Supplementary Information for:

Aging Amplifies the Combined Toxic Effects of Polystyrene Nanoplastics and Norfloxacin on Human Intestinal Cells

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Fig. S 1 shows the fluorescence spectra of pristine and aged PS-NPs before and after swelling. Observations from the results indicate that, for both pristine and aged PS-NPs, the fluorescence intensity increased after swelling, suggesting that TPE successfully swelled into the PS-NPs, endowing them with fluorescent properties. Compared to the fluorescence of pristine PS-NPs, the fluorescence intensity of aged PS-NPs was slightly reduced, indicating that the aging behavior of PS-NPs has some effect on the swelling of TPE into PS-NPs, though the impact is not significant. Therefore, we can conclude that fluorescent aged PS-NPs were successfully prepared.

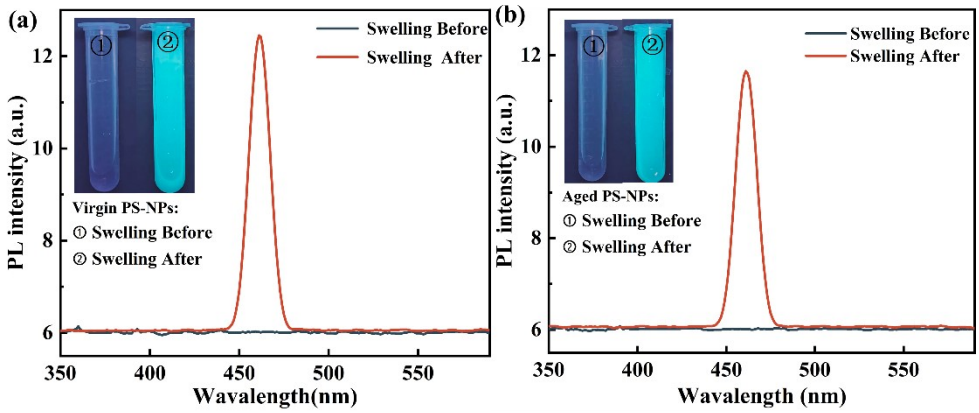

Fig.S 1 Fluorescence spectra of PS-NPs before and after swelling (virgin PS-NPs in (a), aged PS-NPs in (b))

As shown in **Fig. S 2**, before swelling, the original PS-NPs exhibited a spherical shape, while the aged PS-NPs displayed irregular shapes with rough surfaces, cracks, and damage. However, after swelling, there were no significant changes in the shape or particle size of either the original or aged PS-NPs, indicating that the swelling process does not affect the shape or particle size characteristics of PS-NPs. This further demonstrates that the method of incorporating TPE into PS-NPs through swelling is appropriate. Additionally, the particle size distribution and average particle size of the PS-NPs did not show noticeable changes, further confirming that swelling does not influence the particle size of PS-NPs.

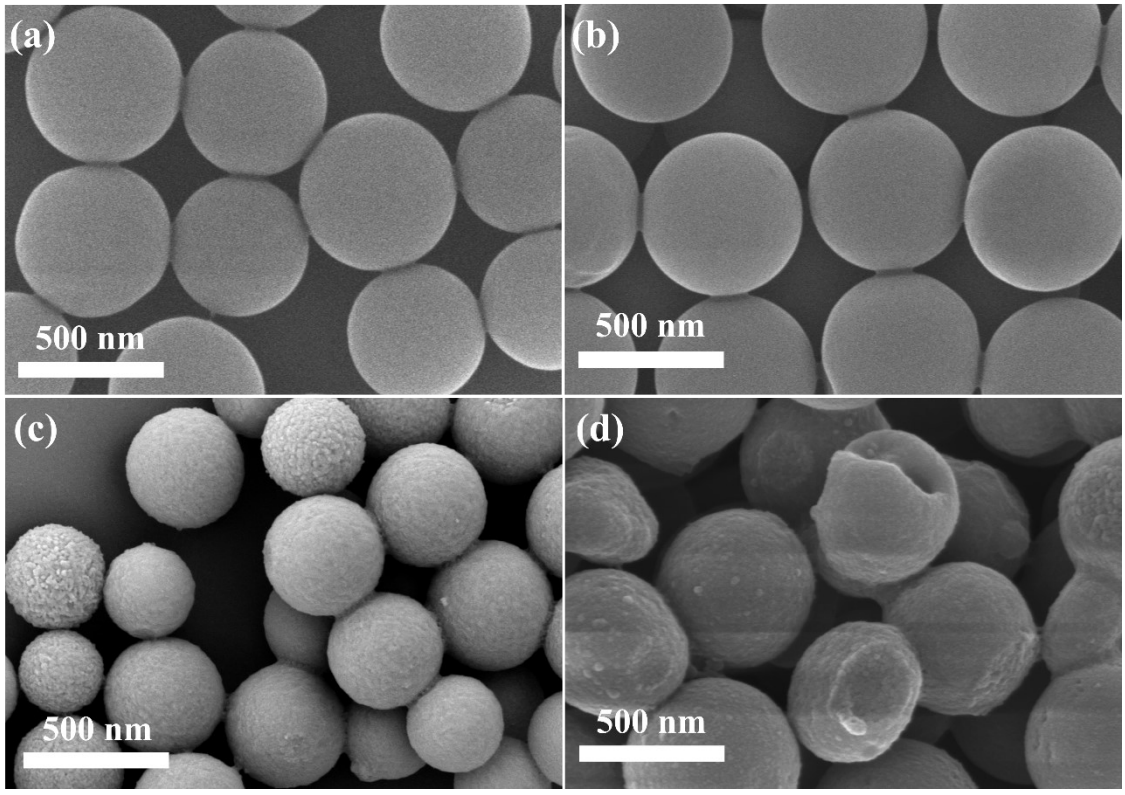

Fig.S 2 SEM images of virgin PS-NPs before and after swelling (a and b) and aged PS-NPs before and after swelling (c and d).

To provide a more intuitive demonstration of the internalization of PS-NPs by Caco-2 cells,

fluorescence microscopy was employed. Human colorectal adenocarcinoma cells were incubated with

PS-NPs at various concentrations (20, 40, 100, 200, and 400 μg/mL) along with NOR (5 μg/mL) for 24

hours, and fluorescence images capturing cellular uptake of NPs were obtained. Bright-field and

fluorescence-field imaging results are presented in**Fig. S 3** and **Fig. S 4**, respectively. The fluorescence

channel images revealed that the cellular uptake of pristine PS-NPs increased significantly with

increasing exposure concentration. At the highest concentration of 400 μg/mL, cells exhibited stronger

brightness, with blue-fluorescent PS-NPs clearly visible in the fluorescence field images. The number of blue-fluorescent PS-NPs within the field of view also increased with higher exposure concentrations, indicating an enhanced uptake of PS-NPs by cells as the concentration increased. Notably, co-

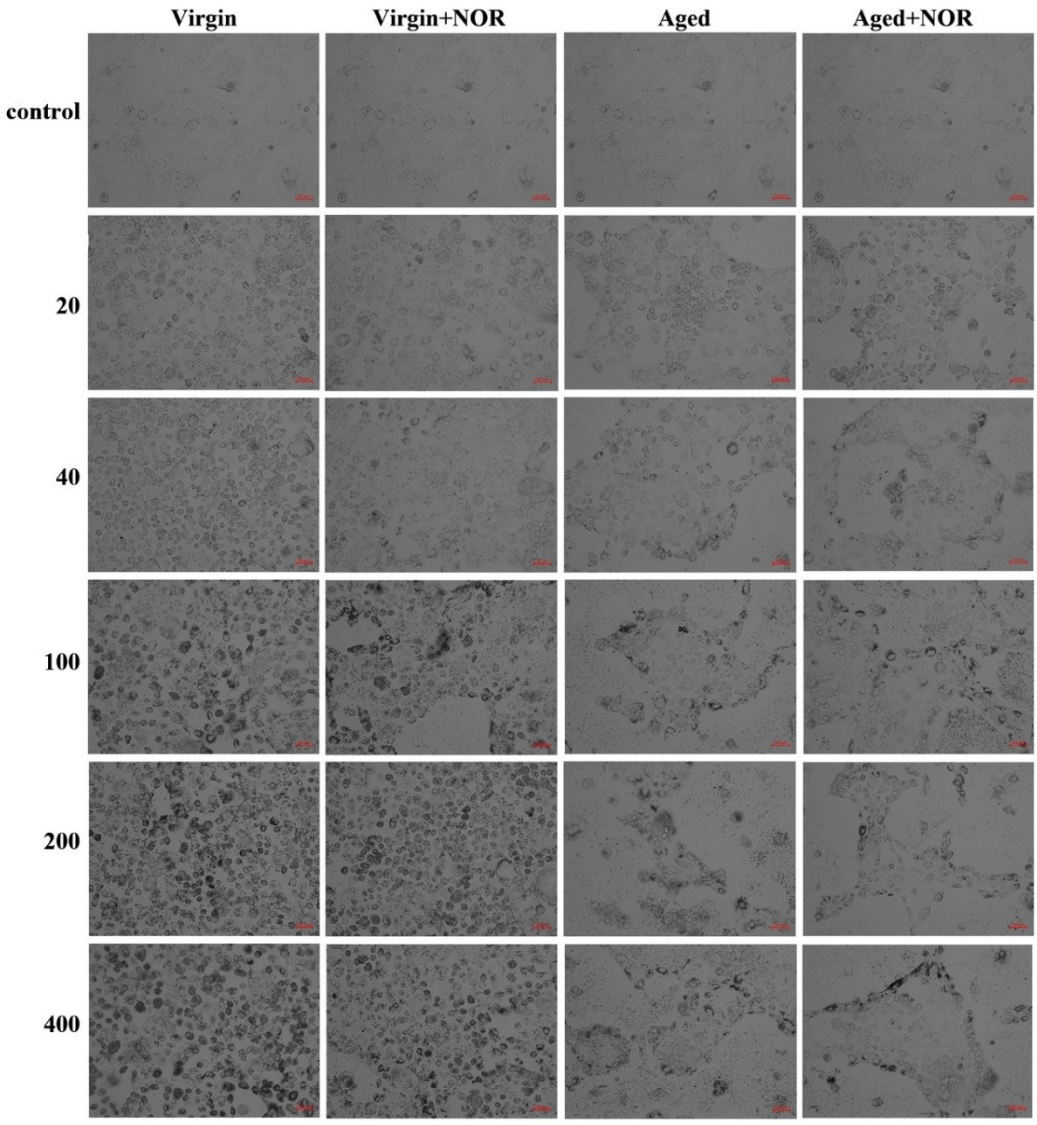

Fig.S 3 Fluorescence microscopy images (bright field) of Caco-2 cells co-incubated with PS-NPs at various concentrations and NOR (5 μg/mL) for 24 hours.

incubation with NOR at 400 μg/mL resulted in visibly lower cell brightness compared to 200 μg/mL, suggesting that NOR significantly inhibited cellular internalization of PS-NPs. Interestingly, the internalization level of aged PS-NPs was markedly lower than that of pristine PS-NPs. However, no significant difference in internalization levels or quantities was observed between aged PS-NPs alone and those co-incubated with NOR.

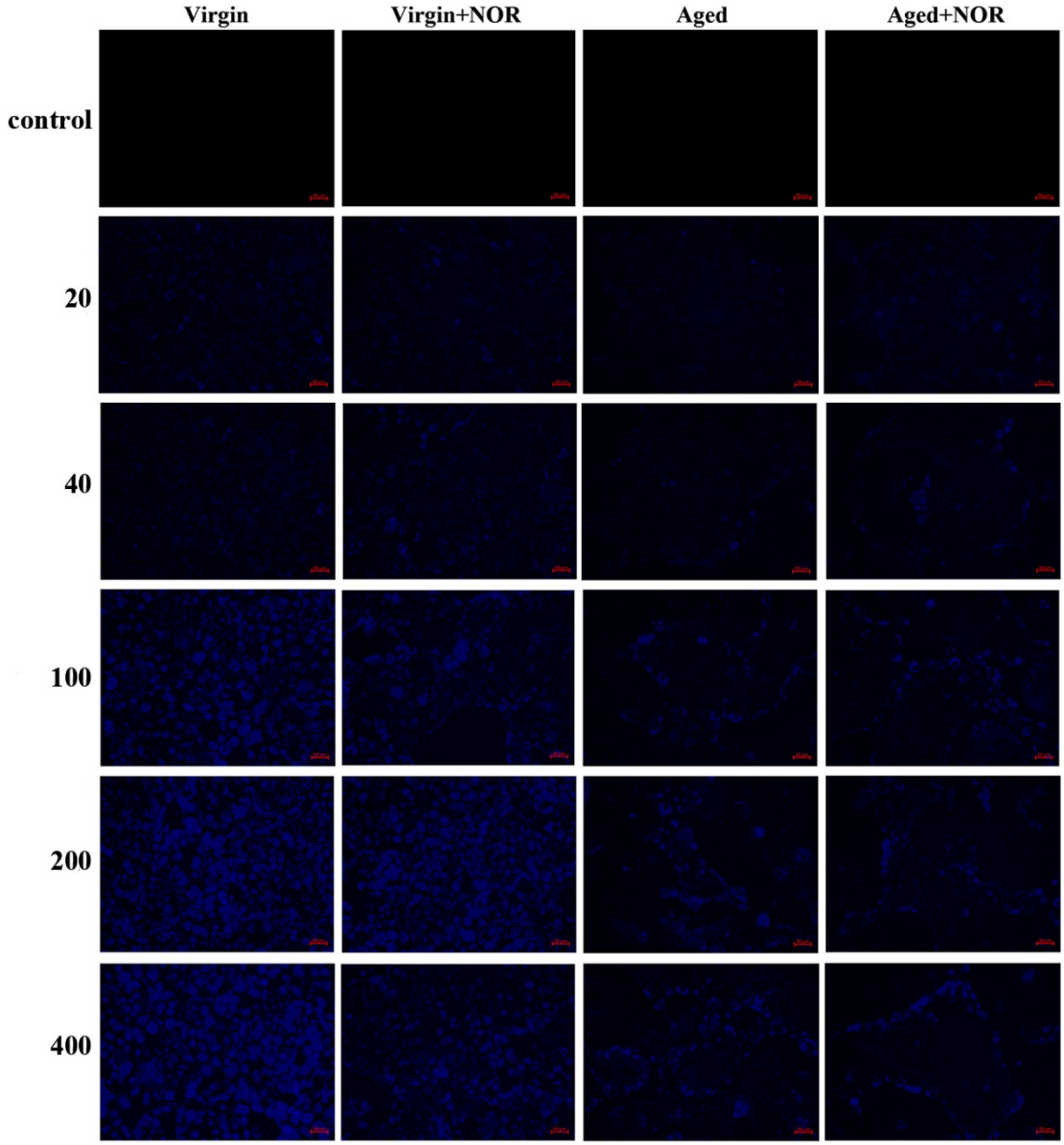

Fig.S 4 Fluorescence microscopy images (fluorescence channel) of Caco-2 cells co-incubated with PS-NPs at various concentrations and NOR (5 μg/mL) for 24 hours.

To establish standard curves for quantitative analysis, both Virgin and Aged PS-NPs and NOR were quantified using fluorescence spectroscopy and UV-Vis spectrophotometry, respectively. A total of 2 × 7 and 1 × 6 independent groups of 1 × 10^6 unlabeled Caco-2 cells were prepared. The cells were first diluted in 5 mL of ultrapure water and subsequently subjected to 30 minutes of sonication to ensure

complete cell lysis. In the 2 × 7 groups, varying volumes of Virgin or Aged PS-NPs were added to achieve final concentrations of 0, 10, 20, 40, 100, 200, and 400 μg/mL, respectively. For the 1 × 6 groups, NOR was added to yield final concentrations of 0, 2.5, 5, 10, 25, and 50 μg/mL. These 20 groups were designated as control groups. The fluorescence intensity of the PS-NPs was measured using a fluorescence spectrophotometer (excitation wavelength: 330 nm; emission range: 350–400 nm; maximum emission peak at 375 nm), and a calibration curve correlating fluorescence intensity with PS-NP concentration was established. For NOR quantification, UV-Vis absorbance was measured at 275 nm, and a corresponding calibration curve of absorbance versus concentration was generated. These standard curves were later employed to quantify the intracellular uptake of PS-NPs and NOR in experimental groups.

As shown in **Fig.S 5**, the standard curves of the three pollutants exhibit a good linear correlation between fluorescence intensity (absorbance) and sample concentration. By using the standard equation, the fluorescence intensity (absorbance) of cells that have taken up the pollutants can be measured, and the results can then be substituted into the linear equation to calculate the concentration of the pollutants.

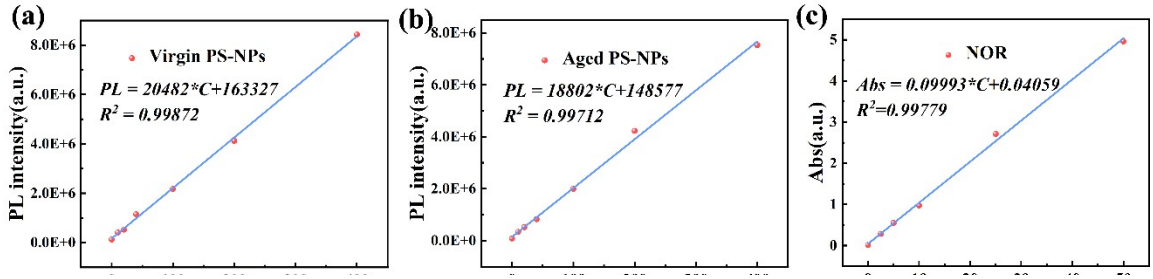

Fig.S 5 Fluorescence intensity (absorbance) - concentration standard curves for PS-NPs or NOR.

As shown in **Fig. S 6**, a clear comparison of cell viability among all five key experimental groups (Virgin, Virgin + NOR, Aged, Aged + NOR, and NOR alone at 5 μg/mL) is presented. Across all concentrations, Aged PS-NPs exhibited significantly higher toxicity than Virgin PS-NPs, regardless of whether exposure was single or co-exposure. These results indicate that simulated aging enhances the intrinsic cytotoxicity of PS-NPs in Caco-2 cells. Furthermore, when PS-NPs were co-exposed with a low, non-toxic concentration of NOR (5 μg/mL), a pronounced synergistic toxic effect was observed. Interestingly, the magnitude of this synergy appeared to be concentration-dependent and differed between Virgin and Aged PS-NPs. Although NOR co-exposure resulted in a greater additional reduction in cell viability at high concentrations of Virgin PS-NPs, the overall toxicity of the Aged PS-NPs combined with NOR remained the highest across all conditions. At high concentrations of Aged

PS-NPs (e.g., ≥200 μg/mL), the particles themselves were highly cytotoxic, inducing substantial cell death. This strong intrinsic toxicity may mask the relatively smaller, additional synergistic effect contributed by NOR, thereby making it more difficult to discern.

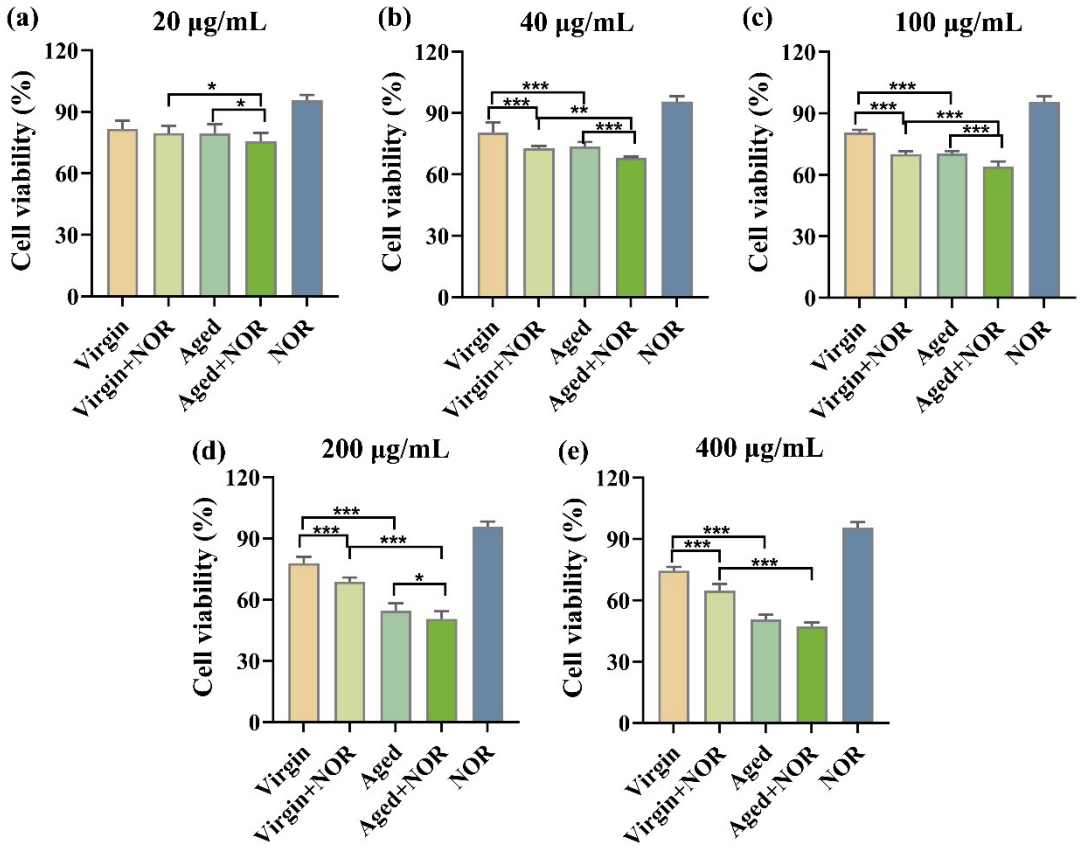

Fig.S 6 Effects of different concentrations (μg/mL) of PS-NPs and 5 μg/mL NOR on the viability of Caco-2 cells at 24 h exposure time: 20 μg/mL (a); 40 μg/mL (b); 100 μg/mL (c); 200 μg/mL (d); 400 μg/mL (e); ; data are expressed as mean ± SD; ⁎ indicates significant difference between control and treatment, ⁎p < 0.05, ⁎⁎p < 0.01, ⁎⁎⁎p < 0.001

As shown in **Fig. S 7**, a clear comparison of ROS levels among all five key experimental groups (Virgin, Virgin + NOR, Aged, Aged + NOR, and NOR alone at 5 μg/mL) is presented. Under co-exposure conditions, the addition of NOR generally led to higher ROS production compared to Virgin PS-NPs alone, with a particularly pronounced effect observed at 400 μg/mL. In contrast, the impact on Aged PS-NPs was more variable. Comparative analysis revealed the most complex aspect of the aging effect: at the lowest concentration (20 μg/mL), Aged PS-NPs induced significantly higher ROS levels than Virgin PS-NPs. Conversely, at intermediate concentrations (40–200 μg/mL), Aged PS-NPs

elicited significantly lower ROS levels. At the highest concentration (400 μg/mL), the trend reversed once again, with aged particles generating higher ROS levels than their virgin counterparts.

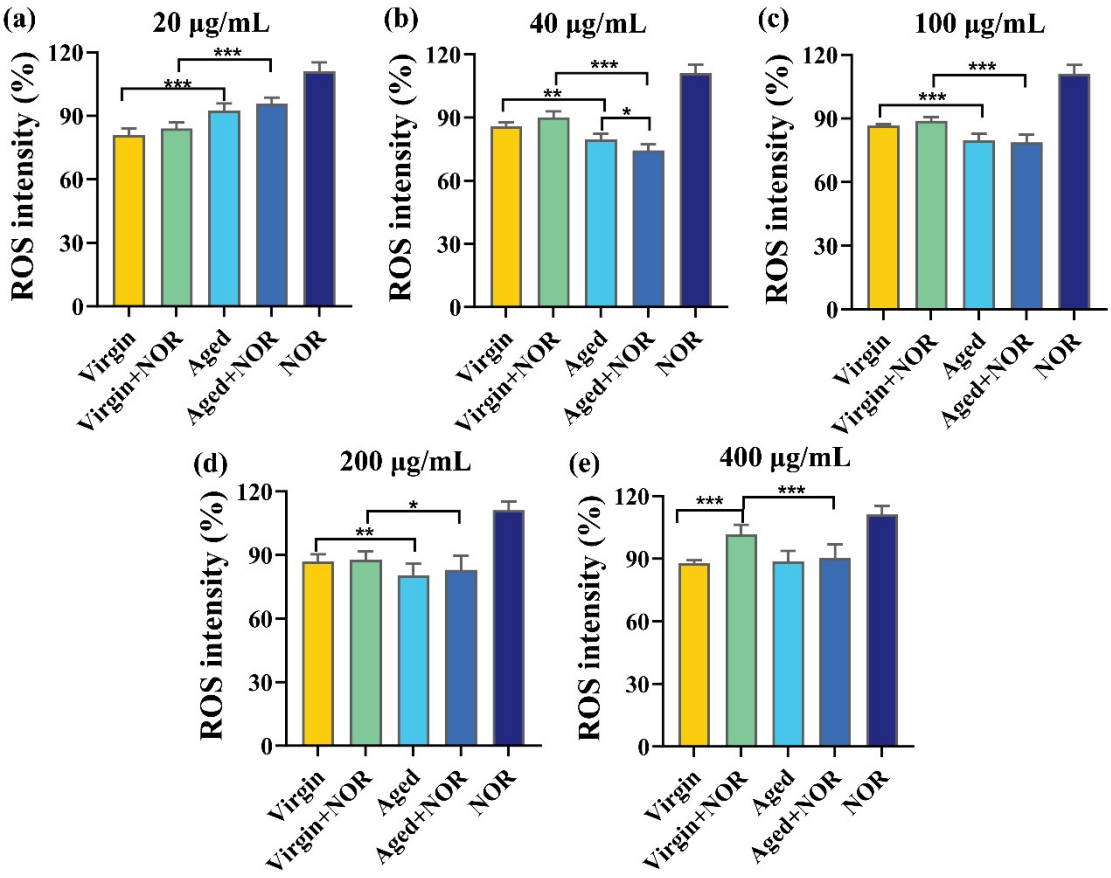

Fig.S 7 Effects of different concentrations (μg/mL) of PS-NPs and 5 μg/mL NOR on intracellular reactive oxygen species (ROS) levels in Caco-2 cells after 24 hours of exposure：20 μg/mL (a); 40 μg/mL (b); 100 μg/mL (c); 200 μg/mL (d); 400 μg/mL (e); data are expressed as mean ± SD; * indicates significant difference between control and treatment, *p < 0.05, **p < 0.01, ***p < 0.001