Laser ablation as a versatile tool to mimic polyethylene terephthalate nanoplastic pollutants: characterization and toxicology assessment

*Davide Magrì1,2****§****, Paola Sánchez-Moreno3****§****, Gianvito Caputo1, Francesca Gatto3,4, Marina Veronesi5, Giuseppe Bardi3, Tiziano Catelani6, Daniela Guarnieri3, Athanassia Athanassiou1, Pier Paolo Pompa3\*, Despina Fragouli1\**

1 Smart Materials, Istituto Italiano di Tecnologia, Via Morego, 30, 16163 Genova, Italy

2 Department of Informatics, Bioengineering, Robotics and Systems Engineering, University of Genova, Via All'Opera Pia, 13, 16145 Genova , Italy

3 Nanobiointeractions & Nanodiagnostics, Istituto Italiano di Tecnologia, Via Morego, 30, 16163 Genova, Italy

4 Department Of Engineering for Innovation, University of Salento, Via per Monteroni, 73100 Lecce, Italy

5 D3-Pharma Chemistry, Istituto Italiano di Tecnologia, Via Morego 30, 16163 Genova, Italy

6 Electron Microscopy Facility, Istituto Italiano di Tecnologia, Via Morego 30, 16163 Genova, Italy

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ABSTRACT: The presence of micro- and nanoplastics in the marine environment is raising strong concern since they can possibly have a negative impact on human health. In particular, the lack of appropriated methodologies to collect the nanoplastics from water systems imposes the use of engineered model nanoparticles to explore their interactions with biological systems, with results not easily correlated with the real case conditions. In this work, we propose a reliable top-down approach based on laser ablation of polymers to form polyethylene terephthalate (PET) nanoplastics, which mimic real environmental nanopollutants, unlike synthetic samples obtained by colloidal chemistry. PET nanoparticles were carefully characterized in terms of chemical/physical properties and stability in different media. The nanoplastics have ca. 100 nm average dimension, with significant size and shape heterogeneity, and they present weak acid groups on their surface, similarly to photodegraded PET plastics. Despite no toxic effects emerged by *in vitro* studies on human Caco-2 intestinal epithelial cells, the formed nanoplastics were largely internalized in endo-lysosomes, showing intracellular biopersistence and long-term stability in simulated lysosomal environment. Interestingly, when tested on intestinal epithelium, nano-PET showed high propensity to cross the gut barrier, with unpredictable long-term effects on health and potential transport of dispersed chemicals mediated by the nanopollutants.

The huge plastic islands floating in the oceans, known as the five gyres, are the symbol of the human impact on the planet, and the emblem of the “Anthropocene”.1,2 Since the beginning of the twentieth century, the development of polymer science and the launch of plastic production have led to one of the greatest revolutions for human society, though, at the same time, to plastics waste accumulation in the marine environment, which is estimated to reach 250 million tonnes in 2025.3,4

Although plastic is considered to be a long-lasting and stable material in the marine environment, weathering processes, such as biotic and abiotic degradation,5,6 induce the breakdown of plastic debris, resulting in the formation of fragments with dimensions ranging from few centimeters to nanometers.3,5 Specifically, UV-light induced oxidation is the most effective abiotic process for the degradation of floating plastics in open waters, while on beaches this phenomenon is enhanced by the contribution of temperature and mechanical abrasion.6–8 There are numerous data on the quantification and characterization of the plastic micrometric fraction in the hydrosphere, while, due to the lack of appropriate methodology to characterize nanomaterials in the environment, there is poor knowledge about the smallest fraction having size <100 nm.5,9,10 These plastic nanoparticles (PNPs) are called nanoplastics. The sub-micrometric fraction deriving from the plastic degradation may penetrate in the marine food chain through the diet11–13 with effects at all trophic levels, *e.g*., traces of such pollutants have been found in protists and zooplankton up to mollusks, fishes, birds and cetaceans.11 Their presence in the food chain may have an impact on humans, and for this reason, numerous studies are underway to investigate their biological effects and define an effective risk assessment.14

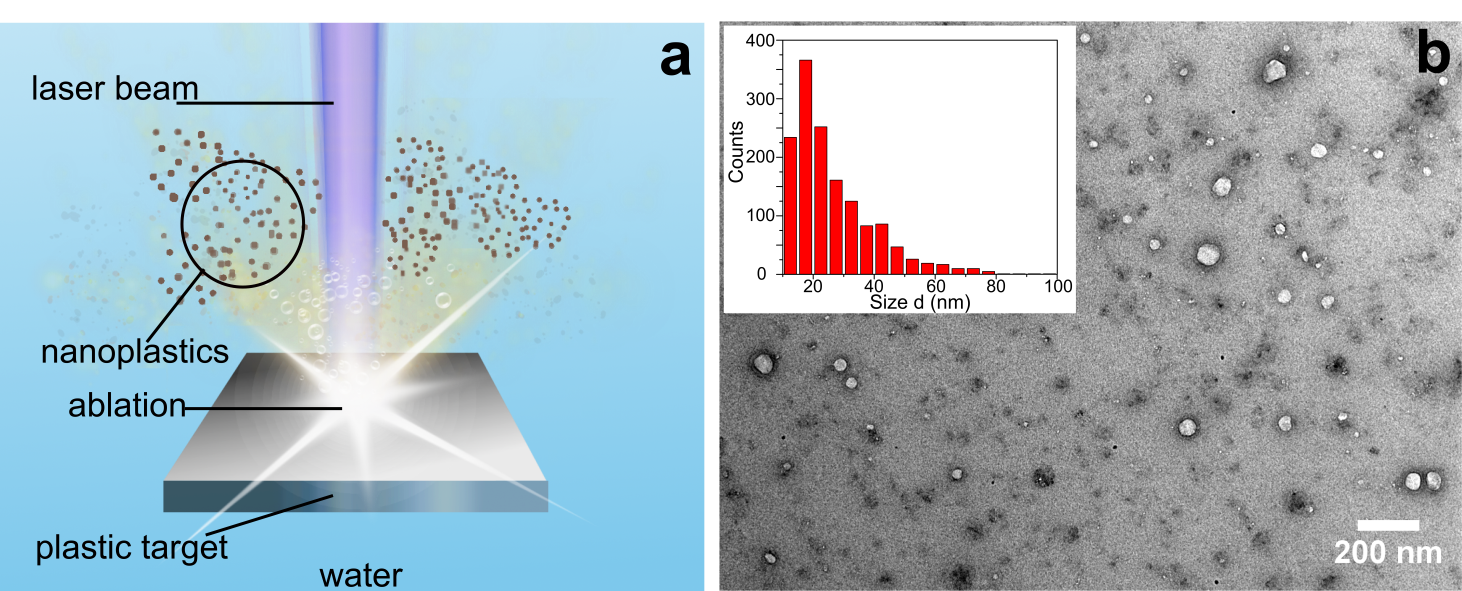
According to the current knowledge, the ingestion of these sub-micrometric particles may elicit negative outcomes on behavior, fertility, hepatic function, immune response, and gene expression pattern,15–20 while lethal effects have been reported only at much higher concentrations compared to the environmental ones.21 Furthermore, the high surface/volume ratio of the smallest plastic debris make them ideal candidates to interact and transfer many hazardous contaminants (polychlorinated biphenyls, organochlorine pesticides, polyaromatic hydrocarbons, *etc*.) to living organisms, increasing their impact and toxicity.22–24

Compared to the microplastics, the smaller size of PNPs (comparable to that of biological components and proteins), associated with their surface characteristics (i.e. surface chemistry and surface energy), may alter their interactions with the surrounding substances and their biological impact.25–27 Several toxic effects for nano and microplastics are described in literature, nevertheless uptake and clearance seem to be the factor mainly influenced by the nanometric dimension of PNPs. Interacting with lipids, PNPs could perturb cell membrane, penetrate by different endocytosis pathways and remain in the organism longer than microsized plastic particles.28–30 As already observed for diverse types of organic and inorganic particles, the smaller size favors their passage through the intestinal barrier.31,32 However, due to the difficulties to identify and isolate the PNPs from the environment, the investigation of their interactions with biological systems and other compounds requires the use of model engineered systems.9 In particular, until now, the studies related to this class of pollutants have been mainly based on the use of monodispersed polystyrene nanospheres, synthesized by colloidal chemistry, following bottom-up approaches.31,33–37 Such protocols typically produce plastic nanostructures with few defects and homogenous chemical composition, substantially different from the PNPs dispersed in the environment, which have irregular shapes and complex surface chemistry, due to the degradation experienced.9 Moreover, the PNPs synthesized by bottom-up approaches could contain solvent residuals, surfactants and other substances used during their synthesis process, that may affect their behavior in aqueous media and their interactions with biological systems.38

The proposed strategy offers the possibility to fabricate diverse types of PNPs that can be used for the realistic investigation of their interactions with biological systems, ranging from cells to organisms, but also with other pollutants. In particular, we present a top-down approach, which, starting from bulk scale materials, allows to obtain nano-sized particles, mimicking a degradation pathway more similar to the one occurring under real environmental conditions. The process, based on the laser ablation of polymer films in water, allows fabricating PNPs avoiding the use of chemicals and precursors. The study is focused on PNPs made of PET, a material largely diffused and mainly used in the food packaging industry for the production of bottles.39 As recently reported, PET microplastics are present in the sea, confirming that such material can degrade and reduce in size when exposed to the environment.40 PET is highly resistant to biotic degradation, therefore the breakdown phenomena are mainly driven by photo-oxidation reactions induced by the UV-light in presence of oxygen, which promote the exposure of carboxyl groups on the polymer surface.6,41,42 We prove that the chemical and physical properties of the as-produced PNPs present similarities with the ones expected to be present in the environment. After the evaluation of the PNP stability in various biological media, detailed studies on their interaction with cells have been performed. In particular, as the food chain was established as the main route of exposure to these pollutants, the biological impact of PET PNPs has been assessed on Caco-2 cells, investigating the PNP uptake and their ability to pass through the intestinal epithelial barrier model.

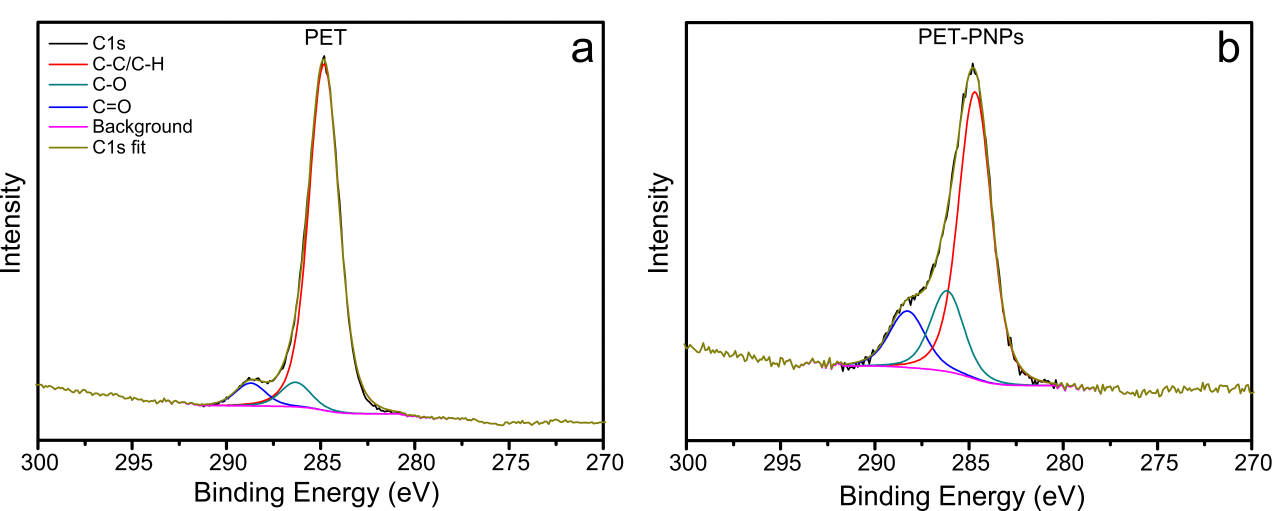
**RESULTS/DISCUSSION**

The main innovation proposed in this work is the use of laser ablation in water as a top-down approach for PNP synthesis, so to develop a model system, which simulates the PNP behavior in natural conditions (Figure 1a). This technique allows to produce nanoparticles without impurities, chemical precursors, and their byproducts.43,44 Laser ablation offers the possibility to control size and polydispersity of the produced samples, as a function of the irradiation fluence, wavelength, and pulses number.45,46 Although this technique has been applied for the synthesis of inorganic nanomaterials (e.g. metals, ceramics and semiconductors), it has been recently proposed for the fabrication of organic polymeric nanoparticles.45,47 Laser ablation in water is a fast and versatile technique and can be applied to several kinds of polymers, preserving their chemical structure even after ablation treatment.45,48 The PET PNPs presented in this study were obtained by shooting 50 irradiation pulses of 248 nm wavelength and 4.5 J/cm2 fluence on a PET solid target. Each ablation cycle had a yield of ~10 µg/ml of PNPs, after the filtration with a 0.2 µm cutoff filter, applied in order to select the nanoscale fraction from the total sample, discarding the micrometric one. The rotavapor treatment allowed to concentrate the PNPs up to 300 µg/ml. As shown by TEM analysis of Figure 1b, the PNPs have an approximatively spherical shape, though some clusters, typical of real PNP samples, were observed in the as-synthesized samples (Figure S1).9 The size distribution of the fabricated PNPs indicated a principal population in the nanoscale, with a diameter of 26.7±14.2 nm (Figure 1b). Their size distribution was also characterized by DLS and Asymmetrical Flow Field-Flow Fractionation (AF4) analysis, and all the results reported an average population with dimensions in the range of few tens to about 100 nm. In particular, concerning DLS, the DH laid at 109.5±13.3 nm, strictly comparable with the gyration radius of 47.1 nm resulting from AF4 analysis, obtained through a sphere model fitting (*cf* experimental details for AF4 analysis and Figure S2 in the Supporting Information).

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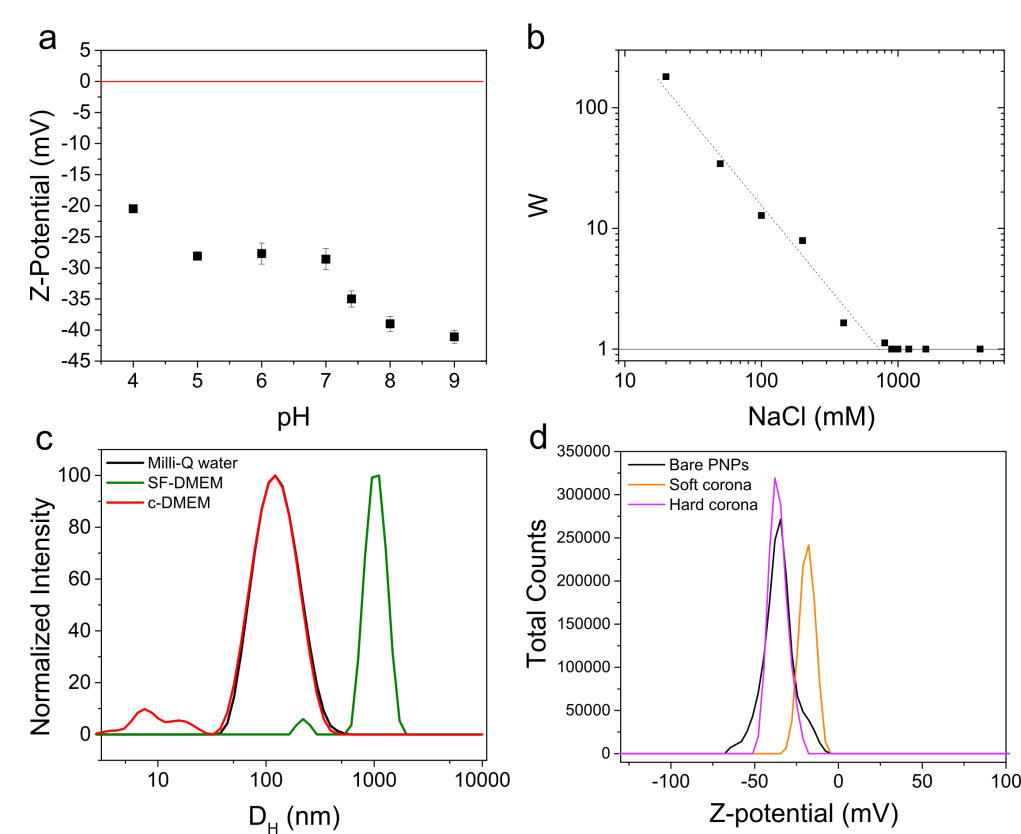
**Figure 1. PNP synthesis. (a)** Schematic representation of the laser ablation procedure applied to produce PET PNPs**. (b)** Representative TEM micrograph and size distribution of PET PNPs.

The evaluation of the surface chemistry of the PNPs is fundamental to correlate their behavior in the aqueous media and the mechanisms leading to the interactions with living organisms.49 To do so, XPS analysis of the pristine polymer and of the formed PNPs was performed (Figures 2a-d and S3). XPS analysis shows that the relative O/C ratio increases from 12.3% for the pristine PET to 32.1% for PET PNPs, indicating a higher degree of oxidation for the PNPs, as determined by the elemental concentration analysis. This is confirmed by the high resolution C1s spectra (Figure 2a-b), which show three different contributions at 284.8, 286.3 and 288.7 eV, corresponding respectively to the C-C and C-H of the phenyl ring, to the C-O, and to the O-C=O bonds.50,51 The increase of the C-O and O-C=O bonds observed in the PNPs, and corroborated by the O1s spectra (Table S1-2, Figure S3), confirmed their higher degree of oxidation and the exposure of new carboxyl groups on the material surface.47,52,53 These findings are in accordance with the formation of carboxyl end-groups, a typical signal of PET that is subjected to photo-degradation promoted by thermo-oxidative and photo-oxidative pathways.6,54



**Figure 2. XPS characterization.** XPS C1s high resolution spectra of the pristine PET (a) and of the as-synthesized PET PNPs (b).

This is further confirmed by the study of the surface charge and size stability of the PET PNPs dispersed in buffer media of different pH values. As shown in Figure S4, the PNPs maintain their original DH within a wide pH range, from 4 to 9, indicating good stability in such conditions. Moreover, as Figure 3a demonstrates, the PNPs are negatively charged and they show lower Z-Pot absolute values at acidic pH compared to those of basic pH, as typically observed in colloidal systems with weak acid groups on their surface.55 These results confirmed the presence of carboxylic acid groups on the surface of the PNPs.

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**Figure 3. PNP surface charge and stability characterization**. Z-Pot of PET PNPs as a function of pH **(a)**. Fuchs factor (W) *versus* NaCl concentration. The dashed line, that fits with the points at low aggregation regime, helps to locate the CCC value **(b)**. Hydrodynamic diameter (DH) distribution of PNPs in Milli-Q water (black) after incubation for 2.5 h in serum-free DMEM (SF-DMEM; green), and in DMEM supplemented with 10% of FBS (c-DMEM; red) **(c)**. Z-Pot of PET PNPs before (black) and after incubation with 10% of FBS: soft corona (orange) and hard corona (purple) **(d).**

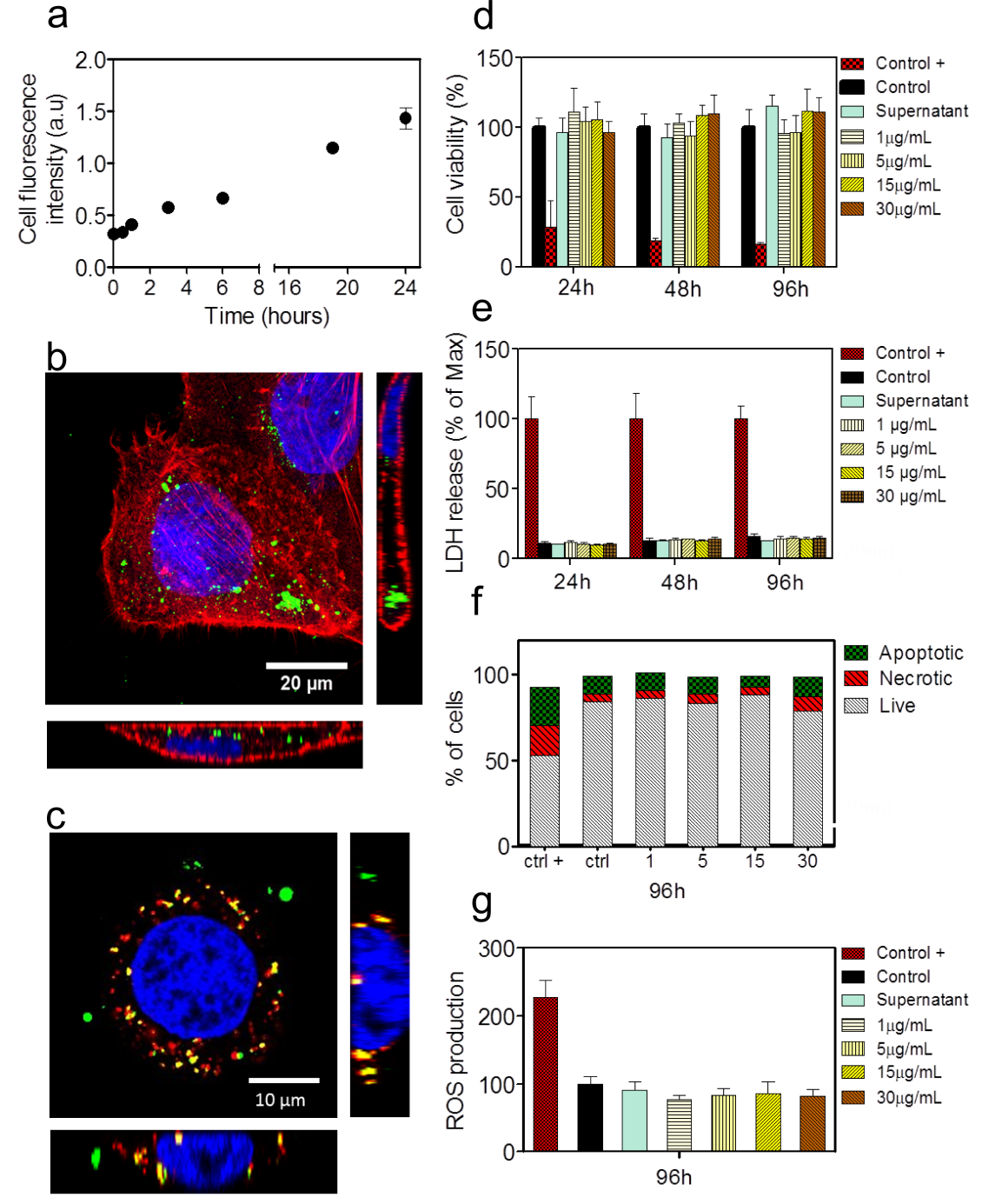
The effect of the salinity on the colloidal stability was then studied, by the analysis of the coagulation kinetics at different NaCl concentrations and calculation of the stability (Fuchs) factor W and, thus, of the CCC (critical coagulation concentration) and CSC (critical stabilization concentration). W is related to the coagulation probability, with W=1 to indicate a completely unstable system, while W=∞ a totally stable one. As shown in Figure 3b, W decreases gradually with increasing the salt concentration until, at around 700 mM, the CCC value is reached. This is a value typically associated with very stable systems, in which the stability is supplied by strong repulsive forces among particles, due to the high surface charge density. Over the CCC point, the PNPs are unstable and they aggregate with a fast rate. The lack of re-stabilization mechanisms given by the so-called hydration forces in high ionic-strength media is a clear indication of the hydrophobic nature of the PET PNP surface.56,57

The stability was subsequently analyzed by DLS in more complex biological media, namely serum-free cell culture medium (SF-DMEM) and DMEM supplemented with 10% FBS (c-DMEM). Figure 3c shows the size distribution of PET PNPs after 2.5 hours of incubation in the different media. Due to the high salt concentration, PNPs were not stable in SF-DMEM, forming large aggregates, up to micron size. On the other hand, the presence of serum proteins in c-DMEM improves the stability of the PNPs, preventing their aggregation. The aggregation kinetics are shown in Figure S5a. The DH of PNPs dispersed in SF-DMEM was higher compared to PNPs in water already at the first time points studied, indicative of fast aggregation kinetics. On the contrary, the PNPs did not change their size in c-DMEM in the timescale of the experiment (up to 72 hours). The colloidal stability of bare PNPs was increased by the formation of a protein surface layer (protein corona). When PNP-protein complexes were isolated by centrifugation and re-dispersed in high NaCl concentrations, they were completely stable. Unlike bare PNPs, that presented a rapid aggregation regime over 700 mM, PNPs incubated with FBS were stable also at the highest salt concentrations studied, up to 4M (Figure S5b). It is well established that protein surfaces tend to be very hydrated and, thus, additional stability is provided by creating a steric hindrance based on structured water molecules strongly bonded to proteins.58 The formation of a protein corona can also be assessed by studying the surface charge of the PNPs before and after incubation with proteins.59 To understand if the adsorbed proteins have high affinity for the PNP surface (hard corona) or, on the contrary, they are loosely associated to the PNPs and in rapid dynamic exchange with the medium (soft corona), a simple method of “pelleting” can be applied,60 followed by the determination of the Z-Pot (Figure 3d). After one hour incubation with 10% FBS, PNPs were centrifuged to pellet the particle-soft-corona complexes, and their surface charge decreased from -35.0±1.2 mV (in water) to -16.0±1.3 mV. When the pellets were washed three times to discard loosely associated molecules, Z-Pot was ≃-36 mV, i.e. similar to the one of bare PNPs, suggesting the absence of proteins strongly adsorbed to the PNP surface (hard corona). This means that, even if the protein corona is responsible of the stability of PNPs in c-DMEM, this protein layer is poorly associated to their surface but confers them a new biological identity that will determine their interaction with cells.59

As shown so far, this realistic model of PNPs presents an optimal combination of characteristics, such as a good size distribution below 100 nm, hydrophobicity, long-term stability in storage conditions (in Milli-Q water, the PNPs were stable for at least 6 months maintaining the same Z-average) and stability in biological media, to interact with living organisms. Since the oral route is the main way of exposure to PNPs (through the food chain), herein we investigated their interaction with the human intestinal epithelium. The Caco-2 cell line, widely applied in toxicity studies for oral exposure scenarios,61 was selected to perform the *in vitro* assays. First, the uptake of PNPs by Caco-2 cells was demonstrated by flow cytometry and confocal microscopy. In order to follow the internalization of PNPs, fluoresceinamine was conjugated to the carboxylic groups of the PNP surface by means of a carbodiimide (EDC) method. It should be mentioned that such process does not alter the PNP characteristics, such as the DH and Z-Pot (Figure S6).

Figure 4a shows the kinetics profile of the PNP uptake, obtained by monitoring the mean fluorescence values from flow cytometry distributions of ≥100.000 Caco-2 cells, treated for different time periods up to 24 hours, with 30 µg/mL of PET PNPs. The cell fluorescence intensity linearly increased without reaching the saturation, likely due to the splitting of PNPs between cells following cell division.62 The cellular internalization of PNPs was further demonstrated by confocal microscopy studies (Figure 4b,c). In particular, the mechanisms of cellular uptake were studied by monitoring the lysosomal localization of internalized PNPs, as shown in Figure 4c. The PNPs are co-localized with lysosomes after 24 hours of treatment, suggesting that the endocytic pathway is the mechanism used by the PNPs to enter the cells, in accordance with previous studies on diverse types of nanoparticles, such as polystyrene and poly(DL-lactide-co-glycolide).63,64

PNPs inside the lysosomes can be degraded, or persist in case of high chemical stability. The biodurability or biopersistence may influence their long-term toxicity after chronic exposure. To explore this aspect, the PNP response to the intra-lysosomal conditions was studied over time, up to 2 months, by utilizing a simplified cell-free assay that mimics the lysosomal fluid. In particular, the PNPs were dispersed in citrate buffer (pH 4.5) and incubated at 37 °C. As shown in Figure S7, the DH value of the PNPs remained stable also for the longest time points and was comparable to that of as-synthesized PNPs stored in water, suggesting that the lysosomal environment (at least in terms of acidic and temperature conditions) is not sufficient to induce PNP degradation.

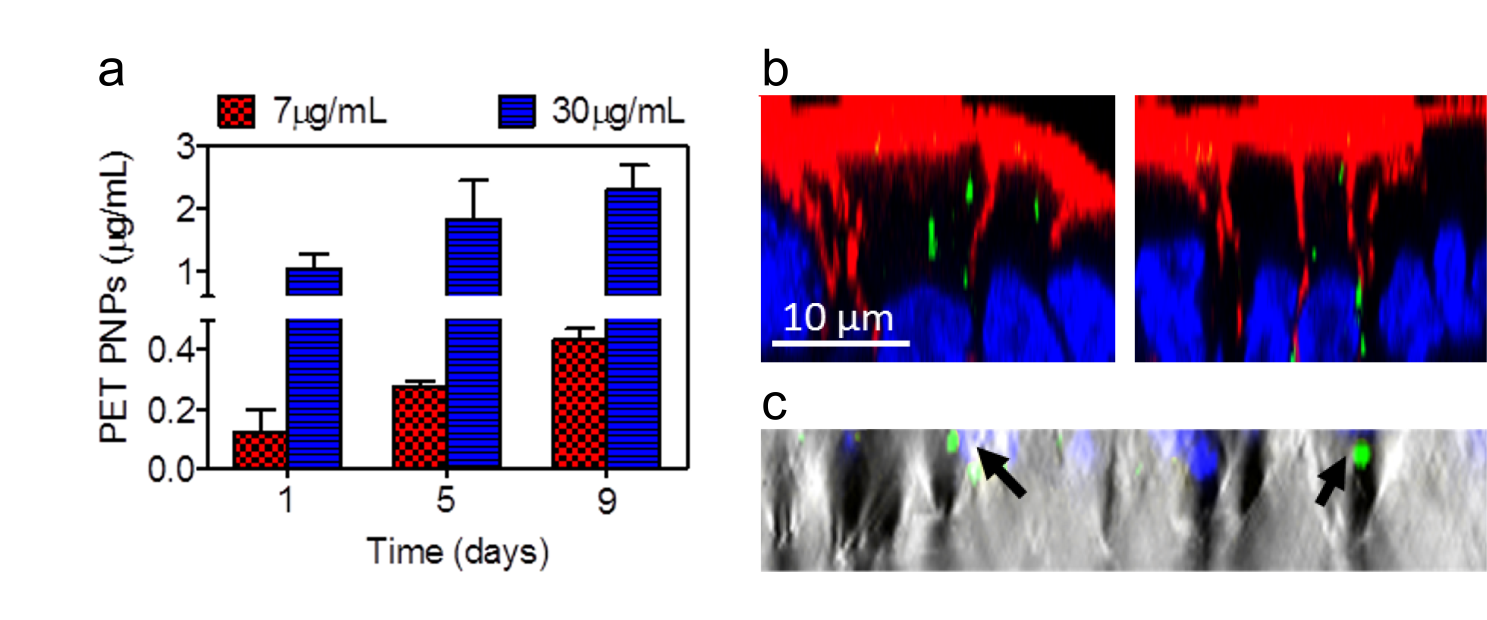
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**Figure 4.** **Uptake, intracellular localization and cytotoxicology of PET PNPs in Caco-2 cell line. (a)** Cellular uptake kinetics of fluorescent PET PNPs by flow cytometry. **(b)** Confocal microscopy of Caco-2 cells incubated for 24 h with fluorescent PET PNPs. Hoechst/nuclei (blue), Phalloidin/actin (red), PET PNPs (green). Lateral boxes represent z-stack projections along x-z and y-z axes. **(c)** Confocal microscopy of Caco-2 cells incubated for 24 h with fluorescent PET PNPs. PET PNPs co-localized with Lysotracker positive organelles of Caco-2 cells. Hoechst 33342/nuclei (blue), Lysotracker (red), PET PNPs (green). Lateral boxes represent z-stack projections along x-z and y-z axes. **(d)** Viability of Caco-2 cells exposed to different concentrations of PNPs for 24, 48 and 96 h. Cell viability of the solution where the PNPs were synthesized was also studied after pelleting the particles (supernatant). Benzalkonium Chloride (BC) (50 µg/mL) was used as positive control. **(e)** Lactate dehydrogenase (LDH) release in cell supernatant after 24, 48 and 96 h treatment with PNPs. Cell lysis solution was used to obtain the maximum LDH release (Positive control). **(f)** Apoptotic, necrotic and live Caco-2 cells after 24, 48 and 96 h of treatment with different concentrations of PET PNPs. DMSO (5%) was used as positive control. **(g)** ROS production by Caco-2 cells after 24, 48 and 96 h of incubation with PNPs. *Tert*-butyl hydroperoxide (TBHP) (100 µM) was used as positive control.

After demonstrating the interaction of PNPs with Caco-2 cells, their toxicity was determined by studying cell viability, integrity of cell membrane, ROS generation, apoptosis and necrosis, and inflammatory response. Caco-2 cells were incubated with 1, 5, 15 and 30 µg/mL of PET PNPs and the toxicological assays were performed after 24, 72 and 96 hours of treatment. To preliminarily assess that the PNP medium does not contain substances that may affect the toxicity results, the chemical composition of the supernatant was investigated by 1H 1D NMR analysis. As shown in Figures S8-9, the concentrations of acetic and formic acids formed in the medium during laser ablation are both reduced resulting sub-micromolar and lower than 5 μM respectively. Therefore, it can be assumed that the results presented below are attributed solely to the interactions of the Caco-2 cells with PNPs and not to the PNP medium contaminations (in any case, in all the cellular experiments presented here, the supernatant was always tested as an additional control). The cell viability was evaluated by an MTS assay and the effect of PNPs on the cell membrane integrity was determined by a LDH assay. Despite the smooth uptake of PNPs, both cell viability and membrane integrity were not affected by the treatment (Figure 4d and 4e). Moreover, PNP treated cells did not show any significant change in apoptosis and necrosis compared to the control group (Figures 4f and S10), in qualitative agreement with previously reported data with 50 nm polystyrene PNPs applied on the same cell line.65 As shown in Figure 4g, cells treated with PNPs did not increase ROS generation, showing similar values to negative controls. Thus, PET PNPs did not induce oxidative stress in this cell line at the concentrations and incubation times studied. Moreover, the exposure of Caco-2 cells to PET-PNPs seems not to affect the production profile of pro-inflammatory cytokine proteins IL-8 and MCP-1 (Figure S11), unlike previous observations with other metal and metal oxide nanoparticle systems.66,67

Despite PET PNPs did not produce short-term toxicity on undifferentiated Caco-2 cells, their potential long-term effects and bioaccumulation cannot be excluded. In fact, overcoming the body barriers, such as gastrointestinal wall, PNPs could reach the blood stream and accumulate into the organism. Hence, considering that the food chain is the main route of exposure to PNPs, a physiologically realistic *in vitro* model of intestinal epithelium was used to investigate the transport of PET PNPs upon ingestion.

The intestinal barrier model was produced by seeding Caco-2 cells on porous filters, as previously reported.68,69 After 21 days of culture, polarized differentiated cell monolayers were obtained with evident formation of the typical tight junctions and microvilli, as shown by TEM imaging (Figure S12). The integrity and confluence of the layers were confirmed by a TEER value of 339 ± 24 Ω cm2.70 Intestinal barriers were then chronically exposed up to 9 days to a PNP suspension containing 7 and 30 µg/mL. The trans-monolayer transport of PET PNPs is shown in Figure 5a, quantified in terms of cumulative transport after 1, 5 and 9 days of treatment. The presence of PNPs in the basal (Bl) medium was found already after 1 day of treatment, and the amount of transported PNPs increased with increasing incubation time. Higher amount of PNPs in the Bl compartment was observed in the inserts treated with 30 µg/mL of PNPs, though the relative transport values were similar at both concentrations. Confocal orthogonal cross sections showed the presence of PNPs inside the differentiated enterocyte-like cells (Figure 5b). A reduction of the PNP uptake was observed, compared to the undifferentiated status (Figure 4b), as previously reported in literature with other organic and inorganic nanomaterials.69,71 Such uptake reduction is likely related to the complex polarized nature of thick epithelial layers. PNPs were also observed in contact with the pores of the filters, confirming their translocation through the intestinal layers (Figure 5c). This was also supported by TEM analysis of the Bl medium, since PET PNPs were observed (Figure S13). These results suggest that PNPs were endocytosed *via* the apical membrane and eventually exocytosed from the basolateral one (namely, transcytosis). The integrity of the barriers was then studied to discard the passage of PNPs through defects of the monolayers and through the paracellular route by disruption of the tight junctions. As shown in Figure S14 a-b, upon chronic incubation, PET PNPs exerted negligible impact on the integrity of the cell layers, confirming the transcytosis as the main mechanism of transport of PNPs through the intestinal epithelium.



**Figure 5.** T**ranslocation of PNPs through the intestinal barrier. (a)** Quantification of PNPs translocated from the Ap to the Bl compartment of the inserts after 1, 5 and 9 days of incubation. **(b)** Confocal fluorescence cross-section images of Caco-2 barriers exposed to PNPs. The PNPs are shown in green; Actin and nuclei were stained with Phalloidin (red) and Hoechst 33342 (blue), respectively. **(c)** Confocal cross-section image of filters where Caco-2 barriers were grown and exposed to PNPs. Fluorescent PNPs (green) were found in contact with the pores (black) of the filters (transmitted light). Cell nuclei are shown in blue (Hoechst 33342).

the cell was previously incubated with LysoTracker

Red (0.75 µ m for 60 min), subsequently exposed to the PNPs

(100 µg mL

–1

for 10 min), and then imaged

Many studies of uptake and translocation *via* intestinal epithelium have been dedicated to a wide variety of polymeric nanoparticles, but only polystyrene has been used to model PNPs up to now.72 Published data on polystyrene PNP uptake have shown high variability mainly depending on particle size, surface properties, and *in vitro* intestinal model.31,64,65,72 In order to clarify the translocation of PNPs, it is necessary to consider the importance of the PNP composition, therefore all the extrapolation based on only polystyrene nanoparticle models have to be taken with caution. Thus, in this study, we improve the knowledge on PNP translocation by studying a different model of PNPs. The study of PET PNPs in a model of intestinal barrier, allowed us to observe the PNP uptake and translocation across the epithelium. This could result in a systemic exposure by reaching the blood stream and spreading in the whole organism. Since the chemical nature of the PNPs allows them to interact with other pollutants, PET PNPs could act as nanocarriers facilitating the passage of toxic substances from the intestinal lumen to the bloodstream.

CONCLUSIONS

In this study, the production of a realistic model of PNPs was achieved through a top-down laser ablation technique. Chemical analysis reinforced the validity of our model, since, in accordance to what has been observed in UV exposed plastic materials in the environment, organic acid groups decorated the surface of laser ablated PNPs. The PNP dispersions showed long-term stability in various biological media and small size, two important factors, which increase the possibility of living organism exposure. The evaluation of the biological impact of PET PNPs upon ingestion on human intestinal cells did not produce any toxic effect on the short-term. However, the PNPs are bio-persistent in lysosomal-like fluid and they are able to efficiently pass through the intestinal epithelia. Since PNPs can adsorb other pollutants from the environment, such as organochlorine pesticides, persistent organic pollutants, polychlorinated biphenyls, further investigations on their ability to act as nano-carriers are needed in future studies.

METHODS/EXPERIMENTAL

***Synthesis and characterization of PET PNPs***

*Synthesis of PET PNPs.*

A top-down physical approach was applied for the formation of the PNPs. In detail, laser ablation of commercial PET films (Goodfellow Cambridge Ltd.) was performed by exposing the samples to a pulsed UV laser irradiation in Milli-Q water, using a KrF excimer laser (irradiation wavelength 248nm, pulse duration of 20 ns, repetition rate of 20 Hz, Coherent-CompexPro 110) coupled with a micromachining apparatus (Optec-MicroMaster). The ablation was carried out with an irradiation fluence of 4.5 J/cm2, by shooting 50 pulses on a 4 cm2 area. To obtain PNPs concentration up to 300 µg/mL, a low temperature (45 °C) rotavapor treatment was performed. In order to remove the big, micrometric sized, particles, the obtained dispersions were filtered (Cellulose Acetate, Ø = 0.22μm, Millipore) before performing all the studies and the characterizations.

*Transmission Electron Microscopy (TEM)*

A transmission electron microscope (JEOL JEM 1011) was used to characterize the morphology and the size of the PNPs and their presence in the biological samples. The images were collected operating at an acceleration voltage of 100 kV and recorded with an 11 Mp fiber optical charge-coupled device (CCD) camera (Gatan Orius SC-1000). The suspension of PNPs was drop casted on ultrathin carbon layered Cu grids (CF300-CU-UL) (Electron Microscopy Science). The PNPs size distribution was determined using the ImageJ software.

*Dynamic Light Scattering (DLS) and Zeta Potential (Z-Pot)*

Hydrodynamic diameter (DH) and Z-Pot of PET PNPs as a function of pH were performed using a Zetasizer Nano S (Malvern Instruments) spectrometer. DLS was studied in Milli-Q water, while for the evaluation of their stability in biological media, DLS was also performed on PNPs in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% of non-heat inactivated HyClone™ Fetal Bovine Serum (FBS, GE Healthcare Life Science). Z-Pot was measured after pouring 10 µl of the PNPs stock (300µg/mL) into 1 mL of a low salinity solution (0.002 M) containing the desired buffer.

*X-ray Photoelectron Spectroscopy (XPS)*

XPS analysis was performed using a SPECS-Lab spectrometer with Al Kα source (hν= 1486.6 eV) operated at 15 kV with an emission current of 10 mA. Charge neutralizer consisting of low-energy (ca. 7 eV) electrons was applied and energy scale calibration was performed by setting the C-C/C-H component of C1s spectrum at 284.8 eV. PNP samples were prepared by drop casting the suspension on a gold substrate dried in vacuum at room temperature. The relative elemental concentrations were estimated from the areas under the C1s and O1s photoelectron peaks weighed by the relative sensitivity factors supplied by the CasaXPS analysis software.

*Colloidal Stability*

PET PNPs were dispersed in simple saline media at different concentrations of NaCl (from 0.002 to 2M). The PNP aggregation kinetics studies were performed spectrophotometrically by monitoring the variation of the optical absorbance (Abs) at λ =570 nm over time. The Abs *versus* time was plotted at every salt concentration, and the slopes of these curves (∂Abs/∂t) enabled the determination of the Fuchs factor (W) defined by the following equation:

Eq.1

where “kf” refers to the fastest aggregation-kinetics rate constant, and “ks” is the rate constant for the slower coagulation regime. The critical coagulation concentration (CCC) and the critical stabilization concentration (CSC), two important parameters in colloidal stability studies, were assessed.55 CCC is the minimum salt concentration at which the most rapid PNP aggregation is observed, and gives information on the surface charge density of the particles. CSC is defined as the minimum salt concentration at which the system begins to re-stabilize when the salinity is increased, and is related with the surface hydrophilicity.

*Protein Corona*

100 μl of PET PNPs (300 μg/mL) were incubated in 1 mL of DMEM supplemented with 10% of FBS for 1 h at 37 °C. The samples were then centrifuged (15 min at 20000g) to pellet the particle−soft-corona complexes. Pellets were re-suspended in 1 mL of Milli-Q water and this procedure was repeated three times to remove proteins with low affinity for the PNP surface. Particle-protein complexes were characterized by DLS and Z-Pot.

***Cell culture conditions***

Caco-2 human intestinal epithelial cells (gently provided by Dr. Isabella De Angelis, Istituto Superiore di Sanità (ISS), Rome, Italy) were cultured with DMEM supplemented with 10% non-heat inactivated HyClone™ FBS, 1% penicillin-streptomycin (Sigma-Aldrich), and 1% non-essential amino acids (Invitrogen) and incubated in a humidified atmosphere with 5% CO2 at 37 °C. Cryopreserved stocks were sub-cultured twice before the experiments.

***Cytotoxicity of PET PNPs in Caco-2 cells***

The effect of PET PNPs on Caco-2 cell was studied by evaluating cell viability measuring their metabolic activity using an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega) and the cell membrane damages by using a lactate dehydrogenase (LDH) assay (CytoTox-ONE™ Homogeneous Membrane Integrity Assay from Promega). The Annexin V-FITC Apoptosis Detection kit (Miltenyi Biotec) was used to detect apoptosis and necrosis of Caco-2 cells after incubation with PET PNPs by flow cytometry, while intracellular ROS generation was measured by using 2’,7’-dichlorofluorescin diacetate (DCFDA) (Invitrogen). For all the assays Caco-2 cells were seeded in 96-well plates at a concentration of 1 x 104 cells/well. Following 24 hours of culture, the cells were exposed to PNPs. Due to the lack of information about the food and environmental concentration, the tests have been performed in a range between 1 and 30 µg/mL, in line with other nanotoxicology *in vitro* studies.38 More experimental details are reported in the supporting information (SI).

***Uptake and intracellular trafficking***

In order to follow the PNPs in the *in vitro* test of uptake and transport, they were fluorescently labelled with 5-Aminofluorescein (Fluoresceinamine, isomer I) (Sigma-Aldrich). The acid groups of the PNPs surface were activated with 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (Thermo Scientific) (20 mM), and then fluorescein amine (50 µg/ml) was covalently coupled. After 2 hours of reaction the PNPs were dialyzed against Milli-Q water, overnight, using a dialysis membrane (MWCO 3500, 1.15 ml/cm, Fisherbrand).

Uptake of fluorescein-PET PNPs by Caco-2 single cells was assessed by flow cytometry and confocal microscopy. Cells were seeded in 12-well plates at a density of 15x104 cells per well one day before treatment. Then, they were treated with 30 µg/mL of PNPs for 30 min, 1, 3, 6, 19 and 24 hours and the fluorescence intensity was measured by flow cytometry. Caco-2 cells (5 x 104) grown on glass coverslips of 12 mm diameter and incubated for 96 hours with PET PNPs, were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.01% Triton X100 for 5 min and blocked with 0.5% bovine serum albumin in PBS for 20 min. They were then stained with 0.1 nM Alexa FluorTM 594 Phalloidin for 30 min and Hoechst 33342 (Thermo Fisher Scientific) at a concentration of 5 µg/mL for 5 min, to localize actin microfilaments and cell nuclei, respectively.

To investigate the subcellular localization, living single Caco-2 cells exposed to fluorescein-PET PNPs (30 µg/mL) for 24, 72 and 96 hours where incubated for 1 hour at 37 °C with LysoTracker™ Red DND-99 (Thermo Fisher Scientific) in DMEM at a concentration of 75 nM. The cells where then washed with PBS and incubated with Hoechst 33342 at a concentration of 5 µg/mL for 5 min to localize their nuclei. Image acquisition was performed by a confocal microscope (Leica TCS-SP5) using a 40x oil objective, with ex of 405, 488 and 561 nm and a resolution of 1024 × 1024 pixels. Image analysis was performed using ImageJ software.

*PNPs characterization in lysosomal-like fluid*

PNPs (30 µg/mL) were dispersed in a 0.1 M citrate buffer (Sigma-Aldrich) presenting a pH of 4.5. The incubation was performed at 37 °C and the DH was monitored through DLS analysis at several time points up to 2 months.

***Intestinal layer formation***

Caco-2 cells were seeded in 12-well plates onto porous Millicell Hanging Cell Culture Inserts (diameter *(d)*: 12mm; surface *(A)*: 1.1cm2; pore size: 0.1 µm) in 0.5 mL of DMEM at a seeding density of 17 x 104 cells/insert in the apical (Ap) side. 1.5 mL of the medium was poured into the basolateral (Bl) compartment. Cells were grown for 21 days and the culture medium was changed every two days to allow the formation of tight junctions and microvilli.68 After 21 days, confluent and differentiated cell monolayers were formed, and the integrity of the epithelia was confirmed by measuring the Trans-epithelial Electrical resistance (TEER) (methods in supporting information file).

***Transport of fluorescein-PET PNPs through intestinal epithelia***

Caco-2 cell monolayers were incubated with 7 and 30 µg/mL of fluorescein functionalized PET PNPs dispersed in phenol red-free 10% FBS supplemented DMEM (Thermo Fisher Scientific). In order to maintain stable the concentration of PNPs in contact with cells, the medium was changed every 2 days up to 9 days. The transport of PNPs through the intestinal epithelium from the Ap to the Bl compartment was quantified after 1, 5 and 9 days of exposure. The fluorescein-PNPs were quantified using a spectrofluorometer (FluoroMax-4, Horiba). The sample solutions, placed in a quartz cuvette with a path-length of 1cm, were excited at a ex of 495 nm. The construction of the calibration line and the measurements were done referring to the peak of emission at a em of 516 nm.

ASSOCIATED CONTENT

**Supporting Information**. Experimental details, TEM image of PET PNP clusters; AF4 analysis; species percentage calculated on the C1s; species percentage calculated on the O1s; high resolution O1s XPS spectra of the pristine PET and of PET PNPs; DLS analysis of the pH influence on particles size and aggregation; aggregation kinetics of PET PNPs in serum free DMEM and DMEM supplemented with 10% of FBS; Z-Average of particle-soft-corona complexes dispersed in different concentrations of NaCl solutions; size distribution of fluorescent PET PNPs and Z-potential distribution of PET PNPs and of fluorescent PET PNPs; 1H NMR spectra of the ablation medium; 1H NMR spectra of the ablation medium before and after the concentration of PNPs in rotavapor and discussion; size distribution of PET PNPs incubated for 2 months in lysosomal-like fluid; flow cytometry scatter plots of Propidium Iodide *versus* Annexin V-FITC labelled Caco-2 cells after treatment with PET PNPs for 96 hours; inflammatory response of Caco-2 cells upon exposure to PET PNPs and discussion; TEM image of differentiated Caco-2 cells; TEM image of PET PNPs in the basal medium; TEER measurements of Caco-2 epithelium and Lucifer Yellow transport across the Caco-2 layer treated with PNPs and discussion.

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AUTHOR INFORMATION

Corresponding Author

\* Dr. Despina Fragouli. E-mail: despina.fragouli@iit.it;

\* Dr. Pier Paolo Pompa. E-mail: pierpaolo.pompa@iit.it;

**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. § These authors contributed equally.

ABBREVIATIONS

PNPs Plastic nanoparticles, PET Polyethylene terephthalate, AF4 Asymmetrical Flow Field-Flow Fractionation, CCC critical coagulation concentration, CSC critical stabilization concentration, TEER Trans-epithelial Electrical resistance, LY Lucifer Yellow.

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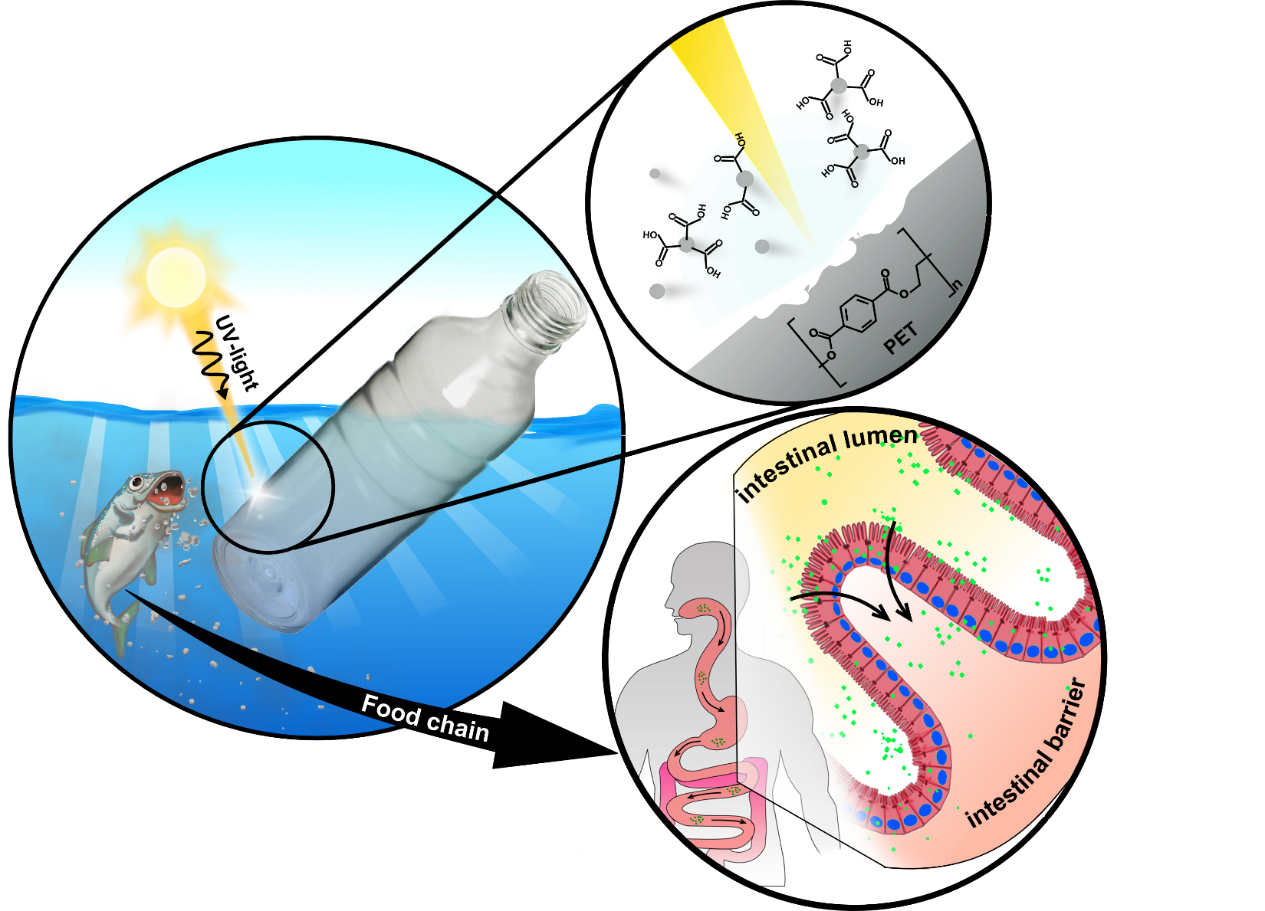
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**TOC Graphic**

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