPs formation, their concentration in the formulation, estimated maximum external exposure (EMEE) and exposure assessment by in vitro dermal absorption studies and also determined by studies in humans—on which systemic exposure was evaluated, regarding a [14C]-para-phenylenediamine-containing oxidative hair dye. A representation of three selected ACIs can be seen in Table 4 below. Concerning to in vivo exposure assessment, the excretion route assumes great importance, once it allows a plausible estimation of systemic BA of hair dye RPs products, metabolites, and other ACIs. This way, plasma, and urine quantification of PPD, metabolites, and RPs were conducted on 16 volunteers. The rate of application was defined, as well as the formulation molecules and their respective concentrations, comprising a mixture of paraphenylenediamine, resorcinol, and meta-aminophenol. A high-sensitive LC-MS/MS method was used to quantify the different ACIs and it was concluded that the main molecule present in the samples was the non-genotoxic acetylated metabolite of PPD—N, N′—diacetyl-PPD.

Table 4. A representation of three selected ACIs based on the opinion building from SCCS.

Oxidative Combination	RP	Concentration (% *w*/*w*) in the Formulation	EMEE % (*w*/*w*) [a]	RP Concentrations Tested in 72 h DP Study (%)	LD in Receptor Fluids (pg/cm^2)	Estimated BA Mean +1 SD (ng/cm^2)	Exposure Per Day (µg/day) [b]
p-toluenediamine (A005) + m-aminophenol (A015)	Trimer A005-A015-A005	0.14	0.07	1.0; 0.3; 0.1	2.0	461.89	9.57
p-aminophenol (A016) + 4-amino-2-hydroxytoluene (A027)	Dimer A016-A027	0.14	0.07	1.0; 0.3; 0.1	2.2	717.79	14.87
N,N-dihydroxyethyl-p-phenylenediamine (A050) + maminophenol (A015)	Trimer A050-A015-A050	0.50	0.26	1.0; 0.5; 0.25	2.8	3.27	0.07

[a]—RP concentration in the final formulation to which consumers are exposed based on kinetic studies (%); [b]—adjusted bioavailable dose × 580 cm^2/28 days; ACI—active cosmetic ingredient; BA—bioavailability; DP—dermal penetration; EMEE—estimated maximum external exposure; LD—limit of detection; RP—reaction product; SCCS—Scientific Committee on Consumer Safety; SD—standard deviation.

5. Legislation and Safety of the NMs Cosmetic Products

As for NMs used in cosmetic products, information can be found in the Cosmetic Regulation (2009/1223/EC), entering into force in 2013, having an article dedicated to NMs used in cosmetics. NM is defined as " an insoluble or bio-persistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm" [42].

More information can be encountered in the Annexes, such as the cosmetic product safety report and assessment—Annex I; an extensive list of ACIs that cannot be used in the cosmetic products—Annex II—or can be used only if the specified restrictions are fulfilled—Annex III; the colorants, preservatives, and UV-filters that can be used for cosmetic purposes are listed, respectively, on Annexes IV, V, and VI [42]. Some adaptations to the methods listed above should be considered for NPs-containing cosmetic products, noting that agglomeration and aggregation can occur; solubility is generally low once NMs are poorly soluble or insoluble materials generally; measure units of toxicological studies can represent a challenge and can be inadequate; uptake and biological profile may be different; this way, an extensive characterization study should be conducted before testing.

As for the legal aspects, they are comprised in European Commission (EC) Cosmetics Directive No. 1223/2009, following the 76/768/EEC directive that is no longer in force. Regarding Directive 76/786/EEC, the ACIs were reviewed for safety according to requirements such as: performing a safety assessment of the final cosmetic product before its release to the EU market; availability of the product information file (PIF) that contains the qualitative and quantitative information of the final cosmetic product, physico-chemical properties, the method of manufacture complying with the GMP and the assessment of the safety from the final product for human health; and also the compliance of labeling requirements. Also, in this directive that is no longer in force the concept of "Responsible Person" is mentioned, regarding non-EU companies placing their cosmetic products in the EU market, for this, they should nominate a "Responsible Person" for the PIF and keep the safety assessment report. Directive No. 1223/2009, replaces the previously mentioned one and contains clearer provisions for the content and format of the safety assessments for the product as well as for the PIF. It contains an extensive list of ACIs that should be avoided in the final cosmetic product on Annex II, followed by a list of substances in annex III that can be used unless under with specific requirements, restrictions, warnings, and instructions contemplated on the table. Specifically, the list of colorants approved for use is contained in Part I of Annex IV and applies to most coloring ACIs named by their corresponding color index number, noting that other ACIs not appearing on the list may be used, if intended, for hair dyeing only [42]. The allowed ACIs regarding the NMs used in cosmetics, are listed in the "Catalogue of nanomaterials in cosmetic products placed on the market, as notified to the European commission by Responsible Persons". This catalogue will be regularly updated, for now, the ACIs found related to hair dyes cited are carbon black used as a colorant, hydrated silica, and silica used to bleach and remove the dye and silica dimethyl silylate that have these two last functions and also is used non-oxidative color [120].

6. Outstanding Concerns

The cosmetic market has grown by leaps and bounds and is expected to continue to expand in the future, considering new technologies, new perspectives and applications, new directions, and new formulations. Many scientific challenges will need time for a successful resolution, and a quick representation can be seen in Figure 6. The ever-increasing number of cosmetic ACIs and products, many of which nanosized materials, should be considered, once the higher the exposition to new chemical ACIs and ACIs, the higher the potential to cause hazardous responses on a consumer level, despite all the safety and risk assessments that are advisable and, hence, comprised. Emphasis has been laid on the limitations of the conventional testing methods available for testing cosmetic ACIs, including those within the nanoscale range [121]. Toxicological and safety assessments need improved models, more efficient and more similar to the interactions of the living system, and a broader-scale methodology allowing to extrapolate more successfully in vitro results to the in vivo landscape [122,123]. Toward this aim, several publications point to interesting strategies complementing the risk assessment procedures, regarding in vitro testing, animal testing in reproducible but more phylogenetic distant animals, in silico approaches, and bioinformatics [10]. To address the expressed concern of animal testing for cosmetic purposes, followed by the animal ban directive, the concepts of the 3"R" emerged strongly, viewed as a conductor with the view of reducing as far as possible the use of animal testing, based on three major concepts: "Replacement"; "Reduction"; "Refinement" [124]. Based on this approach, alternative test methods assume special relevance [125,126]. Digital data processing interfaces could be more and more one of the essential strategies for risk assessment, by structure-activity relationship, in silico methods [10,127], computer modeling of chemical structures, and recurring to high throughput screening (HTS), the latter revealing as a key strategy, frequently mentioned, towards the future [128,129], notwithstanding other developed ideas exploring nanorisk levels [130]. In the near future, the expansion may be broader into new biomaterials, totally biocompatible and biodegradable, and maybe an emphasis on the environment—soil, water—will be not only necessary but also of

unique importance, taking the huge number of studies regarding the environmental impact of those NMs—in the living ecosystems [131,132]; paradoxically, nanotechnology can be applied to counteract this impact, taking to account the recent studies for nanotoxicological modeling [133–135]. Relevance should be given to eco-friendly and green materials as far as possible. As for the case of dyes, and degradation products, with different origins, such as the textile industry and wool staining ones, there are studies regarding their presence in water [136], as water pollutants, and the development of strategies to investigate and to their efficient removal, including NMs-based [137–139], using photocatalytic and chemocatalytic oxidation mechanisms for example. Challenges to adopting new systems for coloring are also plausible ideas to develop in the near future [140].

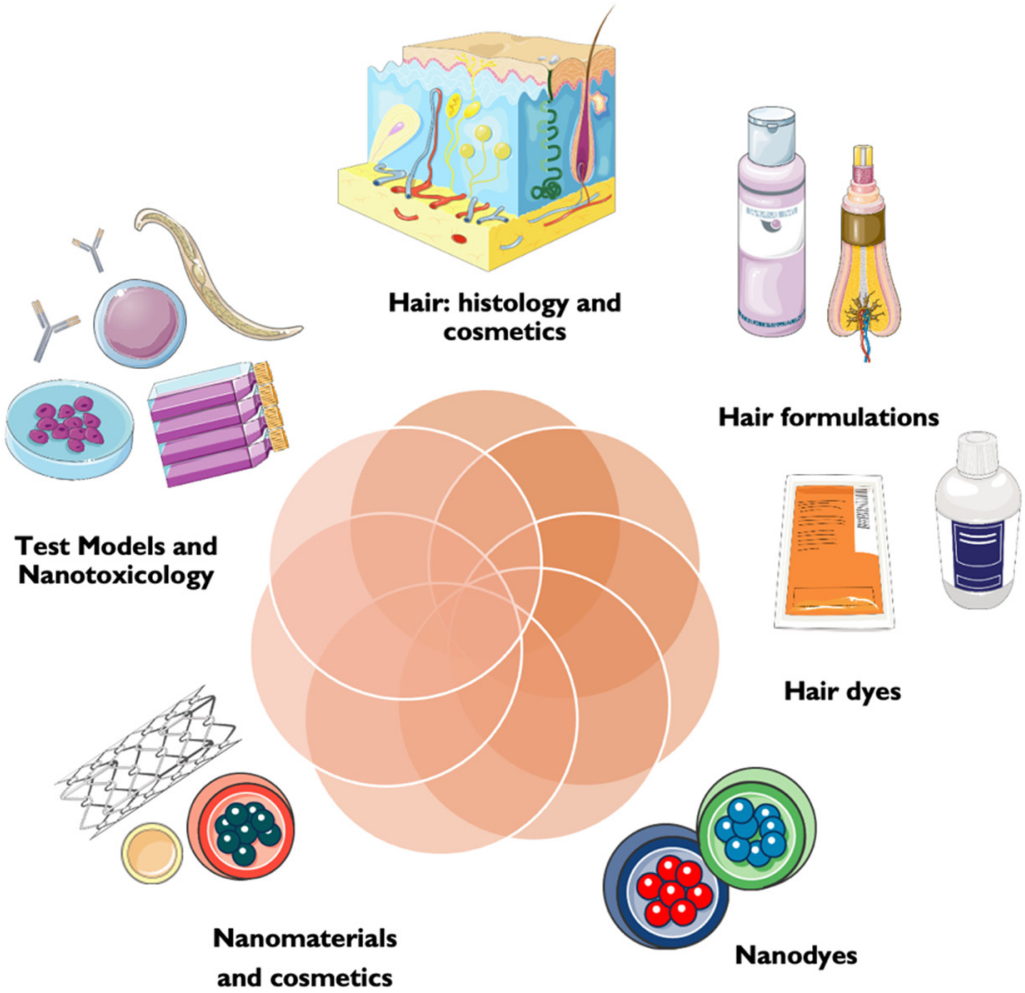

Figure 6. Schematic figure representing the fields where scientific challenges will be faced.

Faster and more reliable methods for NM screening are needed, as well as for the case of dye molecules. Taking in mind the continuous expansion of NMs in the near future, it is crucial to test more and with broader efficiency and approximation to the living complex systems the potential consequences of the human health exposure, the degrees of exposition, the toxicological potential, the underlying molecular mechanisms and cellular interactions of both molecules and NMs, what can be prevented and ameliorated, and the extrapolation to the future—whether short-term or long-term exposition—of nanocosmetics and NMs in general, including bioaccumulation and biodegradability properties and projecting onto safer formulations and products. It is, therefore, evident, that the gap between nanotechnology research and the NM safety, with discoveries and advances yet to be made, shows up in numerous articles regarding nanotechnology, nanosafety, nanotoxicology, and improvement strategies [124,141–143]. New data on absorption, distribution, and

stability in the human body are also needed. Despite all the efforts by researchers, there's still a limitation regarding the testing models: the in vitro to in vivo extrapolation and comparison, hence the importance of refined methods to approximate the two dimensions, even more after the animal ban in the European Union (EU), leading to increase research on alternative methods that could substitute with the fair ability the in vivo ones. There is a long path ahead but surely the walk is going in a good way. The informatic context is also very relevant, where computational nanotoxicology is arising and developing to newer extents. Nano-QSAR models constitute an interesting way of studying the relationship between structure and activity applied to NMs, presenting as more suitable for predicting potential toxicity, depending on the physicochemical characteristics of the NM directly impacting its toxicological profile [144]. Cytotoxicity of metal oxide NPs and the mutagenicity of fullerene were studied recurring to this method [145–147]. QSAR models can also be applied to the general dye molecules consisting in the hair dye formulation, an auxiliary tool to assess the safety of the vast array of molecules existing in the market throughout different cosmetic products, allowing to estimate the toxicological quicker based on the structure–activity relationship equation [148]. Integrative analysis and new economic approaches can support the assessment and open new boundaries concerning toxicology and NMs [149,150]. The following Figure 7 illustrates the expressed concerns.

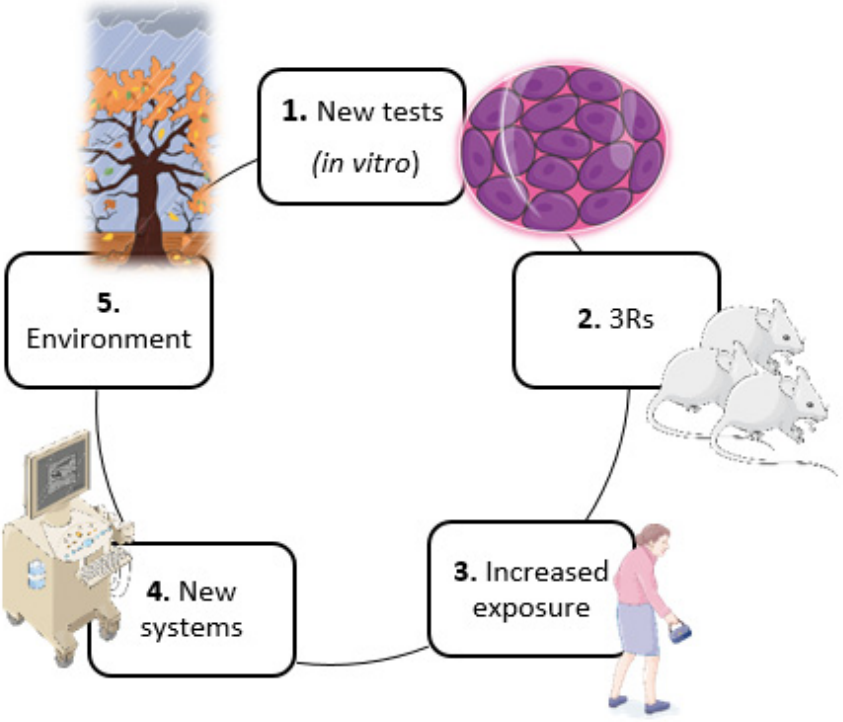

Figure 7. Illustrative representation of the expressed concerns for the use of NMs. 3Rs means: Replacement, Reduction and Refinement. NMs means: nanomaterials.

Following the innovation in the cosmetic industry, bearing in mind the emerging studies on nanocosmetics and nanoformulations, a new kind of hair dye delivery system comes to light. The halloysite clay nanotube can be an interesting way for hair dye targeting, once it would not only allow minimizing the skin contact, the sensitization potential of the dye molecules, and a more directional and effective delivery but also promote a controlled release of the coloring molecule of interest, taking advantage of the sophisticated domains of the nanotechnology and the loading capacity of the halloysite nanoclays, towards a new formulation using cheap, biocompatible and abundant NMs for hair application as a hair dye nanoformulation—the nanodyes—for hair follicle coloring.

7. Conclusions and Future Perspectives

Despite the continuous advances, studies, and knowledge at our disposal there is still a huge challenge ahead for cosmetic and NMs research. The continuous evolution of the engineered NM's field, in the cosmetic industry and formulations themselves, are notable. Nonetheless, it is fair to conclude that there is a significant path to be trilled towards newer, safer, and more efficient formulations.

Much more needs to be studied regarding the complex interactions among the biological systems, nanotechnology, and its NMs. The toxicological effects and health outcomes in living systems are still unclear yet once the continuous exposure studies and the dose-exposure-response remains unknown. Moreover, the ecological impact should be considered and studied. New testing methods should be developed to help elucidate and approximate as far as possible the toxicological pattern of the ACIs and cosmetic NMs in the human body and environment. The new methodological studies should consider the NMs systematically exposure assessment to determine the exposure indexes and safety. In these new methods NMs ACIs composition, the dose variation when in contact with the living system, and possible agglomerations could be important targets to investigate. A database that considers standardized methods, the ACIs, and NPs should be created and accessible to the scientific community. The possible side effects related to the exposure should be available for the consumers and a place to report them could also be developed. Moreover, the optimization of new methods and long-term exposure to NMs could be further considerations.

Furthermore, studying the potential interactions that may arise from exposure, in today's increasingly demanding and challenging world, in which the cosmetic market will continue to exert its force through society from the first cosmetic applications hundreds of years ago, long before the 20th century, and push scientists into new research directions, is an emerging need.

Author Contributions: Conceptualization, S.C.C., F.M.-M., F.V. and A.C.P.-S.; methodology, S.C.C., F.M.-M., F.V. and A.C.P.-S.; software, I.S.-O., I.F.-F., D.P., M.P.-S.; writing-original draft preparation, S.C.C., I.S.-O., I.F.-F., D.P., M.P.-S.; writing-review and editing, A.M., K.D.P., F.R., P.G.M., F.M.-M., F.V. and A.C.P.-S.; supervision, S.C.C., F.M.-M., F.V. and A.C.P.-S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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