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## Stability and dispersibility of microplastics in experimental exposure medium and their dimensional characterization by SMLS, SAXS, Raman microscopy, and SEM

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1           **Stability and dispersibility of microplastics in experimental exposure**  
2           **medium and their dimensional characterization by SMLS, SAXS, Raman**  
3                               **microscopy, and SEM**

4  
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## 17 **Abstract**

18 The plastic production that contributes to the global plastic reservoir presents a major challenge for  
19 society in managing plastic waste and mitigating the environmental damage of microplastic (MP)  
20 pollution. In the environment, the formation of biomolecular corona around MPs enhance the stability  
21 of MP suspensions, influencing the bioavailability and toxicity of MPs. Essential physical properties  
22 including MP stability, dispersibility, agglomeration, and dimensional size must be precisely defined  
23 and measured in complex media taking into account the formation of a protein corona. Using static  
24 multiple light scattering (SMLS), small angle X-ray scattering (SAXS), Raman microscopy, and  
25 scanning electron microscopy (SEM), we measured the particle size, density, stability, and  
26 agglomeration state of polyethylene and polypropylene MPs stabilized in aqueous suspension by BSA.  
27 SEM analysis revealed the formation of nanoplastic debris as MP suspensions aged. Our results  
28 suggest that protein adsorption favors the formation of secondary nanoplastics, potentially posing an  
29 additional threat to ecosystems. This approach provides analytical methodologies by integrating SEM,  
30 SMLS, and SAXS, for characterizing MP suspensions and highlights the effect of the protein corona  
31 on size measurements of micro/nanoplastics. Our analysis demonstrates the detectability of secondary  
32 nanoplastics by SEM, paving the way for monitoring and controlling human exposure.

## 33 **Keywords**

34 Microplastics, nanoplastics, size, dispersibility, polyethylene

35

## 36 **1. Introduction**

37 Global plastic production reached 500 million metric tons in 2020 and contributes to an annual plastic  
38 waste generation of 400 million metric tons and the emission of 2.2 GtCO<sub>2</sub> [1]. With a doubling of the  
39 plastic demand expected by 2050, the total plastic stocks in use, which already amount to 3.2 Gt of  
40 plastic, may increase to 7.7 Gt by 2050 if no measures are taken to reduce this trend. Due to their high  
41 stability and resistance to chemical, physical, and mechanical stresses, most plastics in use are not  
42 biodegradable. Moreover, technical solutions are currently missing to recycle widely used plastics  
43 such as polyethylene and polypropylene [2]. With the continued growth of the plastic sector and the  
44 implementation of new production units worldwide, past and ongoing plastic production pose a major  
45 challenge to our societies to manage plastic waste and to mitigate the environmental damages of  
46 plastic pollution. As the impact of plastic pollution may extend well beyond the end of plastic release  
47 in the environment, a global ‘toxicity debt’ associated with plastic pollution has emerged [3]. Lower  
48 density polymers such as polyethylene (PE) and polypropylene (PP) represent between 25 and 40% of  
49 plastic particles in surface waters, with their ultimate fate remaining unknown, whereas denser  
50 polymers such as polyesters and acrylics are more prevalent in the deep-sea and sediments, which can

51 represent a potential sink for these plastics [8]. Interestingly, the size of buoyant plastic particles vary  
52 in the water column, with smaller particles more susceptible to vertical transport than larger ones, a  
53 process that can be favoured by biofouling [9]. As a result, the mass metrics of plastics (in  $\text{g}/\text{m}^3$ )  
54 decrease more rapidly than the number metrics (in  $\text{number}/\text{m}^3$ ) [10]. Fragmentation of plastic wastes  
55 into microplastics (MPs) by weathering has generated an estimated 15 to 55 trillion MPs in the world's  
56 oceans, putting marine organisms at risk [11]. The reduction of plastic size under the combined effects  
57 of photodegradation by UV irradiation, shear forces, hydrolysis, and biodegradation favors the long-  
58 range transportation of MPs in water and in air, leading to global MP dissemination [12] [13].  
59 Together with polymer ageing, size reduction can favor the release of additives and the sorption of  
60 chemical contaminants by increasing the specific surface area and changing the microstructure of the  
61 particles [14]. Finally, smaller particles may cross biological barriers as evidenced by the discovery of  
62 MPs in human blood and in human placenta [15] [16]. However, there is currently no agreement on  
63 the size limits that define microplastics and nanoplastics. Indeed, the fragmentation of plastic waste  
64 into debris results in a range of particle sizes, where plastic particles with external dimensions below a  
65 certain threshold (either 100 nm or 1  $\mu\text{m}$ , depending on the definition used) transition from  
66 microplastics to nanoplastics. These nanoplastics can be considered a specific category of  
67 nanomaterials.

68 Considering their nature and shape, MPs are defined by ECHA as solid plastic particles composed of  
69 mixtures of synthetic polymers and functional additives. The EFSA defined primary MPs as the  
70 original manufactured products, such as polystyrene pellets, and secondary MPs as particles that  
71 originate from fragmentation. A unique definition was provided for MPs based on their size as '*a*  
72 *heterogeneous mixture of differently shaped materials referred to as fragments, spheroids, granules,*  
73 *pellets, flakes or beads, in the range of 0.1 to 5,000  $\mu\text{m}$ ' [17]. MPs have been recently integrated in  
74 REACH regulation [18]. In the preparation of its Microplastic Act, the European Commission  
75 suggested that the lower limit of 100 nm could be lowered to 1 nm to reduce human exposure to  
76 nanoplastics [19] [20]. However, the 100-nm size limit will probably remain in the final Act until  
77 technical and analytical solutions become available to measure MP concentration in this size range  
78 [21]. The lack of analytic means to characterize MPs may thus prevent the protection of exposed  
79 populations.*

80 In the literature, several definitions have been proposed to conceptualize the presence of  
81 micro/nanoplastics and plastic debris in colloidal suspensions. Recently, Caldwell *et al.* [22] defined  
82 macro- (25-50 mm), meso- (5-25 mm), micro- (<5 mm), and nanoplastics (<1  $\mu\text{m}$ ) following the  
83 recommendations of the Joint Group of Experts on the Scientific Aspects of Marine Environmental  
84 Protection (GESAMP) [23]. While the European Commission defined nanomaterials as < 100 nm [24]  
85 [25], the OECD guidelines recently recommended to extend this size limit to < 1  $\mu\text{m}$  [21].

86 Gigault *et al.* [26] proposed the following categories: nanoplastics (1 nm – 1 µm) with a subdivision  
 87 between nanoplastics (1 – 100 nm) and submicron plastics (100 nm – 1 µm); microplastics (1 µm – 1  
 88 mm); mesoplastics (1 – 10 mm); and macroplastics (> 1 cm). An overview of the size limits used in  
 89 the literature to define micro/nanoplastics and disparities between recommendations from different  
 90 regulatory bodies are presented in **Table 1**. There is no harmonized definition of microplastics. Thus,  
 91 the size limit between micro- and nanoplastics is not well defined and varies between studies.

92 **Table 1.** Disparities of the definitions of microplastics and nanoplastics proposed by the European  
 93 Commission, EFSA, GESAMP, and examples from the literature adapted from Hartmann *et al.* 2019  
 94 [26] and Caldwell *et al.* 2019 [22].

	<b>Macro-</b>	<b>Meso-</b>	<b>Micro-</b>	<b>Sub-micron</b>	<b>Nano-</b>	<b>Ref.</b>
Browne et al. (2007)	> 5 mm		1 µm – 1 mm	n.a.	< 1 µm	[27]
Moore et al. (2008)	> 5 mm		< 5 mm			[28]
Ryan et al. (2009)	> 20 mm	2 – 20 mm	< 2 mm			[29]
Costa et al. (2010)	n.a.		< 1 mm			[30]
<b>European Commission (2011, 2022)</b>	n.a.				<b>&lt; 100 nm</b>	[24] [25]
Desforges et al. (2014)	n.a.		1 µm – 5 mm			[31]
Eriksen et al. (2014)	> 200 mm	4.76 – 200 mm	0.33 – 4.76 mm	n.a.		[7]
Wagner et al. (2014)	> 5 mm	2.5 – 5 mm	20 – 500 µm	n.a.	< 20 µm	[32]
Koelmans et al. (2015)	> 5 mm		1 µm – 5 mm	n.a.	1 – 100 nm	[33]
Andrady (2015)	25 mm – 1 m	1 – 25 mm	1 – 100 µm	n.a.	< 1 µm	[34]
Ivleva et al. (2017)	> 25 mm	5 – 25 mm	1 – 5 µm	0.1 – 1 µm	< 100 nm	[35]
Hernandez et al. (2019)	n.a.		1 – 200 µm	< 1 µm	< 100 nm	[20]
<b>EFSA (2016)</b>	n.a.		<b>0.1 µm – 5 mm</b>	n.a.	<b>1 – 100 nm</b>	[17]
Gigault et al. (2018)	> 10 mm	1 – 10 mm	1 µm – 1 mm	1 – 100 µm	1 – 100 nm	[26]
<b>GESAMP (2019)</b>	<b>25 mm – 1 m</b>	<b>5 – 25 mm</b>	<b>&lt; 5 mm</b>	n.a.	<b>&lt; 1 µm</b>	[23]
Caldwell et al. (2019)	> 20 mm	5 – 20 mm	1 µm – 5 mm	n.a.	n.a.	[22]
<b>OECD (2022)</b>	n.a.				<b>&lt; 1 µm</b>	[21]

95

96 Toxicological studies require model or standard materials that are both reproducible and easy to  
 97 manipulate in order to investigate the effects of defined chemical composition, size, shape, and  
 98 concentration on the biological response at a molecular, cellular or organism level. Standardized test  
 99 nanomaterials in the form of stabilized colloidal suspensions with well-defined sizes provided by the  
 100 Joint Research Centre (JRC) have been extensively used in the field of nanotoxicology for toxicity  
 101 studies, interlaboratory comparisons, calibration of analytical devices, and development of bioassays

102 [36]. However, no such reference materials exist for MP suspensions, with the exception of  
103 functionalized polystyrene spheres.

104 The stabilization of MPs is not straightforward due to the high hydrophobicity of synthetic polymers  
105 such as PE and PP and their low density resulting in fast or instant creaming of the particles from the  
106 suspension. One common method consists in exposing particles to proteins, such as bovine serum  
107 albumin (BSA), which results in the formation of a stabilizing protein corona. The exceptional coating  
108 properties of BSA with respect to PE and PP MPs for the preparation of MP suspensions has been  
109 demonstrated [37]. In the environment, a similar phenomenon spontaneously occurs in the presence of  
110 biomolecules resulting in the formation of an eco-corona [38]. This biomolecular corona enhances the  
111 transport of MPs by changing their mobility [39] and changes their bioavailability, uptake, and toxicity  
112 with respect to living organisms [40]. However, the question of the state and the fate of MPs in such  
113 dispersions is far from being understood. Key physical properties – MP stability, dispersibility,  
114 agglomeration, and dimensional size – must be defined and measured in complex media, such as the  
115 ones used in toxicological studies, by taking into account the formation of both naturally or  
116 intentionally developed corona. This knowledge will help to understand the behavior of  
117 micro/nanoplastics in suspension, provide adapted analytical methods to characterize MPs and develop  
118 reference materials for toxicity studies.

119 The stabilization of MPs in suspension by a biomolecular corona is essential to perform the  
120 dimensional characterization of MPs and to reproduce real-life scenarios of exposure. The dimensional  
121 measurement of particles in suspension is difficult if stability and dispersibility are not ensured. The  
122 main objectives of this study are to complete the dimensional characterization of MPs in suspension  
123 and to improve the current comprehension of the behavior and impact of the corona on size  
124 measurements of micro/nanoplastics. Our specific aims were as follows:

- 125 (i) to examine the role of the corona on the stability, dispersibility, and dimensional size of MPs;
- 126 (ii) to elucidate the influence of the corona on the fate of micro/nanoplastics and the possible release of  
127 secondary nanoplastics;
- 128 (iii) to explore the dimensional size analysis of micro/nanoplastics in suspension.

129 To better understand the interactive effects and mechanisms between the corona and MPs, we focused  
130 our study on two polyolefin MPs, polyethylene and polypropylene, which are widely present in MP  
131 pollution, and a model protein, BSA, which is compatible with toxicity studies. The dimensional size  
132 analysis of MPs in freshly prepared suspension was compared by SEM, SMLS, SAXS, and Raman  
133 microscopy as a function of BSA concentration. The MP suspensions were then aged for 7 days in  
134 shelf storage conditions to evaluate the stability, dispersibility, and size evolution of the MPs over  
135 time.

## 136 2. Experimental

137 The combination of existing methods and the development of novel methodologies are necessary to  
138 fully characterize micro- and nano-plastic suspensions. PE and PP MPs with a corona formed by BSA  
139 were characterized by SMLS, SAXS, SEM, Raman microscopy, and zetametry.

### 140 2.1. Materials

141 Polyethylene and polypropylene MPs are widely present in the environment [8] and as contaminants in  
142 tap water [42]. PE and PP MPs were purchased from Clariant (Germany) in powder form (Ceridust  
143 3610, Ceridust 60050M). The characterization of PE and PP MPs in powder form is provided in [Table](#)  
144 [2](#) [37]. Densities of 0.97 and 0.90 g.cm<sup>-3</sup> for PE and PP respectively were indicated by the  
145 manufacturer. No surface contaminants were detected by XPS [37].

146 **Table 2.** Physical properties of PE and PP microplastics in powder form [37]

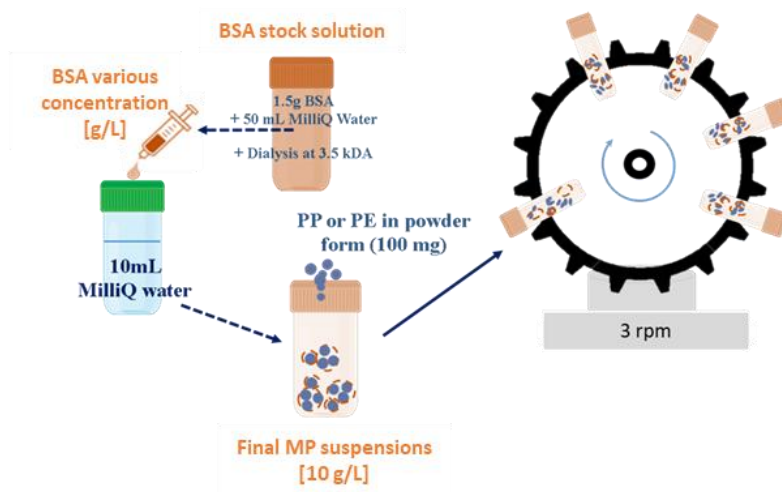
Physical properties	Technique	PE Ceridust 3610	PP Ceridust 60050M
Diameter (μm)	Optical microscopy	4.2	10.0
Surface specific area (m <sup>2</sup> /g)	SAXS	2.5	1.1
Number of crystalline phases	Raman microscopy	3	1

147  
148 BSA was chosen as a model protein to stabilize PE and PP MPs in aqueous suspensions based on its  
149 high affinity for MPs and its good surfactant properties [37] [43]. The isoelectric point of BSA is  
150 around 4.8 and its molecular mass is 66,450 Da. BSA has been widely used for the stabilization of  
151 nanomaterials in the field of nanotoxicology [44], and is a good candidate for the preparation of  
152 reference test materials for the study of MP toxicity. Lyophilized BSA was purchased from Sigma-  
153 Aldrich (France) (reference A7030), dissolved in milliQ water, dialyzed using a porous membrane  
154 with a cut off of 3.5 kDa (Spectra/Por) at 4°C, and centrifuged at 15,000 g for 5 minutes. BSA  
155 solutions were freshly prepared before use, and the protein concentration was checked by UV  
156 spectroscopy.

### 157 2.2. Sample preparation

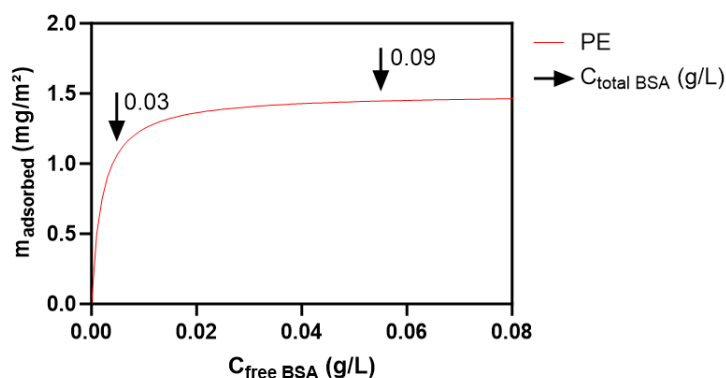
#### 158 2.2.1. Dispersion protocol and formation of a protein corona

159 The dispersion protocol of MPs in BSA solutions adapted from [37] is presented in [Fig. 1](#). PE and PP  
160 in powder form were weighed and dispersed in a BSA solution to obtain final MP suspensions with a  
161 particle concentration of 10 g/L and BSA concentrations of 0.03, 0.09, 0.15, and 0.3 g/L. The colloidal  
162 suspensions were mixed on a rotating wheel at 3 rpm at 20°C for 3 days.



163  
 164 **Fig. 1.** Schematic of the dispersion protocol of polyethylene and polypropylene MPs in BSA solutions,  
 165 adapted from [37]. The final concentration of MPs was 10 g/L and the total concentration of BSA  
 166 varied between 0.03 and 0.3 g/L to form a complete protein corona on PE and PP MPs with increasing  
 167 concentrations of free BSA in solution.

168 The adsorption isotherms of BSA on PE and PP microplastics adapted from [37] are shown in Fig. 2  
 169 and Fig. S1. Note that the available surface is higher for PE than for PP MPs at a fixed MP mass  
 170 concentration of 10 g/L (Table 2). The minimum concentration of BSA (0.03 g/L) was chosen so that  
 171 a quasi-complete corona was formed on both PE and PP particles with a minimal amount of free BSA  
 172 in solution. Higher BSA concentrations (0.09, 0.15, and 0.3 g/L) were chosen to provide an excess of  
 173 free BSA. For PE MPs, there was a 1-fold excess for a total BSA concentration of 0.09 g/L (40 mg  
 174 adsorbed + 50 mg free in solution), a 3-fold excess at 0.15 g/L (40 mg adsorbed + 110 mg in solution),  
 175 and a 6-fold excess at 0.3 g/L (40 mg adsorbed + 260 mg in solution).



176  
 177 **Fig. 2.** Adsorption isotherms of BSA on PE MPs represented by the Langmuir model, adapted from  
 178 [37]. The corresponding concentrations of total BSA are shown by arrows for  $C(\text{BSA}) = 0.03$  g/L and  
 179  $C(\text{BSA}) = 0.09$  g/L.



### 180           **2.2.2. Ageing of MPs suspensions**

181 The use of a purified protein such as BSA allowed us to follow the evolution of MP suspensions over  
182 longer periods. The PE and PP MP suspensions prepared as described above were aged for 7 days in  
183 shelf-storage condition at 20°C without agitation to monitor the release of secondary nanoplastics in  
184 the suspension. The aged suspensions were gently mixed for 10 min on a rotating wheel at 3 rpm  
185 before analysis by SMLS, SEM, and Raman microscopy. No bacterial contamination of MPs or MP  
186 suspensions was observed. The analysis was performed for 4 different BSA concentrations. The same  
187 suspensions were analyzed before and after ageing.

### 188           **2.3. Characterization techniques and measurands**

189 In this study, colloidal stability was evaluated by zeta potential and SMLS. The zeta potential of MP  
190 suspensions was measured as a function of BSA concentration. The SMLS technique allows the  
191 measurement of the Turbiscan Stability Index (TSI) and the creaming rate of MP dispersions, which is  
192 a predictive indicator of colloidal stability. Size can be characterized by measuring the initial mean  
193 size of particle by SMLS and SEM. Each technique has its own dimensional measurand ( $d_{\text{SMLS}}(t=0)$   
194 and  $d_{\text{SEM}}$  respectively) and thus brings complementary information about MP dispersion state, whereas  
195 Raman provides a molecular fingerprint spectrum based on the polarizability of chemical bonds.  
196 Raman microscopy can also provide *in situ* information on the constituent size of MPs in agglomerates  
197 from colloidal suspensions. The Stokes diameter ( $d_{\text{Stokes}}$ ) corresponding to the equivalent diameter  
198 from the particle creaming rate also depends on the particle density, which was experimentally  
199 determined by SAXS.

200 The definitions of the main terms based on ISO standards are provided here for clarity [41]. Stability is  
201 defined as the evolution of the physico-chemical state of MPs over time, whereas dispersibility  
202 represents the property of MPs to be uniformly distributed in a specific medium. An aggregate is  
203 defined as an assemblage of particles in a rigid structure, usually formed in an irreversible process by  
204 covalent bonds. By contrast, an agglomerate is a ‘loosely coherent assembly of particles (or  
205 aggregates) held together by weak physical interactions and with a total surface area virtually equal to  
206 the sum of the surface areas of the constituent particles’ [41].

#### 207           **2.3.1. Zeta Potential and pH**

208 The zeta potential of MP suspensions was measured as a function of BSA concentration on a Malvern  
209 Zetasizer at 25°C in water. Three measurements were done for each suspension. The zeta potential  
210 was calculated using the Smoluchowski method. Data correspond to the mean and the standard  
211 deviation of 3 independent series ( $n = 3$ ). The pH of the suspensions was measured at room  
212 temperature (20°). The pH value corresponds to the mean  $\pm$  standard deviation of 3 independent  
213 replicates.

### 2.3.2. Static multiple light scattering (SMLS)

SMLS is a technique based on static light scattering dedicated to the measurement of the particle mean size in the initial conditions, the creaming rate, and the macroscopic stability of colloidal suspensions [45]. MP colloidal suspensions were analyzed by SMLS with a Turbiscan® Lab (Formulation, France).

Monochromatic infrared light of wavelength  $\lambda = 880$  nm propagated in the MP suspension contained in a cylindrical glass tube. Two detectors collected the light scattered by the medium. The first one is placed at an angle of  $0^\circ$  to the source (i.e., opposite to the incident wave) to collect light transmitted by the medium. The other detector is placed at an angle of  $135^\circ$  to collect the backscattered signal (BS). The highest intensity was measured for backscattered light with both PE and PP suspensions. The first scan measured at  $t = 0$  s was used to evaluate the mean particle size by volume,  $d_{\text{SMLS}}(t = 0)$ , assuming the particle concentration is homogeneous in the suspension.

When particles rose towards the surface, a clarification zone formed at the bottom of the sample favoring the onset of a creaming peak. The peak width as a function of time is proportional to the creaming speed of MPs. It is thus possible to calculate the Stokes diameter,  $d_{\text{Stokes}}(t \neq 0)$ , corresponding to a hydrodynamic diameter, from the particle creaming velocity. In addition to particle diameters, a stability index called TSI (Turbiscan Stability Index) was evaluated from the differences in backscattered intensity at a given time (eq. 1):

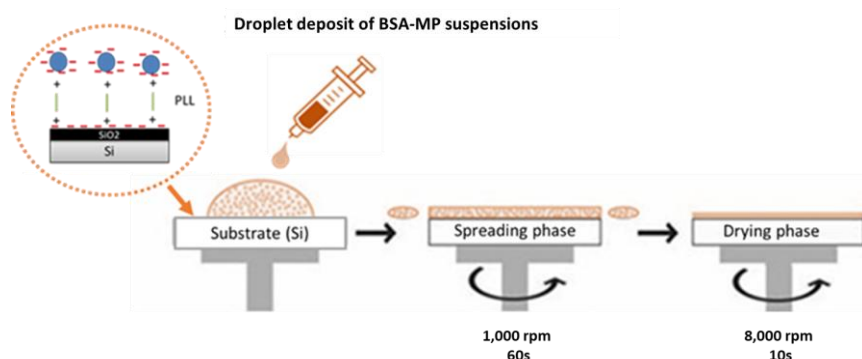
$$TSI(t) = \frac{1}{N_z} \sum_{t_i=1}^{t_{max}} \sum_{z_i=z_{min}}^{z_{max}} |BST(t_i, z_i) - BST(t_{i-1}, z_i)| \quad (1)$$

with  $t_{max}$  the time at which the TSI is calculated and BST the considered signal. Consequently, when the TSI is high, the colloidal suspension tends to be unstable. Each measurement is the average of 3 replicates. The mean values of TSI,  $d_{\text{SMLS}}$ , and  $d_{\text{Stokes}}$  correspond to the mean of 3 independent series ( $n = 3$ ), except for aged suspensions ( $n = 1$ ).

### 2.3.3. Scanning electron microscopy (SEM)

7.5  $\mu\text{L}$  of BSA-PE or BSA-PP suspensions was deposited on a silicon substrate using a LabSpin 6 spin coater system (SUSS MicroTec) (Fig. 3). To improve the deposit of negatively charged particles, the substrate was pre-coated with positively-charged polylysine (PLL) (Serva Electrophoresis, Thermo Fischer scientific). The effect of PLL coating on the quality of MP deposits was evaluated by comparing the SEM images of the same samples, with and without PLL coating of the Si wafer. MPs were imaged using an SEM Zeiss ULTRA-Plus equipped with a Field Emission Gun (FEG) source and in-Lens SE detector. The images were measured using secondary electrons collected by the In-Lens detector at 3 kV at a 3.0 mm working distance. The charge compensator system was used to blow nitrogen gas near the samples and neutralize negative charges. The diameter equivalent to the projected particle surface was measured using Platypus™ software [46] assuming that particles were spherical. We measured MP size by manually adjusting an ellipse on the entire particle using Platypus™ software (Fig. S2). Then the particle edge and corresponding area inside the edge were

250 automatically calculated. The primary size measurement of MPs was done on a stack of 10 to 15 SEM  
251 images to measure a minimum number of 300 particles. The expansion of the distribution  $\sigma$  was  
252 measured in triplicate on MP suspensions with a 95% confidence interval.



253  
254 **Fig. 3.** Schematic of the deposition of micro- and nano-plastic suspensions on silicon wafers by spin-  
255 coating for SEM analysis after pre-coating by PLL.

256 According to our observations, PLL functionalization promoted immediate adhesion of nanoplastics (<  
257 1  $\mu\text{m}$ ) to the silicon wafer.

258 The measurand used for the SEM corresponds to an area-equivalent diameter. For particles with  
259 irregular shapes, an equivalent diameter was calculated from a projected surface area assuming that the  
260 particles are spherical. The average diameter,  $D$ , corresponding to the average of area-equivalent  
261 diameters, and the population number-weighted size distribution were determined. The repeatability  
262 uncertainties related to the measurements ( $u_R$ ) correspond to the standard deviation calculated on the  
263 average diameters of 3 independent replicates.

#### 264 2.3.4. Raman microscopy

265 The agglomeration state of MP suspensions was investigated *in situ* by confocal Raman microscopy.  
266 10  $\mu\text{L}$  of PE and PP BSA-MPs suspensions was deposited between two fused silica substrates (ESCO  
267 Optics, UK) and held in an AttoFluor Cell Chamber (Invitrogen, France) to limit particle motion  
268 during imaging. Raman images were acquired on a WITec alpha300 RA Raman microscope (Oxford  
269 Instruments) with a Zeiss 100x oil immersion objective (NA 1.3) using an excitation wavelength of  
270 532 nm and a grating of 600 g/mm. Exposure time was set to 10 ms and laser power to 10 mW for a  
271 spatial step of 0.3  $\mu\text{m}$ . Raman spectra were treated using WITec Program 5 software to remove cosmic  
272 rays and subtract baseline. Raman images were reconstructed using the True Component Analysis  
273 toolbox.

274 The Raman images of PE were further segmented to measure the size of the constituent particles in the  
275 agglomerates using the SAM segmentation model developed by META [47]. This approach allowed  
276 good segmentation of the Raman images without pre-treatment and preliminary training of the model,  
277 taking advantage of META SA-1B dataset.

278 The same experimental setup was used for Raman imaging and for recording movies of MP  
 279 suspensions with a 100x oil immersion objective and white light illumination in reflection mode.  
 280 Movies were recorded at 25 frames per second for 10 s. Bright field images were analyzed with Fiji  
 281 software (version 1.53t) [48].

### 282 **2.3.5. Small angle X-ray scattering (SAXS)**

283 The particle density of PE and PP MPs were measured in suspension by SAXS using a Xeuss 3.0  
 284 instrument (Xenocs, France) equipped with a copper source and an automated sampler (BioCube with  
 285 robotic arm). Possible limitations in SAXS analysis of MP suspensions are the creaming of the  
 286 particles during measurement, MP loss by adhesion to surfaces, and MP agglomeration. To avoid  
 287 these effects, we used automated sampling and we chose MP suspensions that contained an excess of  
 288 BSA to favor passivation of the tubing and surfaces. MP suspensions were analyzed under vacuum in  
 289 glass capillary tubes ( $d = 1.5$  mm) with a sample-to-detector distance of 1.8 m and a counting time of  
 290 1,800 s. Data correspond to the average of 3 measurements. Data were corrected for the capillary  
 291 thickness and the water signal was subtracted. The density of PE MPs was calculated using the plateau  
 292 value of the Porod limits assuming that particles have a smooth surface.

293 The quantity of interfaces in the suspension were measured by the Porod's limit (eq. 2) [49]:

$$294 \quad \lim_{q \rightarrow \infty} Iq^4 = 2\pi \times \Delta\rho^2 \times \sum_{Porod} VSSA \quad (2)$$

295 where  $\lim_{q \rightarrow \infty} Iq^4$  is the Porod's limit in  $\text{cm}^{-1} \cdot \text{\AA}^{-4}$ ,  $I(q)$  the intensity of scattered light,  $\Delta\rho^2$  the contrast  
 296 in scattering length density (SLD) which depends on the density of the particle  $\rho_{MP}$  and the density of  
 297 the solvent  $\rho_{H_2O}$ , and  $\sum_{Porod} VSSA$  the amount of interfaces per volume unit of dispersion in  $\text{cm}^{-1}$ . No  
 298 difference in SLD was observed between BSA solutions and water in these conditions.

299  $\sum_{Porod} VSSA$  is related to the amount of interface per mass of particle  $\sum_{Porod} SSA$  in  $\text{m}^2 \cdot \text{g}^{-1}$  (eq. 3) :

$$300 \quad \sum_{Porod} VSSA = \sum_{Porod} SSA \times C \quad (3)$$

301 where  $C$  is the mass concentration of particle in solution.

302  $\sum_{Porod} SSA$  in  $\text{m}^2 \cdot \text{g}^{-1}$  was calculated as the ratio of the surface  $S$  of a particle by its mass  $m_{MP}$   
 303 (expressed as the product of its volume  $V$  and density  $\rho_{MP}$ ).  $S$  and  $V$  are then expressed as a function  
 304 of the particle diameter  $d_{Porod}$  to obtain eq. 4:

$$305 \quad \sum_{Porod} SSA = \frac{S}{\rho_{MP} \times V} = \frac{6}{\rho_{MP} \times d_{Porod}} \quad (4)$$

306

## 307 **2.4. Statistical analysis**

308 SEM, SMLS and RAMAN data are presented as mean  $\pm$  standard deviation of 3 replicates. Statistical  
309 analysis was performed with Prism GraphPad Software (v.10.0). Data were tested for normality with a  
310 Shapiro-Wilk test and analyzed by multiple two-way ANOVA with a post-hoc Tukey test. P-values <  
311 0.05 were considered statistically significant.

312 The repeatability uncertainties of SEM measurements were determined following the methodology  
313 developed by Bouzakher-Ghomrasni et al [50]. Briefly, the estimation of the law parameters that best  
314 fits the SEM data was carried out using R-Studio software. The size distribution was estimated using  
315 the Maximum-Likelihood estimation statistical method. Prior to the calculation, a choice has to be  
316 made regarding the theoretical probability distribution that is suitable to represent the size distribution.  
317 Four theoretical models: Gaussian, Lognormal, Gaussian mixture, and Lognormal mixture, were used  
318 to draw the histograms. The probability density function (PDF) of the 4 models was determined for the  
319 measured sizes of the single particles. To discriminate between the 4 models, we used the Akaike  
320 Information Criteria (AIC) and the Bayesian Information Criteria (BIC). The best model is the one that  
321 has a minimum AIC and/or BIC, with the BIC criterion preferred in case of conflict. Lognormal  
322 distribution was selected for the size measurement of MPs by SEM based on this criteria.

323

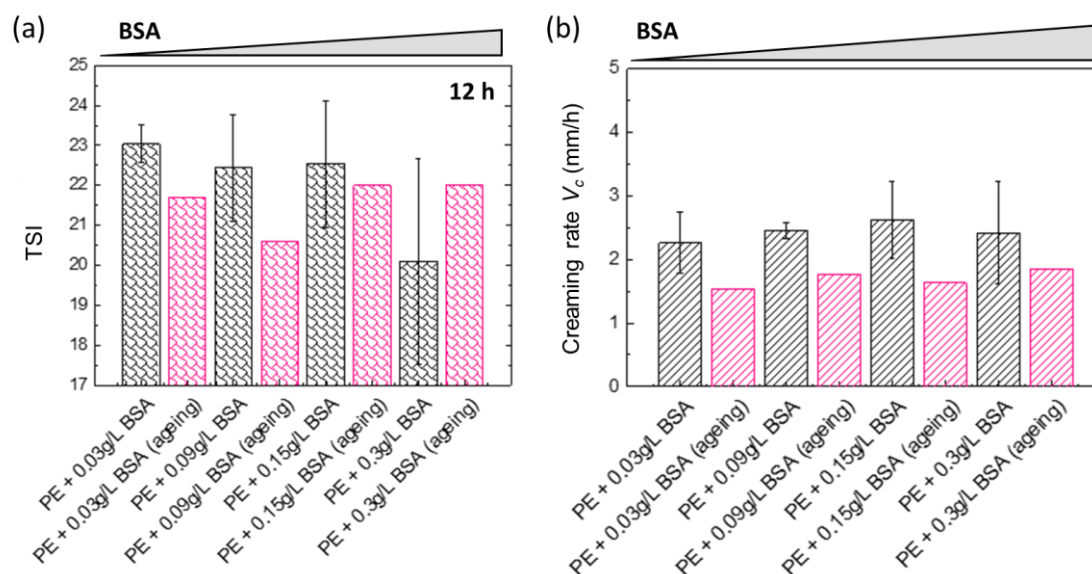
## 324 **3. Results**

### 325 **3.1. Stability of MP suspensions with a BSA corona**

326 PE and PP MPs were stabilized in aqueous solution by the formation of a BSA protein corona,  
327 following previous work by Schwartz *et al.* [37]. The formation of a protein corona is necessary to  
328 obtain MP suspensions, since no particles were detected in solution without protein. First, the stability  
329 of MP dispersions in BSA solutions was assessed by SMLS to evaluate the effect of the concentration  
330 of free BSA in solution. The Turbiscan Stability Index (TSI), which provides macroscopic information  
331 on the stability of colloidal suspensions, was measured after 12 h without mixing (Fig. 4a). No  
332 significant change of the TSI was observed by increasing the concentration of BSA from 0.03 to 0.3  
333 g/L corresponding to a complete protein corona with no excess to a 6-fold excess of free BSA,  
334 respectively (Fig. 2, see 2.2.1). A similar effect was observed for PP MP suspensions (Fig. S3). The  
335 creaming rate  $V_c$  of PE MPs did not vary significantly with BSA concentration, with values between  
336 2.2 and 2.6 mm/h (Fig. 4b). PE MP suspensions with and without an excess of free BSA were aged for  
337 7 days (shelf storage at 20°C) to investigate the release of secondary particles and/or aggregation over  
338 time. After ageing in shelf-storage conditions for 7 days and gentle mixing for 10 min, the TSI and  
339 creaming rate of the colloidal suspensions were measured again. No increase in TSI and  $V_c$  was  
340 observed, confirming the good stabilization of PE MPs by BSA.

341 While the increase in free BSA had no impact on the stability of PE and PP MPs, a higher TSI (TSI >  
 342 32) and creaming rate ( $V_c > 10$  mm/h) were observed for PP compared to PE MPs (Fig. S3). The fast  
 343 creaming of PP MPs was illustrated by pictures taken after 1 and 3 h resting in all the conditions tested  
 344 (Fig. S4). The lower stability of PP MPs suspensions over time despite the formation of a complete  
 345 protein corona could be explained by the larger size of PP particles compared to PE, as reported by  
 346 Schwartz *et al.* [37]. We note that in the case of PP MPs, there was a 9-fold excess of free BSA at a  
 347 concentration of 0.3 g/L (Fig. S1).

348 The surface charge of MPs with a BSA corona was evaluated by zeta potential measurement. PE MPs  
 349 were negatively charged ( $\zeta = -35.9 \pm 1.5$  mV) with no significant change with increasing BSA  
 350 concentration (Fig. S5). A negative zeta potential was also measured for PP MPs covered by  
 351 negatively-charged BSA ( $\zeta = -48.4 \pm 1.4$  mV). The pH of the suspensions did not change significantly  
 352 with BSA concentration ( $\text{pH} = 7.4 \pm 0.1$ ) (Table S1). This is in agreement with the stabilization of  
 353 hydrophobic MPs by adsorbed BSA that acts as a charged surfactant [43] and improves the  
 354 hydrophilicity of the particles.



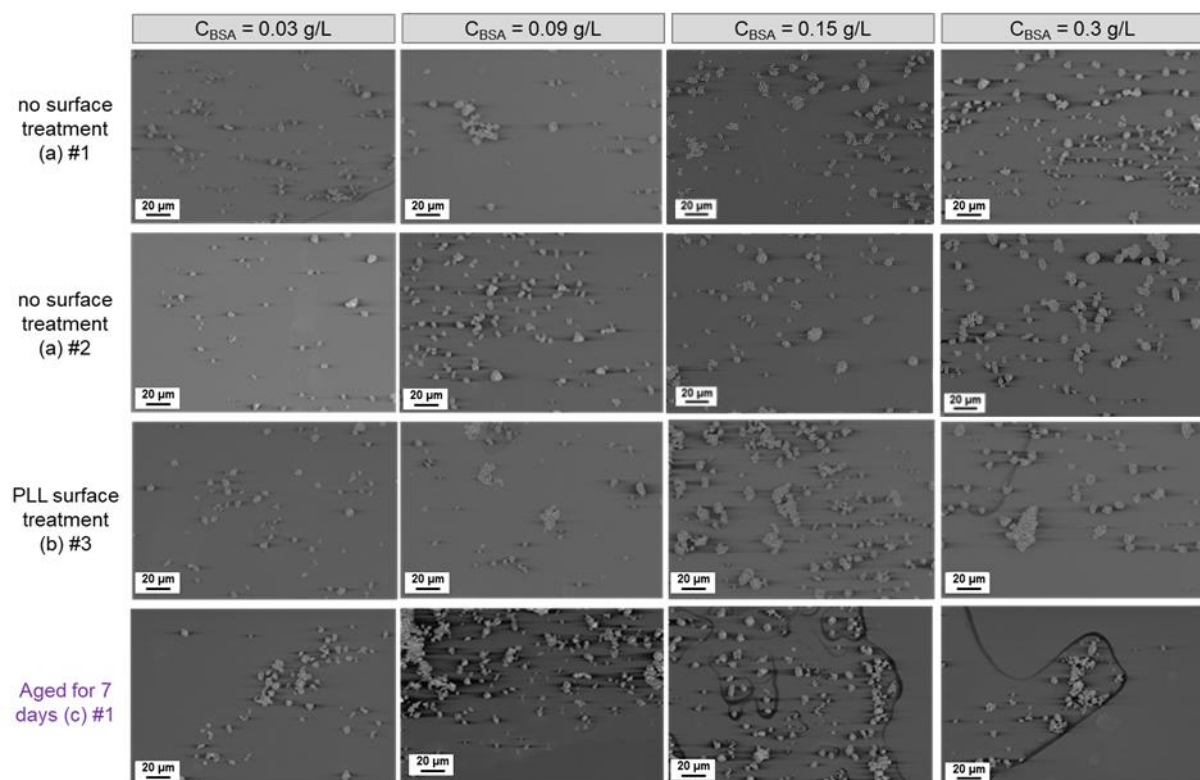
355  
 356 **Fig. 4.** Stability of PE MP suspensions with a protein corona measured by SMLS as a function of BSA  
 357 concentration. (a) TSI (Turbiscan Stability Index) measured at 12 h, and (b) Creaming rate  $V_c$  (mm/h)  
 358 of fresh MP suspensions (grey bars) and MP suspensions aged for 7 days (pink bars).  $C(\text{MP}) = 10$  g/L.  
 359 Values correspond to the mean and standard deviations of 3 independent series ( $n = 3$ ), except for the  
 360 aged series ( $n = 1$ ).

### 361 3.2. Determination of MP dimensional size and dispersibility by SEM

362 The utilization of SEM for characterizing nanomaterials in environmental or biological samples has  
 363 become a standard practice in the field of nanotoxicology [51] [52]. However, this technique is still  
 364 seldom used efficiently for micro- and nanoplastics as it requires specific operating conditions to

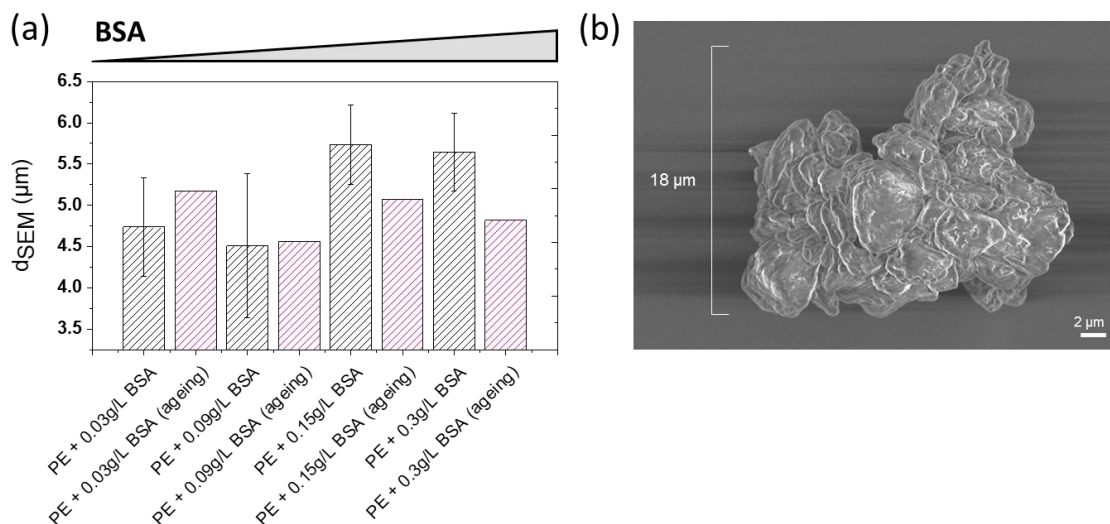
365 prevent any fusion or bubbling of the polymer under the electron beam. In this study, we recorded  
366 high quality SEM images of MPs without carbon coating by using a charge compensator system. The  
367 SEM images of PE MPs deposited on PLL-treated and untreated Si wafers as a function of the  
368 concentration of BSA in the suspension are shown in Fig. 5.

369 The projected diameter of primary particles, either isolated or in agglomerates/aggregates, was  
370 measured after segmentation using PLATYPUS software, as illustrated in Fig. S2. The mean diameter  
371 of PE MPs in suspension,  $d_{SEM} = 4.7 \pm 0.6 \mu\text{m}$  for condition without excess BSA in solution ( $C_{BSA} =$   
372  $0.03 \text{ g/L}$ ) (Fig. 6a) is in good agreement with the diameter of  $4.2 \mu\text{m}$  measured on PE in powder form  
373 (Table 2) [37], confirming that drying of MPs suspensions on a Si substrate did not alter the size  
374 measurement. Good agreement was also obtained for PP MP suspensions with a mean diameter of the  
375 primary particles  $d_{SEM} = 11 \pm 2 \mu\text{m}$  compared to a diameter of  $10 \mu\text{m}$  in powder form (Fig. S6-S7).



376

377 **Fig. 5.** SEM images of PE MPs deposited on silicon wafers as a function of BSA concentration in the  
378 suspension. The conditions presented are (a) no surface treatment, (b) PLL-coated Si, (c) ageing of PE  
379 MPs suspension for 7 days. The series numbers are indicated. Scale bar 20  $\mu\text{m}$ .



380

381 **Fig. 6.** Dimensional size of primary particles of PE MPs with a BSA corona measured by SEM. (a)  
 382 Average SEM diameter as a function of BSA concentration for fresh suspensions (grey) and  
 383 suspensions aged for 7 days (pink).  $n = 3$  for fresh MPs suspensions,  $n = 1$  for aged MPs suspensions.  
 384 (b) Representative image of an agglomerate/agglomerate of PE MPs ( $C_{BSA} = 0.09$  g/L). Scale bar 2  $\mu m$ .

385 The dispersibility of MPs can be qualitatively estimated by the homogeneity and the surface density of  
 386 the isolated particles of the deposits, reflecting the propensity of primary particles to remain dispersed  
 387 and to preserve their original size in suspension. To estimate the homogeneity of the deposit, the  
 388 expansion of the distribution  $\sigma$  was determined for each MP suspension. The smaller the expansion of  
 389 the distribution, the narrower is the particle size distribution.  $\sigma$  was estimated with a 95% confidence  
 390 interval to 0.3 and 0.4 for PE and PP MPs, respectively. For  $\sigma > 0.3$ , the equivalent diameter becomes  
 391 larger due to particle agglomeration/aggregation in suspension. To illustrate this effect, a  
 392 representative SEM image of an aggregate/agglomerate of PE MPs is shown in Fig. 6b. However, few  
 393 large objects were observed in all the conditions tested. The number of aggregates/agglomerates was  
 394 estimated to less than 5% of the total number of objects observed in SEM deposits. The  
 395 characterization of the aggregates/agglomerates by SMLS and Raman imaging will be presented in the  
 396 next sections.

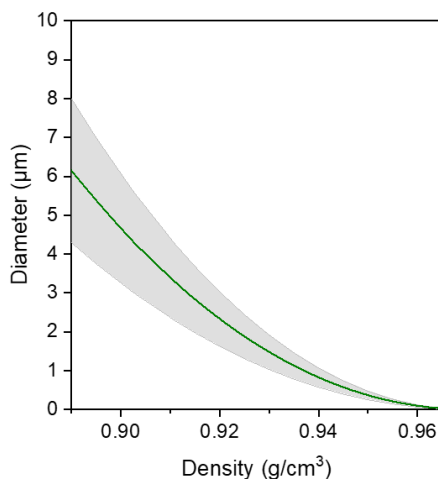
397 Additionally, as same-charge particles and substrates may impact particle deposition, we tested the  
 398 surface treatment of Si wafers by positively-charged PLL before spin-coating of negatively-charged  
 399 MPs suspensions (Fig. 3). No significant change of the dispersibility or primary particle diameter was  
 400 observed with and without PLL-surface treatment, suggesting that this protocol was not necessary for  
 401 the deposition of MPs with a BSA corona in the micron-size range. Besides, no significant difference  
 402 in  $d_{SEM}$  was observed for PE and PP MPs as a function of BSA concentration. Overall, the MP  
 403 diameter and the dispersibility of PE and PP MPs in suspension appeared unchanged with the increase  
 404 of BSA concentrations.



### 405 3.3. Determination of MP density by SAXS

406 The density of MPs is an important factor for the calculation of the Stokes diameter by SMLS, which  
407 is calculated from the particle creaming velocity [45]. We developed a methodology based on SAXS  
408 to precisely determine the MP density in suspension. The advantages of this approach are to take into  
409 account the biomolecular corona and to provide a measurement of the density with a better accuracy  
410 compared to other techniques. The density of PP MPs could not be measured by SAXS due to the fast  
411 creaming and lower stability of the suspensions.

412 Porod's limit depends on the intensity of scattered light  $I(q)$ , the amount of interfaces, and the contrast  
413 in scattering length density (SLD) (eq. 2). The SLD depends on the electron density, hence on the  
414 atomic composition and on the material density. The atomic composition of PE MPs is known, but not  
415 the particle density. The density of the particles can be calculated using the plateau value of the  
416 Porod's limits, as shown by Garcia-Diez et al [53, 54]. The particle diameter  $d_{\text{Porod}}$  can be directly  
417 associated to the measured Porod's limit  $\lim_{q \rightarrow \infty} Iq^4$  and particle density  $\rho_{\text{MP}}$  in  $\text{g}\cdot\text{cm}^{-3}$  (eq. 3, eq. 4)  
418 (see Methods section for detailed calculation). The calculated particle Porod diameter  $d_{\text{Porod}}$  as a  
419 function of the particle density  $\rho_{\text{MP}}$  for PE MPs was plotted for the Porod's limit values of  $4.6 \times 10^{-10}$   
420  $\text{cm}^{-1}\cdot\text{A}^{-4}$  measured for  $C(\text{BSA}) = 0.06 \text{ g/L}$  (Fig. 7). We can notice that our approach is simpler than the  
421 one described in the literature, as the particle diameter was determined by SEM independently from  
422 the SAXS measurements. The uncertainty on the density is given here by the minimum and maximum  
423 Porod's limits values of  $3.6 \times 10^{-10} \text{ cm}^{-1}\cdot\text{A}^{-4}$  and  $5.9 \times 10^{-10} \text{ cm}^{-1}\cdot\text{A}^{-4}$  respectively measured on 3  
424 independent replicates. We chose MP suspensions that contained an excess of BSA to favor the  
425 passivation of the tubing by BSA during automated sampling and to limit MP loss and/or aggregation.  
426 This is shown by the deviation observed at  $C(\text{BSA}) = 0.03 \text{ g/L}$ , while good consistency was observed  
427 at higher BSA concentrations (Fig. S8).  $d_{\text{Porod}}$  was assimilated to  $d_{\text{SEM}}$  as both techniques are sensitive  
428 to the electron density of the material. Using  $d_{\text{SEM}}$  in each condition and the observation that the  
429 particles are relatively smooth based on SEM imaging of the surface (Fig. S9), we could deduce a  
430 density range for PE MPs in suspension corresponding to an average density  $\rho_{\text{MP}} = 0.90 \pm 0.01$ . The  
431 particle density measured by SAXS significantly differ from the value of  $0.97 \text{ g}\cdot\text{cm}^{-3}$  given by the  
432 manufacturer for the HDPE source material (without indication on the method used). Our SAXS data  
433 indicate that the density of PE particles is closer to  $0.90 \text{ g}\cdot\text{cm}^{-3}$ , suggesting that the MPs are composed  
434 of LDPE rather than HDPE. The difference between our results and the provider's ones may be due to  
435 the processing of an initially high density raw material into microparticles that may decrease the  
436 particle density, possibly through the increase of the amorphous or low cristallinity phases identified  
437 by Raman microscopy [37].



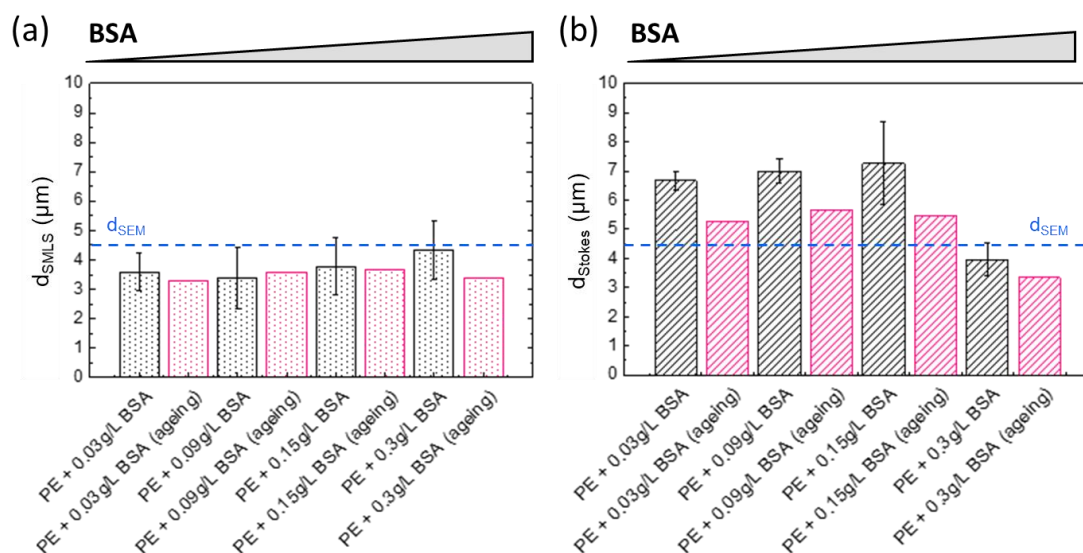
438

439 **Fig. 7.** Calculated MP diameter as a function of MP density determined from the Porod's limits  
 440 measured by SAXS for PE MPs suspensions with  $C(\text{BSA}) = 0.06 \text{ g/L}$ . The average of 3 independent  
 441 replicates is shown (green line). Minimum and maximum values of Porod's limits are represented by  
 442 the grey area.

443 A difference of  $< 1\%$  of the density was obtained between  $C(\text{BSA}) = 0.06 \text{ g/L}$  and  $C(\text{BSA}) = 0.3 \text{ g/L}$   
 444 (Fig. S8). This difference can be explained by the uncertainty in the concentration of MP suspensions  
 445 used to calculate the amount of interface. Indeed, the mass of a complete BSA corona represents only  
 446  $0.2\%$  ( $0.01 \text{ pg}$  per particle) and  $0.05\%$  ( $0.3 \text{ pg}$  per particles) of the mass of PE and PP particles,  
 447 respectively. The effect of the BSA corona on the measurement of MP density in suspension is thus  
 448 negligible. Note that this measurement cannot be performed without a protein corona to stabilize MPs  
 449 in aqueous suspension. By combining SEM and SAXS, this methodology allows the measurement of  
 450 the density of MPs in suspension with a sensitivity that is difficult to achieve by other techniques.

#### 451 **3.4. Determination of MP dimensional size and Stokes diameter by SMLS**

452 Two diameters can be deduced from the SMLS measurements:  $d_{\text{SMLS}} (t = 0)$  and  $d_{\text{Stokes}} (t \neq 0)$  (Fig. 8,  
 453 Fig. S10).  $d_{\text{SMLS}}$  was measured at the initial time point from the light scattering properties of the  
 454 particle suspension, while  $d_{\text{Stokes}}$  was measured during the evolution of the dispersion with time based  
 455 on its hydrodynamic properties [45]. The mean diameters of PE MPs measured *in situ* by SMLS and  
 456 *ex situ* by SEM were  $d_{\text{SMLS}} = 3.6 \pm 0.6 \text{ }\mu\text{m}$  and  $d_{\text{SEM}} = 4.7 \pm 0.6 \text{ }\mu\text{m}$  for  $C(\text{BSA}) = 0.03 \text{ g/L}$ . The mean  
 457 diameters of PP MPs were  $d_{\text{SMLS}} = 10.1 \pm 1.3 \text{ }\mu\text{m}$  and  $d_{\text{SEM}} = 10.9 \pm 2.2 \text{ }\mu\text{m}$  for the same conditions.  
 458 The difference between  $d_{\text{SEM}}$  and  $d_{\text{SMLS}}$  may arise from the anisotropy of the particles as  $d_{\text{SEM}}$  is related  
 459 to a projected surface whereas  $d_{\text{SMLS}}$  is associated with volumetric information. No significant  
 460 difference in  $d_{\text{SMLS}}$  was observed with different concentrations of free BSA in solution. Moreover, no  
 461 effect of ageing was observed in any of the conditions tested (Fig. 8a), confirming the good stability of  
 462 PE MP suspensions after storage and re-suspension.



463

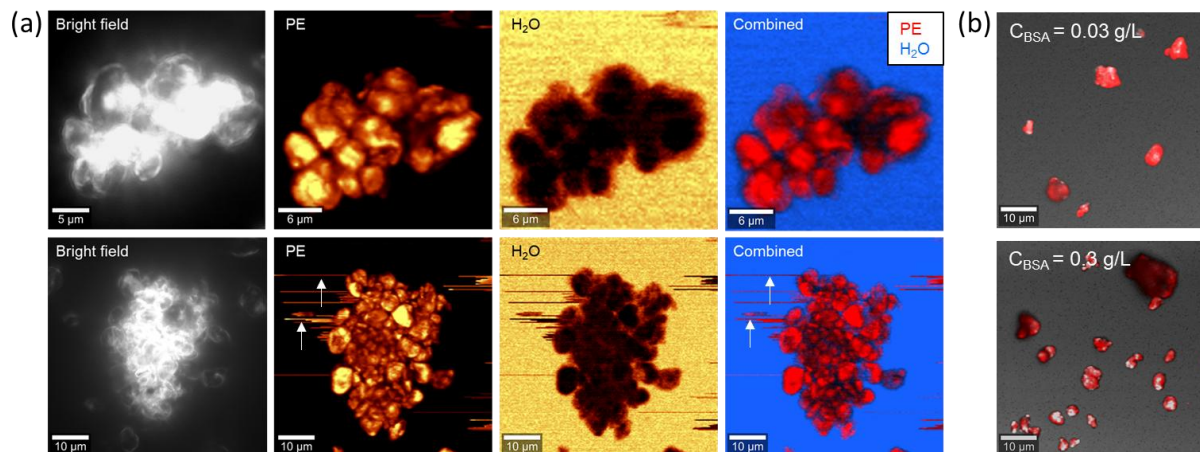
464 **Fig. 8.** *In situ* dimensional size analysis of PE MP suspensions by SMLS as a function of BSA  
 465 concentration. (a) Average SMLS diameter measured at  $t = 0$  and (b) average Stokes diameter  
 466 measured at  $t \neq 0$  for fresh suspensions (grey) and suspensions aged for 7 days (pink).  $n = 3$  for fresh  
 467 MP suspensions,  $n = 1$  for aged MPs suspensions. The average SEM diameter measured for  $C(\text{BSA}) =$   
 468  $0.03 \text{ g/L}$  is reported on the dotted blue line.

469 The Stokes diameter of PE MPs,  $d_{\text{Stokes}} = 6.7 \pm 0.3 \text{ }\mu\text{m}$  for  $C(\text{BSA}) = 0.03 \text{ g/L}$ , is not significantly  
 470 different from  $d_{\text{SMLS}}$  and  $d_{\text{SEM}}$ , except for the highest concentrations of BSA (Fig. 8b). This result  
 471 suggests that agglomeration or aggregation took place in the suspension during the experimental time  
 472 of 24 hours. Interestingly, this effect did not hamper the stability of the dispersion as evidenced by the  
 473 low creaming rate, suggesting that MP density is the main determinant of particle creaming. In the  
 474 case of PP MPs,  $d_{\text{Stokes}}$  was equivalent to  $d_{\text{SEM}}$  and  $d_{\text{SMLS}}$ . However, fast creaming suggests that the  
 475 largest particles have been removed from the suspension by creaming during the experimental time  
 476 (Fig. S10b). Note that stability, dispersibility, and the average diameter of PE MPs did not change  
 477 after 7 days of ageing in shelf-storage conditions.

### 478 3.5. *In situ* analysis of particle-particle interactions by Raman microscopy

479 To further investigate the formation and nature of MP agglomerates or aggregates in the suspensions,  
 480 *in situ* observation of particle assemblages with sizes  $> 15 \text{ }\mu\text{m}$  was conducted by Raman microscopy.  
 481 Large objects were observed in PE suspensions only, while only single particles were detected in PP  
 482 suspensions in the same experimental conditions. The Raman images revealed a high degree of  
 483 hydration with a layer of water separating loosely bound particles that assembled in dynamic  
 484 agglomerates (Fig. 9a). The deconvoluted Raman spectra are shown in Fig. S11. The same  
 485 organization of hydrated agglomerates was observed for all BSA concentrations (Fig. S12). The  
 486 horizontal stray lines visible on some Raman images are due to the binding or detachment of particles  
 487 to/from the agglomerate during scanning. This was confirmed by the observation of highly dynamic

488 particle-particle interactions by video-microscopy in reflection mode (Movies S1-S4). Overall, these  
489 results show that PE MPs do not form tight and covalently-bound aggregates, but highly dynamic and  
490 hydrated agglomerates that can appear and disappear through transient particle-particle interactions  
491 inside the suspension.



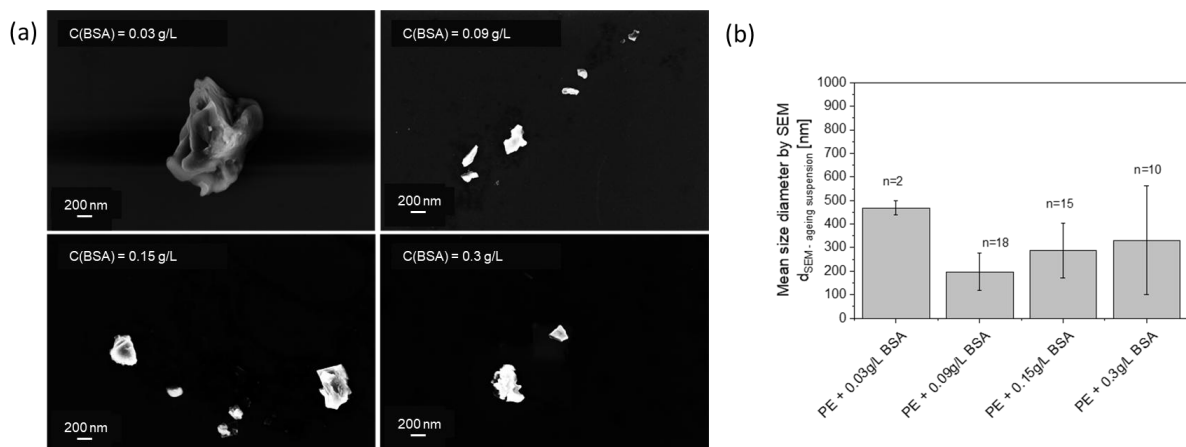
492  
493 **Fig. 9.** *In situ* analysis of PE MPs agglomerates by Raman microscopy. (a) From left to right: bright  
494 field image, Raman image of PE, Raman image of H<sub>2</sub>O, combined Raman image of PE (red) and H<sub>2</sub>O  
495 (blue). A small and large agglomerate are shown for the same concentration  $C(\text{BSA}) = 0.3 \text{ g/L}$ . Scale  
496 bar 6 μm (top) and 10 μm (bottom). White arrows indicate the binding or detachment of particles  
497 to/from the agglomerate during scanning. (b) Raman images of PE (red) from dry deposits on Si  
498 wafers from PE suspensions with  $C(\text{BSA}) = 0.03 \text{ g/L}$  (top) and 0.3 g/L (bottom). Scale bar 10 μm.

499 The size of the primary particles in the agglomerate can be estimated after segmentation of the Raman  
500 image of PE (Fig. S13) and compared to the diameter of single PE particles from the dry deposits (Fig.  
501 9b). The size analysis was not performed on single particles in suspension due to Brownian motion  
502 that impairs Raman imaging. The size analysis of the primary particles in the agglomerates *in situ*  
503 compared to particles from dry deposits confirmed that the agglomerates are composed of primary  
504 particles of the same size, excluding the trapping of smaller or larger MPs in the agglomerates.

### 505 3.6. Identification of secondary nanoplastics released during ageing

506 The analysis of PE MP suspensions by SEM revealed the presence of nanoplastics ( $d < 1 \text{ μm}$ ) in  
507 suspensions aged for 7 days without agitation (Fig. 10). Particles with irregular shapes and a diameter  
508 between 200 and 500 nm were clearly identified for  $C(\text{BSA}) \geq 0.03 \text{ g/L}$ . The fraction of nanoplastics  
509 is significant and could be underestimated due to the loss and/or the agglomeration of the smallest  
510 particles during deposition. By contrast, no nanoplastics were observed in fresh suspensions or in PP  
511 MP suspensions aged under the same conditions. Our results clearly suggest that the ageing of PE MPs  
512 with an excess of BSA can lead to the release of nanoplastics debris. BSA acts as a surfactant and can  
513 favor the dissociation of nanoplastics through the reorganization of the polymer chains, followed by  
514 the stabilization of the released particles in suspension by fast protein adsorption that increases the

515 surface hydrophilicity and charge. The difference in the behavior of PP and PE particles may be  
516 related to the polycrystalline nature of PE chains that forms subdomains within each particle [37].



517  
518 **Fig. 10.** Identification of nanoplastics from PE MP suspensions by SEM after ageing of the  
519 suspensions for 7 days in BSA solution. (a) SEM images and (b) size analysis of PE nanoplastics as a  
520 function of BSA concentration. A total number  $n = 45$  nanoplastic particles were analyzed. Scale bar  
521 200 nm.

522 These observations confirm that SEM is a method of choice for the dimensional size characterization  
523 of micro- and nano-plastics and provides evidence of the influence of the corona on the release of  
524 nanoplastic debris from soft polymeric microparticles in suspension.

525

## 526 4. Discussion

### 527 4.1. Influence of the corona on the dimensional analysis of MPs

528 The analysis of MP suspensions is not possible without a biocorona because the MPs do not mix or  
529 enter the aqueous solution due to their high hydrophobicity. Indeed, no particles were detected in the  
530 suspension without BSA. The deposition of micro or nanoplastics on SEM substrate for single particle  
531 analysis also requires the stabilization of MPs in suspension. Similarly, the measurement of MP  
532 density by SAXS is not possible on the dry powder.

533 The adsorption of BSA on nanomaterials has been widely studied in the literature, for example by  
534 Norde et al on silica nanoparticles [55], by Yoon et al and Baier et al on polystyrene microspheres [56,  
535 57] to cite just a few examples. Depending on surface chemistry, surface charge, and particle size,  
536 both enthalpic ( $\Delta H_{\text{ads}}$ ) and entropic ( $T \cdot \Delta S_{\text{ads}}$ ) contributions can drive BSA adsorption through  
537 electrostatic interactions, hydrogen bonding, protein conformational changes ( $\Delta H_{\text{ads}}$ ), or hydrophobic  
538 interactions mediated by the desorption of water molecules ( $T \cdot \Delta S_{\text{ads}}$ ). Notably, the adsorption of  
539 negatively charged BSA on negatively charged particles, including functionalized polystyrene  
540 microspheres, was reported [57]. In this case, clusters of basic residues such as arginine may facilitate

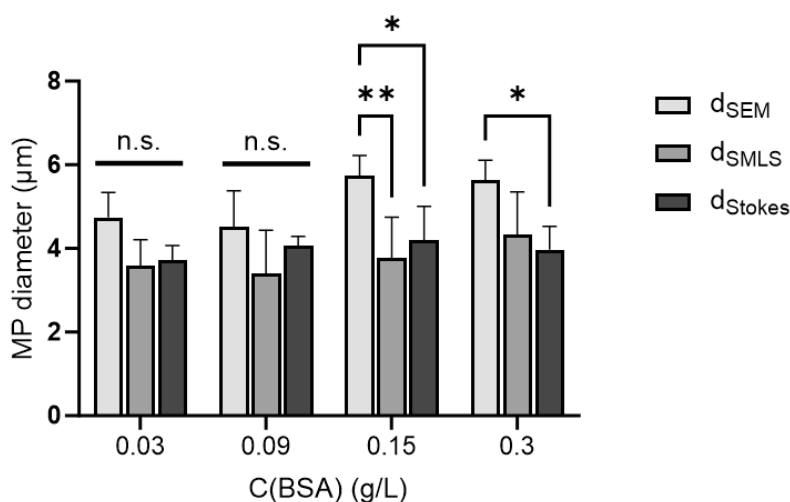
541 electrostatic interactions and H bonding [58], while hydrophobic interactions predominate at low  
542 surface charge for functionalized polystyrene [56].

543 More recently, the adsorption of BSA on PVC MPs [59] and polyolefin MPs [37] was investigated by  
544 isothermal calorimetry (ITC), vibrational spectroscopy, and synchrotron-radiation deep UV  
545 fluorescence microscopy. The thermodynamic analysis showed that electrostatic interactions and  
546 conformation changes play a role in the adsorption of BSA on PVC [59]. Label-free imaging of  
547 adsorbed BSA by deep UV fluorescence microscopy showed that the protein quality, expressed in  
548 terms of surface coverage and homogeneity at a single particle level, was different between PE and PP  
549 MPs. BSA formed a more homogeneous and denser protein corona on PP MPs compared to PE [37].  
550 This is in agreement with the higher amount of adsorbed BSA on PP MPs ( $1.5 \pm 0.1$  mg/m<sup>2</sup> for PE,  $2.6$   
551  $\pm 0.2$  mg/m<sup>2</sup> for PP) [37].

552 Based on the mass of adsorbed BSA, we can estimate the number of adsorbed molecules to  $1.4 \times 10^{16}$   
553 and  $2.4 \times 10^{16}$  molecules/m<sup>2</sup> for PE and PP MPs respectively. The hydrodynamic radius of BSA in pure  
554 water is 3.9 nm [60]. Assuming a spherical protein, the available surface for one BSA molecule within  
555 the protein corona can be estimated to 73 nm<sup>2</sup> on PE MPs and 42 nm<sup>2</sup> on PP MPs. By comparison, the  
556 average surface of one BSA molecule in water corresponds to 48 nm<sup>2</sup>. This calculation confirms that  
557 BSA forms a fully packed corona on PP and a looser corona on PE MPs that can be evaluated to one  
558 and up to 2 layers of adsorbed proteins. It is in agreement with the study of Yoon et al, who calculated  
559 an adsorbed layer thickness of BSA on functionalized microspheres of 4.6 to 7.1 nm corresponding to  
560 a monolayer of side-on or end-on BSA molecules [56]. Thermodynamic analysis and simulations of  
561 protein adsorption [61] could provide information on the driving forces and molecular mechanisms of  
562 protein-MP interactions in future work.

563 In this study, we developed methodologies to characterize the dimensional size of PE and PP MPs  
564 with a BSA corona and investigate the effect of the protein corona on the size analysis of micro- and  
565 nano-plastic suspensions. The diameters of MPs in suspension  $d_{SEM}$ ,  $d_{SMLS}$  and  $d_{Stokes}$  measured by  
566 SEM and SMLS using the density values determined by SAXS are compared in Fig. 11 and Fig. S14.  
567 The full data sets are presented in Table S2-S3. The diameters measured by SEM and SMLS ( $t = 0$  s)  
568 are very similar, which suggests that suspensions are mostly composed of constitutive particles with  
569 few agglomerates. The increase of free BSA concentration in solution does not significantly change  
570 the average SEM and SMLS diameters of PE and PP MPs, suggesting that the initial stabilization of  
571 microplastics in suspension by the protein corona is not altered. At higher BSA concentrations,  
572 variability in size analysis between the different techniques is observed, suggesting differential  
573 behaviors and the onset of instability in the suspensions. While a complete corona is formed in all the  
574 conditions, these results suggest that the excess of free BSA may favor agglomeration, particle-particle  
575 interactions and dynamics in the suspension, a process in which a transient, low affinity, soft protein  
576 corona may be involved [61]. It is worth noting that the dimensional analysis of MP suspensions also

577 provides the opportunity to measure density through its integration with specific surface  
578 measurements, such as SAXS. The density of PE MPs in suspension was measured by combining  
579 SEM to determine the average particle diameter and SAXS to determine the Porod's limit of the  
580 particles.



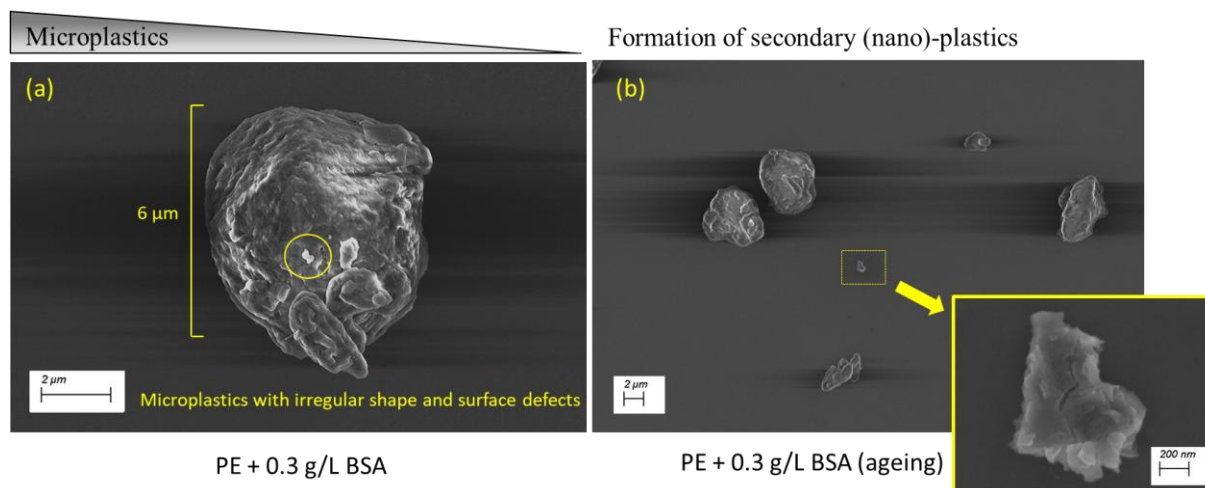
581  
582 **Fig. 11.** Comparison of SEM, SMLS, and Stokes diameters of PE MPs measured in the experimental  
583 medium as a function of BSA concentration (n = 3) \* p < 0.05, n.s. non-significant.

584 In toxicological studies, stability and dispersibility of MPs are key parameters that govern the  
585 exposure of cells in vitro and organisms in vivo. The number and size of particles that come into  
586 contact with cells/organisms depend on the creaming or sedimentation, and aggregation or  
587 fragmentation during exposure. The control of MP stability and dispersibility in suspension by BSA  
588 combined with their dimensional characterization provides a useful framework to evaluate the dose of  
589 exposure. Note that this study was performed at 20°C with purified BSA. The same methodology  
590 could be applied to the study of the size, stability, and dispersibility of various types of MPs in other  
591 media at lower or higher temperature.

#### 592 **4.2. Release of secondary nanoplastics and potential consequences**

593 In the recent report '*Nanoplastics – State of knowledge and environmental and human health impacts*'  
594 mandated by the European Commission [62], the occurrence and the formation of secondary  
595 nanoplastics were discussed. Environmental studies revealed the presence of nano-sized (< 1 µm)  
596 plastics debris generated from secondary sources in the atmosphere and in aquatic environments [63,  
597 64]. The identified sources of secondary nanoplastics are physical abrasion [64], degradation  
598 processes, and ageing [65]. Nowack et al showed that textiles are a significant source of nanoplastics  
599 released by abrasion during washing [66]. The release of nanoplastic debris by the ageing of PE MPs  
600 in suspension identified in this study reminds us that PE and PP are indeed called plastics because of  
601 their mechanical plasticity. This property originates from the mobility of the polymeric chains inside

602 the material. As a consequence, MPs are dynamic materials that are prone to evolution under  
 603 mechanical, thermal, or chemical stress. The observation of secondary nanoplastics formed in aged  
 604 suspensions without heating, photodegradation, or mechanical stress suggests that an equilibrium  
 605 between a high and a low specific surface area form of the same material is observed here. According  
 606 to this model, the excess of BSA would shift the equilibrium towards the high specific surface area  
 607 form. Thus, the fraction released from plastics through erosion of their surfaces produce fragments of  
 608 microplastics ( $d > 1 \mu\text{m}$ ), but also particles smaller than  $1 \mu\text{m}$  in size, which we define here as  
 609 nanoplastics ( $d = 1 \text{ nm} - 1 \mu\text{m}$ ). To date, the characterization of nanoplastics remains challenging. The  
 610 number of nanoplastic fragments increased with increasing exposure time of MPs in BSA media  
 611 **without mechanical stress** (Fig. 10b). A variety of plastic fragment sizes, morphologies and shapes  
 612 were found in our experiments. Debris released in the suspension could be described as small and  
 613 irregular fragments and cracked particles (Fig. 12b). PE MPs produced a higher number of small  
 614 fragments including nanoplastics (200 – 500 nm) compared to PP MPs under the same experimental  
 615 conditions. Our observations detailed in this study reveal the potential release of nanoplastics if we  
 616 consider a size threshold of  $1 \mu\text{m}$  for this type of nanomaterial. The release of nanoplastics could result  
 617 from a continuous mechanism in spatiotemporally dynamic media with specific physico-chemical  
 618 properties, including ionic strength gradients [67, 68]. Bonacci et al suggested that solid-solid contacts  
 619 play a role in the ageing mechanisms of dense colloidal suspensions at rest [69]. This contact-  
 620 controlled ageing may concern a whole range of materials, including MPs.



621  
 622 **Fig. 12.** Illustration of the release of secondary (nano)-plastics after ageing in BSA solution: (a) PE  
 623 MPs before ageing showing irregular shapes and surface defects, (b) PE MPs and NPs after ageing.

624 The release of nanoplastics is also critical in terms of pollution as an increase in specific surface area  
 625 may favor the release of toxic additives or the adsorption of toxic contaminants such as  
 626 perfluoroalkyls (PFAS) [70]. As nanoplastics are stabilized by a corona, the sorption of contaminants  
 627 will also depend on the exchange between adsorbed proteins and free contaminants that directly  
 628 depends on the affinity of the contaminant for the surface and the concentration of the contaminant in



629 the environment. As proteins are adsorbed in a non-covalent way with a moderate affinity, the  
630 exchange of small hydrophobic pollutants, such as bisphenols or perfluoroalkyls, which could further  
631 enter the polymeric structure by diffusion, may occur.

632 Compared to extensive studies on abiotic mechanisms, few occurrences of MP fragmentation by biotic  
633 processes have been reported. Yet, the fragmentation of ingested MPs into nanoplastics was evidenced  
634 in aquatic organisms, such as in the Antarctic krill *Euphausia superba* [71] and in the freshwater  
635 amphipod *Gammarus duebeni* [72] that were able to rapidly fragment PE MPs. The formation of a  
636 biocorona has also been shown to favour particle size reduction through dissolution of cobalt  
637 nanoparticles in biological media [73]. We further demonstrated in this study that nanoplastic debris  
638 were formed in PE MP suspensions without mechanical stress, a process that is favoured by the  
639 formation of a biocorona on MPs. Together, these results suggest that biotic processes that are not  
640 limited to specific enzymatic degradation of synthetic polymers may play a crucial and understudied  
641 role in the fate of MPs in organisms.

#### 642 **4.3. Methodologies for the metrology of MP suspensions**

643 Despite the identification of many emerging sources of nanoplastics in the environment, there is a gap  
644 between the ability to sample, isolate, detect nanoplastics on one hand, and the possibility to  
645 characterize and quantify nanoplastics on the other hand [22, 74]. Nanoplastics size is below the  
646 instrumental limit of detection of infrared spectroscopy and coherent Raman microscopy. For this  
647 reason, few studies have provided images of nanoplastics. It is therefore crucial to explore new  
648 methodologies to analyze nanoplastics.

649 SEM, TEM, FTIR and Raman microscopy, and to a lesser extent fluorescence microscopy, have been  
650 used for the identification and size measurement of microplastics [62]. Quian et al recently  
651 demonstrated the detection and identification of nanoplastics using Stimulated Raman Scattering [75].  
652 This approach has significantly lifted the limitation of Raman microscopy in terms of spatial  
653 resolution and sensitivity for the detection of nanoplastics. DLS (Dynamic Light Scattering) is the  
654 reference method for the study of nanoparticles stability in suspension, although the size range of the  
655 instrument and the low stability of MPs in aqueous suspensions make it more difficult to apply to MPs  
656 compared to manufactured nanomaterials [74]. In this study, we developed the analysis of MP size and  
657 stability using SMLS and the analysis of MP density in suspension by SAXS. Lee et al used SMLS to  
658 monitor the sedimentation of polystyrene microspheres in methanol during synthesis [76]. This  
659 technique was also used to study the aggregation of core-shell polystyrene-iron oxide nanoparticles in  
660 Pickering emulsions by Han et al [77]. There are few reports on the analysis of other types of MPs by  
661 SMLS to our knowledge. Even if SEM is common for imaging MPs, the European Commission report  
662 on nanoplastics [62] underlined the current limitations of SEM analysis due to quantification  
663 difficulties, sample preparation, and charging effect. We solved these problems by improving sample  
664 deposition on silicon wafers and preventing charging effects by flowing nitrogen gas near the surface

665 to neutralize the charge effect. Using this method, we successfully measured nanoplastics size by SEM  
666 with high spatial resolution. The PE and PP MPs chosen in this study have a spherical shape, even if  
667 they are not perfect spheres (such as polystyrene microspheres). It would be interesting to extend the  
668 dimensional analysis of MPs by SEM to MP fibres to characterize the release of synthetic textile fibres  
669 of various lengths and diameters in the environment [78].

670 Despite the formation of a protein corona on PP MPs, the fast creaming rate of the particles and the  
671 lower stability of the suspensions prevented the accurate measurement of PP density by SAXS. We  
672 also observed differences between replicates that suggests that instability was triggered by other  
673 mechanisms. Future work could further investigate the molecular mechanisms that drive such  
674 instability, depending on the exposure medium and experimental conditions.

#### 675 **4.4. Towards a unified definition of nanoplastics**

676 Plastic pollution takes three forms, depending on the size of the pieces of plastic found in the  
677 environment. These are macroplastics, microplastics, and nanoplastics. Various definitions are  
678 employed to conceptualize the presence of micro/nanoplastics and debris in marine, freshwater, sludge  
679 and environmental ecosystems as well as in food or drinking water. Significant differences exist in the  
680 definition on size. The lack of global standardization of nomenclature, harmonization of test methods  
681 for dimensional characterization, and reference materials complicates the situation. The 'grey' area  
682 regarding the class boundary between micro and nanoplastics is undoubtedly due to the high level of  
683 equipment required to observe nanoplastics. Collaboration between research teams, involving  
684 nanoplastics specialists, could provide an initial solution.

685 It is necessary to employ multiple methods for detection and characterization, and methods available  
686 to date for micro and nanoplastics may not be appropriate to detect and quantify nanoplastics [22, 35,  
687 74]. Moreover, standardization of size class limits is needed to facilitate the exchange of results  
688 between teams, enabling comparisons that will facilitate the understanding of the phenomena. SEM is  
689 a method of choice that provides data on nanoplastic morphology, information that is still missing.  
690 Additionally, this method can be combined with chemical analysis strategies by EDX (Energy-  
691 Dispersive X-ray spectroscopy) and EBSD (Electron Backscatter Diffraction) [26].

692

## 693 **5. Conclusions**

694 Using SEM, SMLS, SAXS, and Raman microscopy, we developed methodologies for the dimensional  
695 size analysis and the physicochemical characterization of PE and PP MPs with a BSA corona in  
696 suspension. We completed the dimensional characterization of microplastics stabilized by a protein  
697 corona, including the measurement of Stokes diameter and particle density. The analysis of the effect  
698 of the biocorona on the dimensional characterization of MPs revealed that ageing of MPs in  
699 suspensions resulted in the release of secondary nanoplastics. SEM can be used for *ex situ* dimensional  
700 analysis of micro- and nano-plastics, with the advantage of existing normalized methods for particle

701 analysis, where it showed good agreement with size analysis of suspensions by SMLS. MP density  
702 could be determined in suspension with high sensitivity by SEM combined with SAXS to determine  
703 the particle diameter and Porod's limits respectively. Moreover, SEM was successfully applied to the  
704 detection of nanoplastic fractions that would have been obscured by other methods due to the small  
705 fraction of nanoplastics. The identification of nanoplastic debris in MP suspensions suggests that an  
706 excess of BSA favors the release of nanoplastics with a high specific surface area, potentially posing  
707 an additional threat to ecosystems. This observation strongly supports the adoption of a unified  
708 definition of nanoplastics and the inclusion of nanoplastics with a maximum size threshold of 1  $\mu\text{m}$  in  
709 future regulations. In addition to SEM, we can also foresee the possible use of EDX and EBSD for  
710 their characterization.

## 711 **Declaration of Competing Interest**

712 The authors declare no competing interest.

## 713 **Data availability**

714 The datasets produced in this study are available from the corresponding authors upon request.

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