Prospects on the nano-plastic particles internalization and induction of cellular response in human keratinocytes

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Research

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Abstract

Background

Today cosmetic usage becomes customary in both sexes to improve their appearance and increase societal visibility. In this study, we have isolated nano-sized plastic particles ranging between 30 to 300 nm from the commercial face-scrubs and investigated its effects on keratinocytes.

Results

Initially, nano-plastics adsorbed protein molecules and formed protein corona, thereby mimicked as protein aggregates, which then triggered the macropinocytosis activity. As a result, corrosion and degradation of plastic particles were observed. Concurrently, nano-plastics concentration-dependent cytotoxic, cytostatic, and cytoprotective activities were found in the keratinocytes. Additionally, a single dose of nano-plastics exposure resulted in the ROS mediated down-regulation of cell growth and proliferation inhibition followed by autophagy, finally, premature aging in HaCaT cells.

Conclusion

At the outset, this work provides insights into the nano-plastics concentration-dependent regulatory, cytoprotective, and cytotoxic effects in HaCaT cells. Further signifies the crucial need for new regulation in nano-plastics usage in cosmetics.

Background

The constructive practice that strengthens one's outward appearance is called a fashion process. Cosmetics are the primary object of the fashion process that enriches a person's appearance more beautiful, attractive as well as appealing to attain improved social visibility [1, 2]. Cosmetic usage is certainly not new; some archaeological evidence attests that cosmetics were quite prevailing used for self-beautification, religious/ nobility purposes among men and women in ancient India (2500 BC) [3] and Egypt (4000 BC) [4]. Today cosmetics are enormously and routinely used by both genders of all age group for beautifying their appearance (makeup and lipsticks), cleansing body parts (soaps, shower gels, bubble bath, shampoos, face scrubs, toothpaste, shaving foam, etc.), correcting body odors (perfumes, deodorants, antiperspirants, etc.) and protecting/ maintaining good condition (sunscreens, toothpaste, etc.).[5, 6, 7] Formerly, the cosmetics and personal care products are composed with natural exfoliates such as pumice, aniseed, coconut, almonds, oat flour, fruit stones, and husk of apricot, bamboo, pecan nut, and walnut shell[5] alongside the generalized compositions (soap, surfactant, alcohol, oil, wax, water, and inorganic pigments). Of late, the nano/micro-sized plastic particles (polyacrylate, polyethylene, polyethylene terephthalate, polypropylene, polymethyl methacrylate, polystyrene, polyurethane, nylon,
teflon, etc.) have replaced the natural exfoliates owing to the economical, versatile, durable, lightweight, and corrosion-resistant properties[5, 6, 7, 8, 9, 10].

In order to fortify the exfoliation and cleansing properties, an extensive amount of plastic ingredients are added intentionally into the cosmetics and personal care products (CPCP). According to European Chemical Agency (ECHA) and United Nations Environment Programme (UNEP) reports, more than 500 different types of nano/micro/macro-plastic ingredients weighing above 9300 tonnes are added in CPCP every year in the European Union countries alone[7, 11]. Among the polymer types, polyethylene particles are enormously (about 93%) added in cosmetics[7] because of its abrasiveness, film-forming, and viscosity controlling capacity, followed by polypropylene due to bulking and viscosity property and polystyrene due to film-forming property. Tills now, there is no specific data available on the size-frequency of plastic particles used in cosmetics. However, few studies encountered 5 µm to 2 mm size plastic particles from major brands of cosmetics, of which many brands consisted monodispersed particles, but some products contained polydispersed particles with a large portion of small particles[5, 9, 12]. Recently Hernandez et al.,[13] and our group [14, 15] have isolated nano-plastics (NPs) with less than 100 nm size range from facial scrubs. According to Hernandez et al., one gram of face scrub contains more than 300 billion nano-sized plastic particles equivalent to 300 µg[13]. The per capita of polyethylene microplastics (MPs) exposure to the US population via CPCP was determined to be 2.4 mg/person/day[16]. The emission per capita of polyethylene MPs in the UK population ranges between 40.5–215 mg/person/day, suggesting the annual environment release of around 86 tonnes from facial exfoliation alone [9].

Numerous studies testified the plastic particles in CPCP are the primary contributors to the aquatic plastic pollution and demonstrated the MPs exposure stimulates adverse effects in numerous marine and some terrestrial organisms. For example, altered growth and reproduction in rotifer, amphipod, and copepod [17, 18, 19], altered cellular functions in blue mussel and sea bass[20, 21], reduced feeding activity in lugworm[22], fibrosis, congestion and inflammatory infiltration in earthworms[23] and activation of the immune response in mouse[24]. Therefore, the plastic particles are recognized as the second most poisonous agent affecting the environment and ecology during the second UN Environmental Conference in 2015 [25]. This proclamation ultimately raises the concern about the direct exposure of plastic particles to human skin via cosmetics and recommends an empirical investigation on the effects of nano-plastic on skin cells.

The skin is the extraordinary and most substantial organ of the human body, made up of epidermis, dermis, and subcutaneous layers. It serves as a physical barrier against the fluctuating environmental factors especially, pollutants, toxic substances, UV and ionizing radiation. The epidermis is the external layer composed of 90–95% keratinocytes and fewer melanocytes and Langerhans’ cells[26]. Hence, keratinocytes are the first cells that frequently confronted with toxic chemicals and pollutants, which might have elicited it to establish spectacular mechanisms to recognize and differentiate the biological and external substances, respectively[27]. Direct application of nano/micro-plastics containing cosmetics and persistence on the skin for a long time under various environmental conditions could provide a higher
chance for NPs to invade the skin via percutaneous absorption. However, the absorption and persistence rate of NPs on the skin, and its effect has not yet been studied. Currently, several recent studies focussed on the NPs entry via ingestion or inhalation route; nevertheless, the definite impact on human health is not known[28]. Hence, the present study intended to determine the NPs entry and its implications on keratinocyte cells using the nano-sized plastic particles isolate from cosmetics.

Results And Discussion

Isolation and identification of Nano-plastic particles (NPs) from face scrubs

The HR-SEM observation of mNPs and wNPs isolated from major brands of facial scrubs for men and women, respectively, showed the majority of the mNPs are spherical with a smooth surface and few irregular aggregates (Fig. 1a), in contrast, the wNPs are irregular with sharp edges (Fig. 1b). The resulted amorphous nature of the NPs could be due to the homogenization, emulsification, and heating process in cosmetic manufacturing. The particle size measurement displayed a broad size distribution in the mNPs ranging from 30–300 nm (maximum particles are in 100 ± 20 nm size) in diameter and a narrow distribution (90–130 nm in diameter) in the wNPs. Here the increase in mNPs grain size (> 200 nm) was observed as a result of agglomeration during the drying process. The Raman spectra (Fig. 1c) of mNPs and wNPs showed characteristic Raman shifts at 1062.02, 1132.10, 1299.05, and 1444.69 cm⁻¹ corresponding to the C-C stretching, CH₂ twist and CH₂ bending vibrations of polyethylene particles, respectively.[29, 30] Since, polyethylene particles are the most prominently (i.e., 93%) used polymer type in cosmetics, it could be the highest fraction in the isolated mNPs and wNPs. As a result, the polyethylene peaks surpassed the Raman shift of other polymers such as polypropylene, polyethylene terephthalate, polymethyl methacrylate, nylon, etc.[7] This observation indicates both face scrub contain nano-sized plastic particles, particularly of polyethylene plastic-type. Therefore the effects ensue in this study could be due to the polyethylene NPs.

Impact Of NPs On Keratinocytes Viability

Enormous usage of cosmetics on skin fetches a direct interaction of NPs with keratinocytes; this could provoke the physiological, biochemical, and pathological responses in cells. To unveil this, we exposed HaCaT cells to different concentrations of PSNPs, mNPs, and wNPs for six consecutive days. Resulted viability graph shows an evident cell viability loss at the higher concentrations of mNPs and wNPs (Fig. 2), but no pronounced inhibition by PSNPs and low concentrations of mNPs and wNPs. Remarkably the wNPs exhibited prominent inhibition at higher concentrations, and hence the concentration range was limited to 250 µg/ mL. The observed cell death at high concentrations could be due to the cell damage produced by NPs via physical interaction. Besides, the adsorbed materials and additives used for plastic production may also be augmented the cytotoxic effect[31]. Herein, the pristine PSNPs did not cause acute toxicity in cells as reported by the previous in vitro studies[31, 32, 33]; however, a noticeable
increase in the cell viability over control was recorded after 48 hrs of exposure. The cell viability rate increased the overall viability of PSNPs and lower concentrations of mNPs and wNPs exposed cells than the control. The observed growth pattern in the cells exposed to pristine NPs certainly harmonized the assumption of chemically rather inert polymers are not expected to produce pronounced cellular toxicity per se. The sudden increase in overall cell viability in NPs exposed cells will be discussed below.

**Oxidative Stress Generation In The HaCaT Cells**

In addition to the cell viability, the unspecific stress induced by NPs within the cells was estimated using reactive oxygen species (ROS) assay. Upon observation, the ROS level reached a maximum at 48 and 72 hrs post-NPs exposure, followed by a steady downward stream at 96, 120, and 144 hrs was recorded (Fig. 3). This indicates the NPs interference in cells was high during 48 and 72 hrs, later due to either defensive action against the generated ROS or invaded NPs or destruction of cells, the ROS level was gradually reduced [34, 35]. During metabolism, ROS can be produced naturally in keratinocytes, but it can be rapidly removed by enzymatic (catalase, superoxide dismutase, thioredoxin-reductase, glutathione peroxidase, and GSH-reductase) or non-enzymatic (ascorbic acid, tocopherol, ubiquinol, and GSH) anti-oxidants [36, 37]. As soon as the ROS production overwhelms the anti-oxidant defense, the equilibrium between pro-oxidant and anti-oxidant becomes affected, leading to the oxidative damage in nucleic, lipids, and proteins, followed by the destruction of cells [36, 38, 39, 40]. On the contrary, low level of ROS acts as signaling molecules that promote cell proliferation [41], while the intermediate level of ROS elicits several biological responses such as autophagy, senescence, etc., which leads to apoptosis and inflammation [42, 43]. Because of these phenomena, increased toxicity at the high concentration of mNPs and wNPs, increased viability in the PSNPs as well as low concentration of mNPs and wNPs was observed [42, 43, 44]. We suspect that the PSNPs and low / sub-lethal concentrations of mNPs and wNPs might have stimulated the ROS mediated biological responses in cells. Before perceiving this, there are two queries, how the cells with spectacular differentiation mechanisms recognize and engulf the NPs, and how the internalized NPs interfere with the cellular process at certain time points ought to be answered.

**Mechanism Of Cell Uptake**

For scrutinizing the NPs internalization, the fluorescently labeled polystyrene NPs (FLPS) was exposed to the cells for 12, 24, 48, 72, 96, 120, and 144 hrs. Fluorescence microscopy examination showed minimal accumulation of FLPS at 12 and 24 hrs and a maximum internalization at 48 and 72 hrs post-exposure, but no further accumulation was observed at 96 and 120 hrs, which indicates the interruption in internalization process. The halt in the internalization process could be due to the commencing of the ROS defense mechanism against oxidative stress. On the other hand, the exclusion experiment displayed a gradual decrease in the FLPS concentration in cells at 96 and 120 hrs and complete exclusion of FLPS at 144 hrs (Fig. 4). It appears that the gradual reduction of FLPS in cells could be due to the ROS
mediated decease of cells or the rapid exclusion of internalized FLPS or both. As a result of the increased NPs internalization at 48 and 72 hrs post-exposure (Fig. 4), the elevated ROS level in PSNPs, mNPs, and wNPs exposed cells was observed in the ROS assay. Additionally, the recorded reduction in the ROS level could be due to the exclusion of NPs from cells/ blocking of NPs internalization/ the defensive anti-oxidant activity by the cells/ cell death. The results presented herein suggest that the NPs internalization is not an immediate process; indeed, the NPs required an incubation time to achieve recognition and internalization by cells.

We strongly suspect that the spectacular differentiation mechanisms of keratinocytes recognized the NPs as the external substance and prevented its entry for up to 12 hrs. Later due to the alteration or modification in their surface property, the NPs might have attained cell’s recognition. As described in our previous study, the NPs adsorbs biological macromolecules, especially proteins on its surface known as corona formation, thereby mimic as protein aggregates [15]. To verify the corona formation on the NPs surface, the pristine PSNPs were introduced into the DMEM medium and incubated for 6, 12, and 24 hrs, and then the particles were examined under HR-TEM [15]. Resulted TEM micrographs (Fig. 5) showed the corona formation (Fig. 5a, b) as well as the adsorption of biomolecular aggregates (Fig. 5c-e) on PSNPs surface and mNPs (Fig. 5f). The total number of coronated particles and corona thickness was high in 24 hrs of exposure than in 12 and 6 hrs. Generally, the plastic particles adsorb organic and inorganic substance quite rapidly; herein, the corona formation was delayed due to the limited availability of biomolecules in DMEM, and hence the FLPS internalization commenced after 24 hrs (Fig. 4). The corona formation rate and corona thickness are directly proportional to the biomolecule availability; for instance, the multi-layered protein corona with few 100 nanometers size was achieved in less than 2 hrs in the human serum albumin, human blood, and plasma [15]. Therefore, the corona formation and internalization process can be quite rapid in the human and animal systems. These observations so far evidenced that because of the adsorbed protein and other molecules, the NPs imitate as protein/biological aggregates and succeed the cellular recognition, thereby, trigger the internalization mechanism.

As a result of false identity, the cells might have internalized the coronated-PSNPs, -mNPs and -wNPs through macropinocytosis, an ideal mechanism that internalizes protein aggregates [45, 46, 47, 48]. Yet the macropinocytes activation mechanism by protein aggregates remains unidentified [49]. The HR-TEM micrograph (Fig. 6) clearly illustrates the binding of coronated NPs on keratinocytes surface (Fig. 6a, b) triggers the pinocytic cups/ large membrane ruffles production (Fig. 6c, d), which then folds back on the cell surface along with the coronated NPs and extracellular uid (Fig. 6e) and finally produces a membrane surrounded intracellular compartment (Fig. 5f) [50]. Having settled with the internalization process, first, the fusion of the lysosome with macropinosomes was examined under CLSM (Fig. 7). For this experiment, FLPS that emits green fluoresce (at 485 nm) while exciting at 441 nm, and neutral red (a lysosomal probe) [51] which emits red fluoresce (at 610 nm) while at 541 nm excitation [52] were used. Under CLSM, the intense green and red fluorescence were observed respectively from the FLPS containing macropinosomes (Fig. 7f) and neutral red stained lysosomes (Fig. 7g). The stratified images of red and green channels depicted macropinolysosomes (yellow color) generated through the fusion of lysosomes
with macropinosomes (Fig. 7h). Further, the accumulation of lysosomes was noticed around macropinosomes, which indicates the fusion attempt. The formation of macropinolysosomes represents the commencement of degradation of protein covered NPs.

The Fate Of NPs During Macropinolysosomal Activity

Generally, the toxic substances and undigested materials from the macropinolysosomes are expelled from cells through exosomes or naked or by cell death in an extreme situation. To determine the fate of PSNPs subjected to macropinolysosomal action, the culture medium was collected and examined under HR-TEM (Fig. 8) during 72 and 96 hrs post-exposure (based on the FLPS exclusion assay). It is essential to mention that the culture medium (with PSNPs) was removed and replaced with the fresh medium (without PSNPs) at 48 hrs post-exposure. Resulted electron micrographs presented partly damaged (Fig. 8b-f), disintegrated (Fig. 8g-j), and enlarged PSNPs (Fig. 8m-o) due to various enzymatic actions in the macropinolysosomes. The corrosion of PSNPs within the macropinolysosomes may lead to leaching of styrene molecules and additives, which can be subsequently released into the cells. The release of styrene molecules from PSNPs treated cells was determined using gas chromatography along with the styrene standard [53] (data not presented). Further, the corrosion and release of styrene molecules into the cell raises concerns about the easily dischargeable endocrine-disrupting compounds such as bisphenol A, nonylphenol, and octylphenol used in plastics production [54, 55, 56, 57]. The enlargement in PSNPs could be due to the protein-mediated coalescence effect between the coronated PSNPs under the protein-rich environment [15]. All the above observations clearly confirm the surface modifications of NPs eventually triggers the internalization followed by the lysosomal activity, ROS generation, and subsequent elicitation of oxidative stress-mediated cellular responses.

The Cytoprotective Response Of NPs Exposed Cells

The absence of significant cytotoxicity in the cell exposed to PSNPs and low concentration of mNPs and wNPs clearly indicates the exertion of cytoprotective mechanisms, especially inhibition of cell proliferation, senescence, and autophagy. For perceiving this, the cells were exposed to the biological effective single dose of lethal and sub-lethal concentrations of NPs. Primarily, the cell proliferation inhibition in the NPs internalized cells was determined using the cumulative population from succeeding cultures. Viable cell count indicated a gradual and concentration-dependent decrease in the population of PSNPs, mNPs, and wNPs internalized cells after 72 hrs (Fig. 9a). However, during the microscopic examination, the trypan blue uptake in the NPs internalized cells was measly observed. This clearly signifies that the cells are certainly seized proliferation but metabolically active and yet remain viable. Among the NPs, significant growth inhibition was observed in wNPs, followed by mNPs and PSNPs. The relative difference of proliferation index calculated from the NPs internalized cells and control cells exhibited a concentration and NPs physical properties dependant inhibition in the proliferation index (Fig. 9b). Additionally, the observed effects so far from styrene molecules justify the possible contribution
of leachates from macropinolysosomes in the cellular responses. Further, the results are in close
correlation with the MTT assay signifying a single dose of NPs can cause the cytostatic effect in cells.

In general, the cells under permanent growth arrest can eventually turn into senescent cells [58, 59]. The
HaCaT cells exposed to PSNPs (10, 100 & 500 µg/mL), styrene (10, 100 & 500 µg/mL), mNPs (1, 50 &
100 µg/mL), wNPs (1, 25 & 50 µg/mL) and H$_2$O$_2$ (25, 50 & 100 µM) for 48 hrs were washed and then
incubated with NPs free medium. At 72 hrs post-exposure, the cells were stained for SA–β–gal activity
and examined under a microscope (Fig. 10). Microscopic analysis revealed the concentration-dependent
increase in the percentage of SA–β–gal–positive cells in PSNPs (37.5 ± 1.0, 44.5 ± 0.4 & 68.9 ± 1.0%),
mNPs (15.5 ± 0.6, 43.4 ± 0.3 & 55.3 ± 1.2%), wNPs (37.9 ± 1.1, 59.2 ± 0.5 & 73.5 ± 0.0%), styrene (34.2 ± 0.1,
67.6 ± 0.4 & 72.2 ± 0.5%) and H$_2$O$_2$ (71.7 ± 1.5, 85.4 ± 1.0 & 86.0 ± 1.3%) treatment, respectively.
Additionally, the typical senescent morphology, i.e., enlarged and flattened cells, as well as cytoplasmic
granules accumulation, was detected in the NPs, styrene, and H$_2$O$_2$ treated cells [60, 61]. Certainly, the
cellular senescence can be accompanied by the following phenotypic alterations such as chromatin
modification, metabolic refinement, high autophagy activity, and production of proinflammatory
secretome [58]. Among the phenotypes, autophagy, a genetically regulated bulk degradation process, was
detected in the NPs treated cells (Fig. 11). Autophagy is a cell survival mechanism where the damaged
cytoplasmic organelles and long-lived proteins can be degraded with the help of lysosomes [62, 63].
Recent studies on keratinocytes indicated that autophagy is associated with the increased anti-apoptotic
effects [64, 65, 66] and plays a crucial role in keratinocytes' biology and pathology. The keratinocytes
exposed to mNPs (Fig. 11a-d) and wNPs (Fig. 11e-h) displayed a series of autophagy structures namely,
autophagosomes (Fig. 11a,f), autophagolysosome (Fig. 11b,c,g) and autolysosome (Fig. 11a,d,e,h).
These structures evidence that the damaged intracellular organelles are actively engulfed by
phagophores eventually turns into autophagosomes; after maturation, the autophagosomes are fused
with lysosomes and produced autophagolysosomes/autolysosomes where the damaged organelles are
actively digested. The observed proliferation inhibition, senescence, and autophagy certainly prove the
cell’s determination to maintain cellular homeostasis via cytoplasmic and organelle turnover against a
low level of ROS stress. The results further emphasize that all those ROS mediated molecular pathways
may be interconnected [64, 67]. However, the increased level of cytoprotective events could also trigger
inflammation and apoptosis [42, 43, 65, 67].

Recent immense attention paid on the cytotoxicity of micro/nano-plastics in human and animal models
evidenced that plastic particles produce oxidative stress in cells at low concentration and cytotoxic at
higher concentrations [68, 69]. In the present study, we have provided insights into the concentration-
dependent regulatory, cytoprotective, and cytotoxic effects in HaCaT cells produced by smaller plastic
particles. Herein, we present three lines of evidence that may help to close the existing knowledge gap on
the NPs internalization and cell response. First, plastic particles mimic as protein aggregates via protein
corona formation on their surface that triggers and accelerates the internalization process. Second, the
internalized NPs are subjected to the lysosomal activity, which damages the NPs leading to the plastic
molecules and additives release into the cells, thereby accelerate oxidative stress. Third, the ROS
mediated down-regulation of cell growth and proliferation inhibition induces autophagy, followed by premature aging of cells. Therefore the increased use of nano/micro- plastic particles containing cosmetics may lead to premature aging in the skin cells, which may result in pigmented stains and deep wrinkles, and further increase the risk of skin cancer [70, 71].

**Conclusion**

While the number of cosmetic products with the increased amount of nano/micro sized-plastic particles continue to increase, no relevant studies on the internalization and accumulation of NPs on skin cells and its cytotoxic effects have been conducted. Therefore, in the present study, we used HaCaT cells to evaluate the potential risk of NPs exposure on human skin. To the best of our knowledge, this is the first study that demonstrates the protein corona-mediated entry of NPs followed by potential disintegration by lysosomal activity and release of additives along with the extensive ROS production in cells, which may lead to concentration-dependent cytoprotective and cytotoxic effects. Even though the exposure concentrations to human in every single use has not precisely calculated, the concentrations used herein were still orders the magnitude of realistic human exposure. Collectively, the findings will shed additional light on the effects of NPs on the human cells. At the outset, our finding emphasizes the significant need for new regulations and guidelines in the nano/micro-plastics usage in CPCP.

**Materials And Methods**

**Materials, reagents and cell line**

The polystyrene NPs- 100 nm (PSNPs) (Catalog # 108821-10, Corpuscular Inc., NY, USA), and fluorescently labeled polystyrene NPs (FLPS- 200 nm) (Fluoresbrite® Yellow Green 0.20 µm Microspheres, Polysciences, Inc, Warrington, PA) were commercially procured. Styrene standard (45993, 250 mg) and 2', 7'-Dichlorofluorescin diacetate (DCFDA) were obtained from Sigma Aldrich (St. Louis, MO). DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride) and MTT (3-[4, 5-dimethylthiazol-2-yl] -2, 5-diphenyl tetrazolium bromide) were procured from HiMedia, India. Senescence β-Galactosidase Staining Kit (#9860, CST) was purchased from Cell Signaling Technology (Beverly, MA, USA).

Human keratinocytes (HaCaT cells) were maintained in DMEM (Dulbecco's Modified Eagle's Medium, HiMedia) containing 4.5 g/l glucose and sodium pyruvate supplemented with 10% FBS, 2 mM L-glutamine, 100U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B at 37 °C under a 95% humidified incubator with 5% CO₂. All the cell culture studies involved in this study were performed using the same conditions.

**Isolation Of NPs From Face Scrubs**
NPs were isolated from commercial face scrubs meant for men and women, respectively, using sequential filtration steps[13]. Herein, face scrubs was selected due to the rapid isolation and significant recovery of plastic particle alone compare to other cosmetic products (this data has yet to be published). Briefly, 0.2 g samples from 10 replicates of each brand were diluted separately in 10 mL of filter-sterilized ultrapure hot water, subsequently filtered through 20–25 µm (Grade 2 Whatman®), 2.5 µm (Grade 41 Whatman®), 0.45 µm (Acrodisc® syringe filter) and 0.2 µm (Acrodisc® syringe filter) filters. The filtrates obtained from 0.2 µm filter were pooled together with the respective brands and dried under desiccator. Resulted powders were subjected to Fourier Transform Raman Spectroscopy (FT-Raman) (PerkinElmer 1600 instrument, USA) and HR-SEM (Carl Zeiss Evo 18 SEM, Germany) analysis. Hereon, the extracted NPs were abbreviated as mNPs and wNPs, where m symbolized for men and w is for women. Since the brand names are not of particular relevance to the study, the samples were mentioned as face scrubs for men and women.

**PSNPs, mNPs And wNPs Interaction With Keratinocytes**

**Cytotoxicity assay**

HaCaT cells (1 × 10^4) were seeded in 96 well plates and incubated. After achieving confluence, the DMEM was replaced with fresh medium containing different concentrations of PSNPs (25, 50, 100, 250, 500 µg/mL), mNPs (25, 50, 100, 250, 500 µg/mL), wNPs (5, 10, 50, 100, 250 µg/mL) into respective wells and incubated. After every 24 hrs interval, the cell viability was measured using MTT assay. Herein, the experiment was carried out six consecutive days because the majority of the MPs/NPs experiments on the marine and terrestrial life were conducted for 24 to 48 hrs.

**Determination of intracellular oxidative stress**

The reactive oxygen species (ROS) production in HaCaT cells exposed to various concentrations of PSNPs, mNPs, and wNPs was measured for six consecutive days using DCFDA assay. Briefly, the NPs treated cells were washed twice with PBS at every 24 hrs intervals; 10-µM DCFDA was added and incubated for 60 min. In the case of ROS production in cells, the non-fluorescent DCFDA oxidized into green fluoresce 2', 7' –dichlorofluorescein (DCF). The level of fluorescence is directly proportional to the oxidative stress in the cells[72]. After 60 min, the cells were washed with PBS twice and measured for fluorescence under spectrofluorometer (JASCO FP-8300, Japan) using the excitation and emission wavelengths of 495 and 525 nm, respectively. Additionally, hydrogen peroxide (25, 50, 100, 250, 500 µM) was used as the positive ROS indicator, and the analytical standard styrene was used to assess the effects of styrene molecules.

**Degree Of NPs Cellular Internalization And Exclusion**

**Determination of NPs internalization**
To study the NPs internalization, retention, and elimination, herein, we used the green fluorescent FLPS particles. The HaCaT cells \((8 \times 10^3)\) were exposed to 1:500 dilutions of FLPS and incubated. After every 24 hrs interval, the cells were washed thrice with PBS, fixed with 3.7% formaldehyde, stained with DAPI, and examined under a fluorescence microscope (EVOS™ FLoid™ Cell Imaging Station, Life Technologies, Carlsbad, CA).

Another set of washed cells were supplemented with fresh medium (without FLPS) and incubated for the evaluation of retention and exclusion activity. Post-incubation, the cells were washed, fixed, and stained for microscopic examination.

**Mode of NPs internalization in cell**

The keratinocytes exposed to the PSNPs were fixed with 2% glutaraldehyde, harvested, and then embedded in resin for ultra-thin sectioning under ultra-microtome (Leica ultra-cut UCT UC7, Austria)[73]. The ultra-thin sections were placed onto a copper grid and stained with uranyl acetate (4%) and lead citrate (1%), and observed under the high-resolution transmission electron microscope (HR-TEM) (Technai, G2 20 Twin, FEI, USA).

**Lysosome labeling with neutral red**

The keratinocytes exposed to FLPS were washed thrice with PBS and immersed with the neutral red solution (2 mM) for 5 min. Then the cells were washed thrice with PBS, mounted and observed under confocal laser scanning microscope (CLSM) (Zeiss LSM 710, Carl Zeiss, Germany)[52].

**Determination of NPs exclusion from cells**

The cell-free medium from the PSNPs exposed cells was collected and centrifuged at 4000 rpm for 5 min. The pellet was dissolved in ultra-pure water, drop coated immediately onto a TEM grid, air-dried, and observed under HR-TEM. In a similar fashion, the PSNPs incorporated in the sterile DMEM were also examined under HR-TEM[15].

**Assessment Of Cellular Response Against Internalized NPs**

**Trypan blue cell proliferation assay**

For determining the proliferation rate of NPs internalized cells, trypan blue staining was performed, where the viable cells remain unstained, and the dead cells uptake blue color[74]. Briefly, HaCaT cells \((3 \times 10^4)\) were seeded in a 24 well plate and treated with different concentrations of PSNPs, mNPs, wNPs, styrene, and H\(_2\)O\(_2\). Here, H\(_2\)O\(_2\) was used as a positive control for cell proliferation inhibition. After the required internalization time, the culture medium was replaced with DMEM (without NPs) at every 24 hrs interval and incubated. At every 24 hrs interval, the cells were harvested, stained with 0.4% trypan blue, and counted in triplicates using a hemocytometer. Cell proliferation rate was calculated from the triplicate experiments using the following equation;
where i is the generation number, and Ni is the number of events in generation i.

**Senescence Associated β-galactosidase Assay**

For assessing the cellular senescence, HaCaT cells \((5 \times 10^4)\) were exposed to PSNPs (10, 100 & 500 µg/mL), styrene (10, 100 & 500 µg/mL), mNPs (1, 50 & 100 µg/mL), wNPs (1, 25 & 50 µg/mL) and \(\text{H}_2\text{O}_2\) (25, 50 & 100 µM) for 72 hrs. After incubation, the cells were washed with PBS, fixed using paraformaldehyde (3.7%), and stained for senescence-associated beta-galactosidase (SA-b-gal) using Senescence β-Galactosidase Staining Kit according to the manufacturer’s instructions. Five phase-contrast images were randomly captured using Carl Zeiss inverted microscope (Axio Vert.A1 FL, Carl Zeiss, Germany). The number of SA-β-gal positive cells (stained in blue) in respective concentrations was calculated and quantified using ImageJ 1.8.0 software (NIH).[35]

**Abbreviations:**

CLSM - Confocal Laser Scanning Microscope; CPCP - Cosmetics and Personal Care Products; DMEM - Dulbecco’s Modified Eagle’s Medium; FLPS - fluorescently labeled polystyrene NPs; HR-TEM - High-Resolution Transmission Electron Microscopy; mNP- NPs isolated from men’s facial scrubs; NPs/MPs - Nano-plastics/ Microplastics; PSNP- PolyStyrene Nano-Plastics; ROS - Reactive Oxygen Species; wNP - NPs isolated from women’s facial scrubs

**Declarations**

**Ethical Approval and Consent to participate:**

Not applicable

**Consent for publication:**

Not applicable

**Availability of data and materials:**

Not applicable

**Competing interests:**

The authors declare no competing financial interests.
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P.M.G., N.C. and PR., conceived the idea and designed the experiments; P.M.G. and K.S.T. carried out the experiments wrote the paper; J.T. and A.M. contributed materials characterization, analyzed data; all authors discussed the results and commented on the manuscript.

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**Figures**
Figure 1

Characterization NPs isolated from cosmetics
Figure 2

Cytotoxic effect of NPs on the HaCaT cells at different time intervals
Figure 3

NPs mediated ROS generation in the HaCaT cells
Figure 4

Time-dependent internalization and exclusion of FLPS particles. (Scale bar; 60 µm)
Figure 5

Protein-corona formation on NPs surface
Figure 6

Mechanism of NPs internalization in HaCaT cells
Figure 7

Lysosomal activity on the internalized NPs
Figure 8
The fate of NPs in macropinolysosomal process

Figure 9

NPs induced growth inhibition and the relative difference in the proliferation index (PI).
Figure 10

NPs induced cellular senescence in the HaCaT cells. a. control, b& c. 25& 50 µM of H2O2, d-f. 10, 100 & 500 µg/mL of PSNPs, g-i. 10, 100 & 500 µg/mL of styrene, j-l. 1, 50 & 100 µg/mL of mNPs, m-o. 1, 25 & 50 µg/mL of wNPs, respectively.
Figure 11

Accumulation of autophagy structures in keratinocytes exposed to mNPs and wNPs.