Enhancing Passive Transport of Micro/Nano Particles into Cells by Oxidized Carbon Black

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ABSTRACT: Uses of micro-/nano-sized particles to deliver biologically active entities into cells are common for medical therapeutics and prophylactics and also for cellular experiments. Enhancing cellular uptake and avoiding destruction by lysosomes are desirable for general particulate drug delivery systems. Here, we show that the relatively nontoxic, negatively charged oxidized carbon black particles (OCBs) can enhance cellular penetration of micro- and nano-particles. Experiments with retinal-grafted chitosan particles (PRPs) with hydrodynamic sizes of 1200 ± 51.5, 540 ± 29.0, and 430 ± 11.0 nm (three-sized model particles) indicate that only the sub-micron-sized particles can penetrate the first layer of multilayered liposomes. However, in the presence of OCBs, the micron-sized PRPs and the two submicron-sized PRPs can rapidly enter the interiors of all layers of the multilayered liposomes. Very low cellular uptakes of micro- and submicron-sized PRPs into keratinocytes cells are usually observed. However, in the presence of OCBs, faster and higher cellular uptakes of all of the three-sized PRPs are clearly noticed. Intracellular traffic monitoring of PRP uptake into HepG2 cells in the presence of OCBs revealed that the PRPs did not co-localize with endosomes, suggesting a nonendocytic uptake process. This demonstration of OCB’s ability to enhance cellular uptake of micro- and submicron-particles should open up an easy strategy to effectively send various carriers into cells.

INTRODUCTION

Drug carriers in particulate forms have been used to solve problems on solubility, stability, and sustained release of drugs and have been applied in both therapeutic and prophylactic purposes. The carrier function is the delivery of various cargoes to targeted cells, and once they reach the targeted cells, it is expected that the carriers should be able to enter those cells and then release drug molecules to perform the intended functions. It has been known that nanoparticles can enter cells through active and passive processes, depending on their physical and chemical properties including size, shape, surface charge, and surface chemistry. Some nanoparticles can enter cells via a nonendocytic pathway (passive transport), whereas many others are taken up into cells by active transport processes in which they have to face cellular elimination and digestion by lysosomal pathway. Nanoparticles with very small size and positive charge have been observed to pass through cell membranes by generating membrane hole or membrane deformation, causing toxicity to cells. The use of amphiphilic molecules (often sold as transfection reagents) that can effectively disrupt phospholipid bilayer membrane assembly and thus allowing many cargos to pass through the membrane is one of the popular strategy used in many in vitro experiments to bring macromolecules, such as polynucleotides and proteins, into cells. Differently, reports on enhancing cellular penetration of micro-/nano-particles are mostly limited to the use of positively charged materials to fabricate into or to decorate onto the particles. Cell-penetrating peptides are positively charged materials that have been used for this purpose. Nevertheless, there are numbers of carrier systems that cannot be easily decorated with the positively charged moieties; therefore, a simpler means to improve their cellular penetration ability is needed. Ability to send particles into cells will allow the study on cellular metabolism of the particles or materials. Local therapeutic applications of carriers such as topical drug delivery or local prophylactic use, such as vaccine antigen delivery, can also benefit from an ability to enhance the cellular uptake of particles.

We have reported that oxidized carbon nanoparticles (OCSs) can interact with lipid bilayer membranes and can deliver peptide nucleic acids to the nucleus of mammalian cells via endocytosis with endosome leakage. Distinct superiority of OCSs over oxidized carbon nanotubes and graphene oxide sheets in passing through the phospholipid bilayer membrane has been demonstrated in both artificial cells and real cells. Recentely, we have also prepared new OCSs from commercially

Supporting Information

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available carbon black particles and showed that these oxidized carbon black particles (OCBs) can directly deliver big functional proteins across cell membranes via a nonendoctytic pathway. Here, we report that these nonimmunogenic, relatively nontoxic OCBs can outstandingly enhance the penetration of both micro- and submicron-sized particles across phospholipid bilayer membranes. We also demonstrate this finding in both artificial cells (giant liposomes) and real cells. The work also includes intracellular traffic of the particles delivered into cells with the aid of the OCBs.

**RESULTS AND DISCUSSION**

Preparation and Characterization of OCBs. OCBs (Supporting Information Figure S1) could be successfully prepared by oxidizing carbon black with sodium nitrate and potassium permanganate in strong acid, as previously described. Scanning electron microscopy (SEM) images indicate spherical morphology with the size of 130 ± 29.27 nm, agreeing well with their hydrodynamic size of 127 ± 1.35 nm obtained from dynamic light scattering (DLS) technique. The zeta potential of the particles in water is −34 ± 1.75 mV. Verification of the particles was carried out by identifying functional groups of the particles by Fourier transform infrared spectroscopy and Raman spectroscopy (Supporting Information Figure S2). The particles consist of epoxy, carboxyl, and hydroxyl functional groups and possess carbon to hydrogen to oxygen molar ratios of 1.0:0.27:0.64 as deduced from combustion-elemental analysis (see Supporting Information Figure S2 for the OCB model particle).

Retinal-Grated Chitosan Particles. Here, we used the PRPs having various sizes as model particles to investigate an ability of the OCBs to bring particles into cells. We selected these particles because of a few reasons. First, the particles are auto-fluorescent, thus allowing us to monitor them under a confocal laser fluorescence microscope (CLFM). Second, it has been known that chitosan is the polymer that possesses some cellular uptake character, therefore an ability to enhance the particles made from chitosan should demonstrate real interesting efficiency enhancement. Third, because the PRPs (or the so-called pro-retinal particles or PRPs) can be used for therapeutic aspect inside the cells, enhancing their cellular uptake efficiency should have a direct impact for their applications. We prepared the particles as previously described and used step-wise centrifugation to separate them into three different sizes (Figure 1), 1000 ± 82.5, 500 ± 22.7, and 390 ± 15.5 nm, as estimated from their SEM images. These sizes corresponded to their hydrodynamic sizes in water of 1200 ± 51.5, 540 ± 29.0, and 430 ± 11.0 nm obtained from DLS analysis. All three-sized PRP particles possess similar zeta potential of approximately 50 ± 0.5 mV.

Penetration of PRPs into Cell-Sized Liposomes. The different sized PRPs were tested for their ability to penetrate across the lipid bilayer membrane using artificial cells (cell-sized liposomes, see Supporting Information Figure S3 for the lipid structures used for the liposome construction). The use of the giant liposome makes it possible to focus only on passive transport across the membrane with no involvement from active trans-membrane mechanism. We prepared the artificial cells with dioleoyl L-α-phosphatidylcholine using the hydration technique as previously described. We then incubated the cell-sized liposomes with PRPs and monitored fluorescence signals of PRPs at the inside and outside of the liposomes as a function of incubation time, using a CLFM. We observed that the 540 nm PRPs and the 430 nm PRPs were adsorbed on the surface of the liposomes after 30 min of incubation and the two-sized particles could penetrate into the inside of the liposomes after 45 min incubation (Figure 2). In the case of the 1200 nm PRPs, the fluorescence signal of the particles on the liposomes was undetectable even after 90 min of incubation, thus implying minimal to no interaction between the 1200 nm PRPs and the liposomes. These results indicate that the 430–540 nm-sized PRPs penetrate phospholipid bilayer membranes more effectively than the 1200 nm PRPs. It should be noted here that most of the prepared liposomes are multilayered liposomes and we observed that during the 90 min incubation time, the penetration of the 430/540 nm PRPs took place only at the first layer. In other words, we did not observe significant penetration of the 430/540 nm PRPs into the interior of the smaller liposomes inside the big liposomes.

Using OCBs to Deliver PRPs into Cell-Sized Liposomes. Here, we tested whether OCBs could increase the lipid bilayer membrane penetration of the PRPs. OCBs and PRPs were mixed at the mass ratio of OCBs/PRPs of 1:4 and then the mixture was incubated with liposomes; the fluorescence signal of the PRPs was monitored by CLFM. The results show that in the presence of OCBs, the fluorescence signals of the 1200 nm PRPs could be observed at the surface of the liposomes within 5 min after incubation and at the inside of the liposomes after 30 min of incubation (Figure 3). Comparing with the above experiment which was carried out without OCB in which no PRP signal was observed at/in the liposome after 90 min of incubation, here OCBs not only enabled the 1200 nm PRPs to associate quickly with the surface of the liposome but also facilitated their penetration into the liposomes’ interior. A similar experiment on the 540 and 430 nm PRPs with OCB addition showed fluorescence signals of the PRPs at the surface and at the inside of the liposomes after only 5 min of incubation (Figure 3). Comparing with 45 min requirement for the PRPs to penetrate the liposomes when there was no OCB (Figure 2), here the enhancement in penetration rate was very obvious. Therefore, we conclude that OCBs can enhance the association rate between the giant liposomes and the PRPs of all sizes and can increase the degree of liposome penetration for all three-sized PRPs.
As shown and discussed above that during the 90 min incubation time, the 430/540 nm PRPs (without OCB) could penetrate only the first layer of the lipid bilayer membrane and therefore could not get into the smaller liposomes located at the interior of the big liposomes. Nevertheless, the addition of OCBs into the system could enable the penetration of all three-sized PRPs across the second-layer liposomes inside the first-layer liposomes. In other words, smaller liposomes inside the big liposomes were also filled with PRPs when OCBs were added (Figure 3, 430 and 540 nm PRPs at 45–90 min).

To investigate whether OCBs directly interacted with PRPs, here we incubated OCBs with the 1200 nm PRPs (at the mass ratio of OCBs/PRPs of 1:4) and monitored the penetration of PRPs into liposomes without (Figure 2) and with OCBs (Figure 3).
ratio of OCBs/PRPs of 1:4, similar to that used in the above experiments) and subjected the mixture to SEM imaging. It should be mentioned here that if adhering between the two particles was taking place, size change should be observable. Previously, we have used this technique to compare an ability to be adsorbed onto OCB’s surface, of different materials.

The SEM image shows no change in size and morphology of the 1200 nm PRPs (Supporting Information Figure S4), implying that OCBs neither directly adhere to the surface of PRPs nor cause the deformation or size change to the PRPs. To confirm this, we have performed the DLS analysis of PRPs, OCBs, and the mixture of OCBs and 540 nm PRPs at 1:4 wt ratio. The results reveal the unimodule size distribution with the average sizes of 531 ± 33.1 and 141 ± 15.2 nm for PRPs and OCBs, respectively. The bimodule size distribution with the averages of 531 ± 29.1 and 122 ± 13.3 nm was observed for the PRP + OCB mixture (Supporting Information Table S1). Interestingly, the average zeta potential value of the mixture system (OCB to PRP at 1:4 wt ratio, zeta potential of 48 ± 1.5 mV) resembles that of the pure PRPs (50 ± 0.3 mV). This is quite unexpected. To further investigate on this point, we evaluated size and zeta potential of the mixture with a higher ratio of OCBs to PRPs. The bimodule size distribution with maxima at 122 ± 18.1 and 531 ± 24.5 nm was observed (Supporting Information Table S1). Even at a higher ratio of OCBs to PRPs, we still observed no size change of the PRPs. Zeta potential of PRPs was also unaffected by the increase in the OCB concentration. These results indicate that the negative zeta potential OCBs were not significantly adhering to the positive zeta potential PRPs.

We speculate the hydration shell of OCB and PRP particles act as a barrier that inhibits the direct contact of the two particles. We previously reported that small amphiphilic phospholipid molecules could be adsorbed on to the surface of OCBs. It is due to the effective adsorption of lipid molecules, which disrupt the local bilayer structure, so that the OCBs can directly induce transient leak on the phospholipid bilayer membranes. The positively charged phospholipid molecules are small and contain hydrophobic tails that repel water molecules. As a result, adsorption of the lipid molecules on the OCBs can take place in water (not so strong hydration shell around phospholipid molecules). However, PRP particles are a result of polymer self-assembly with entanglement, thus disruption is much harder. In other words, to find non-particulate PRP polymeric chain (disrupted from the particles) with less hydration shell is very unlikely in the water.

**Cellular Uptake of PRPs.** To test if OCBs could also facilitate the penetration of particles across cell membrane, we first tested for the cytotoxicity of OCBs in keratinocyte cells at various OCB concentrations using the MTT assay. The result indicates no toxicity under our experimental conditions at OCB concentrations of up to 30 μg/mL (Supporting Information, Figure S5). Next, we investigated effects of the OCBs on an ability of PRPs to penetrate the cell membrane. Keratinocytes were incubated with the PRPs under two different conditions, with (Figure 4, row 1–4) and without the OCBs (Figure 4, row 5–8), for 24 h. After incubation, the cells were washed and fixed and fluorescence signals of the PRPs in the cells were observed using CLFM. Fluorescence images of the cells incubated with each of the three-sized PRPs without OCB (Figure 4 row 2–4) barely showed fluorescence signals of PRPs in the cells. This result indicates that without OCB, all three-sized PRPs could not significantly penetrate into cells. Interestingly, the fluorescence signals from PRPs inside the keratinocyte cells were very obvious when OCBs were present (Figure 4 row 6–8). Without an addition of OCB, the numbers of keratinocyte cells with detectable PRP fluorescence signal were ~9.5%, for all three-sized PRPs. In the presence of OCBs, the numbers of keratinocyte cells with PRP fluorescence in their interior were ~93.6% for the 1200 nm PRPs and ~100% for the 540 and 430 nm-sized particles. These results clearly imply that OCBs can deliver both 1200 and 540/430 nm-sized PRPs into the cells. These results indicate that the PRPs (at the highest tested concentration of 2 μg/mL) are nontoxic to keratinocytes even under the condition that the OCBs help increasing their cellular uptake. This nontoxicity of the combined materials in the keratinocytes together with the increased cellular uptake of the PRPs should have an impact on dermatological applications of the pro-retinal nanoparticles. As a result, we next tested for the toxicity of PRPs and OCBs on the three-dimensional (3D) human skin models (EpiSkin, EpiSkin Research Institute, Lyon, France). The results revealed the cell viabilities of higher than 50% upon the treatments with OCBs (at the skin

![Figure 4. Cellular delivery of PRPs by OCBs. Confocal fluorescence microscopic images of keratinocyte cells after being incubated with media (row 1), 1200 nm PRPs (row 2), 540 nm PRPs (row 3), 430 nm PRPs (row 4), OCBs (row 5), 1200 nm PRPs plus OCBs (row 6), 540 nm PRPs plus OCBs (row 7), and 430 nm PRPs plus OCBs (row 8) for 24 h. Cells morphology images from phase contrast mode are in column A; fluorescence signals of (4’,6-diamidino-2-phenylindole) (DAPI; λex/λem of 405/450 nm, blue color) are in column B; fluorescence signals of PRPs (λex/λem of 488/510 nm, green color) are in column C; and merged images of cell morphology, DAPI fluorescence, and PRP fluorescence signals are shown in column D.](image-url)
coverage of 0.96 μg/cm²), PRPs (at the skin coverage of 0.064 μg/cm²), or the OCB/PRP mixture (at the skin coverage of 0.96 μg/cm² for OCBs and 0.064 μg/cm² for PRPs), whereas the cell viability decreased to 8.8 ± 0.5% upon the treatment with 5% sodium dodecyl sulfate (SDS, a positive control, used at the skin coverage of 1.6 mg/cm²). The result here indicates possible application of the OCBs as a nontoxic cellular penetration enhancer for micro-/nano-particulate form of therapeutic agents.

**Intracellular Trafficking.** We investigated an effect of the OCBs over the intracellular trafficking of PRPs using human liver cancer cell lines (HepG2). We used the 540 nm OCBs as representative OCBs. The HepG2 cells were incubated with the 540 nm PRP particles (in the presence and absence of OCBs) for 30 and 60 min, then the PRP locations in the cells were identified through the particles’ auto fluorescence signals, whereas the locations of nucleus, endosomes, and lysosomes compartments were determined through the fluorescence signals of the dyes specific to these three organelles (DAPI for nuclei, early endosome-RFP for endosomes, and lysotracker for lysosomes). CLFM images of HepG2 cells incubated with PRPs (no OCB) for 30 min (Figure 5 row 2) showed no signal of PRP fluorescence in the cells. However, after 60 min of incubation (Figure 5 row 3), the signals of PRP fluorescence were detected at the same locations of fluorescence signals from endosome specific dyes, implying that the PRPs were in the endosomes inside the cells. This indicates that some PRPs were endocytosed into the cells.

When the cells were incubated with PRPs plus OCBs for 30 min, the fluorescence signal of PRPs were detected in cytoplasm and nucleus of the cells (Figure 5 row 4 and Supporting Information video showing a 3D view of the cell’s interior with fluorescence signals indicating locations of PRPs in red, nucleus from DAPI in blue, endosomes from the tracker dyes in green and lysosomes from the tracker dyes in magenta, and Figure S6 in Supporting Information). In addition, here PRP fluorescence locations were related to neither the locations of endosomes nor the locations of lysosomes. These results imply that the cellular uptake of PRPs in the presence of OCBs is faster and more effective than that in the absence of OCBs. More importantly, with OCBs, the cellular uptake of PRPs does not take place via endocytic pathway.

**CONCLUSIONS**

Here, we show that the OCBs not only can speed up the association between 1200, 540, and 430 nm-sized PRPs with phospholipid bilayer membranes of giant liposomes but also can facilitate the PRP penetration across the membrane of the liposomes. Without OCB, the submicron-sized PRPs can slowly penetrate only the first layer of the multilayered liposomes, whereas the micron-sized PRPs cannot penetrate the liposomes. In contrast, in the presence of OCBs, both submicron-sized and micron-sized PRPs can enter all layers of the multilayered liposomes. We also show that without OCB, micron-sized PRPs could not be taken up into keratinocytes, and submicron-sized PRPs could, in a small degree, be taken up into keratinocytes. However, in the presence of OCBs, all of the three-sized PRP keratinocytes could effectively get into keratinocytes. Without OCBs, PRPs enter HepG2 cells via endocytosis; however, in the presence of OCBs, PRPs enter cells via a nonendocytic process and can further translocate to the cells’ nucleus. Last, OCBs and OCBs plus PRPs are nonirritating when tested on the 3D skin model. We anticipate that these abilities of OCBs bring micro- and nano-sized particles into cells via a nonendocytic process, to be a starting point for applications of OCBs as a cellular penetration enhancer for various particulate materials.

**EXPERIMENTALS**

**Penetration of Micro/Nano Particles into Cell-Sized Liposomes.** Two conditions of PRP penetration were observed in this experiment. First, no OCB: the liposome suspension in water was mixed with PRPs (1200 ± 51.5, 540 ± 29.0 or 430 ± 11.0 nm, final concentrations of liposomes and PRPs were controlled at 0.25 mM and 100 μg/mL, respectively). Second, with OCB: the liposome suspension in

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*Figure 5. Intracellular trafficking of the 540 nm PRPs. CLFM images of HepG2 cells after being incubated with media for 60 min (control, row 1), with PRPs for 30 (row 2), and 60 (row 3) min, with PRPs plus OCBs for 30 (row 4) and 60 (row 5) min: fluorescence signals from DAPI (λex/λem of 405/450 nm, blue, column A), PRPs (λex/λem of 488/510 nm, red (pseudo-color), column B), early endosome tracker dyes (λex/λem of 559/584 nm, green (pseudo-color), column C), lysosome tracker dyes (λex/λem of 650/668 nm, magenta (pseudo-color), column D), and cell morphology from phase contrast mode (column E), and the merged images of cell morphology, DAPI, early endosome tracker dyes and lysosome tracker dyes (column F).*
water was incubated with the mixture of OCBs and PRPs (ratio of OCBs/PRPs as 1:4) at final concentrations of liposomes and OCBs/PRPs mixture of 0.25 mM and 100 μg/mL, respectively. After mixing, the suspension was added dropwise onto the glass slide with a silicon chamber. Then, the liposomes in the suspension were observed under a CLFM (Nikon Digital Eclipse C1-Si, equipped with Plan Apochromat VC 100×, BD Laser (Melles Griot, Carlsbad, CA, USA), a Nikon TE2000-U microscope, a 32-channel PMT-spectral-detector, and Nikon-EZ-C1 Gold Version 3.80 software) with λex/λem of 488/510 nm.

**Cellular Uptake and OCB Delivery of Micro/Nano Particles. Keratinocyte Cell Culture. Preparation of Condition Medium.** Mouse embryo fibroblast cells lines (3T3, purchased from American type culture collection, ATCC) were cultured in the mixture of Dulbecco’s modified Eagle medium (DMEM, HyClone, Logan, UT, USA) with 10% (v/v) of fetal bovine serum (FBS, Gibco BRL Laboratories, Grand Island, NY, USA), 1% of l-glutamine (HyClone), and 1% of penicillin–streptomycin (Gibco). After cells were completely grown, the cells were treated with 10 μg/mL mitomycin C (Sigma-Aldrich, St Louis, MO, USA) in DMEM without serum for 2 h at 37 °C, 5% CO2. The mitomycin C-containing DMEM was removed and washed twice with phosphate-buffered saline (PBS), and successively added to 10 mL of DMEM/F12 (HyClone, Logan, UT, USA) medium with 10% (v/v) of FBS, 2.5 μg/mL NaHCO3, 0.5 μg/mL hydrocortisone (Sigma-Aldrich), 1% of l-glutamine, and 1% of penicillin–streptomycin. The cells were cultured at 37 °C, 5% CO2 for 24 h. The culture medium was collected and centrifuged twice at 1000 rpm for 5 min. Then growth factor [5 mg/mL human insulin, 20 ng/mL epidermal growth factor (EGF), Gibco] was added into the supernatant for use as keratinocyte culture medium.

**Preparation of Feeder Cells.** 3T3 cells were cultured in the mixture of DMEM with 10% (v/v) of FBS, 1% of l-glutamine, and 1% of penicillin–streptomycin. After the cells were completely grown, the cells were treated with 10 μg/mL mitomycin C in DMEM without serum at 37 °C, 5% CO2 for 2 h. After the cells were completely grown, the cells were treated with 10 μg/mL mitomycin C in DMEM without serum at 37 °C, 5% CO2 for 2 h. The 3T3 cells were trypsinized using 0.25% of trypsin/ethylenediaminetetraacetic acid (EDTA) solution at 37 °C, 5% CO2 for 2 min. To stop the reaction, DMEM-high glucose, 10% (v/v) of FBS, 1% of l-glutamine, and 1% of penicillin–streptomycin were added and centrifuged at 1000 rpm for 5 min. Then growth factor [5 mg/mL human insulin, 20 ng/mL epidermal growth factor (EGF), Gibco] was added into the supernatant for use as keratinocyte culture medium.

**Preparation of Keratinocyte Cells.** Keratinocytes (American type culture collection, ATCC) were grown in the presence of feeder cells in the mixture of DMEM/F12 medium with 10% (v/v) of FBS, 2.5 μg/mL NaHCO3, 0.5 μg/mL hydrocortisone, 5 mg/mL human insulin, 20 ng/mL EGF, 1% of l-glutamine, and 1% of penicillin–streptomycin at 37 °C, 5% CO2 for 10–14 days. Then, the cells were trypsinized using 0.25% of trypsin/EDTA solution and used afterward in the experiments.

**Cytotoxicity Test (MTT Assay).** Keratinocytes were seeded into 96-well plates coated with 10 μg/mL collagen type I at density of 1 × 104 cells/well in the condition medium at 37 °C, 5% CO2 for 24 h. After removal of the condition medium, cells were incubated with OCBs at concentrations of 0.1–30.0 μg/L in the condition medium, for 48 h. After incubation, 10 μL of PBS containing 1 mg/mL MTT solution was added to each well and the plates were incubated for 4 h at 37 °C. After that, the medium was removed from the wells and isopropanol (200 μL/well) was added to dissolve formazan crystals. The cells were subjected to absorbance measurement at 540 nm by a microplate reader (Varioskan LUX, Thermo Fisher Scientific Inc., MA, USA). All conditions were tested in triplicate. Cell viability was calculated using the equation below (eq 1).

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\text{Cell viability} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}} \times 100
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**Cellular Uptake and OCB Delivery of Micro/Nano Particles into Keratinocytes.** Keratinocytes were seeded into 24-well plates on collagen type I-coated cover slips at a density of 1 × 105 cells/well in the condition medium at 37 °C, 5% CO2 for 24 h. First, we investigated the cellular uptake of micro/nano particles in the absence of OCBs. PRP particles (1200 ± 51.5, 540 ± 29.0 and 430 ± 11.0 nm) were added to cells at the final concentration of 2.0 μg/mL. Second, the cellular uptake in the presence of OCBs was observed by treated keratinocytes with PRP and OCB mixtures. The final concentrations of PRPs and OCBs were 2.0 and 30.0 μg/mL, respectively. Then, all of the test plates were left at 37 °C, 5% CO2 for 24 h. The cells, that were washed three times and replaced medium with fresh PBS, were fixed by adding 500 μL of 4% paraformaldehyde and let stand at room temperature for 10 min before being washed with PBS. Then, they were incubated with 200 μL of 0.01 mg/mL DAPI solution for 3 min (to stain nuclei of the cells) and washed with PBS before being subjected to fluorescence microscope analysis (Zeiss Observer Z1, Carl Zeiss Microscopy Ltd., Cambridge, UK.).

**Cellular Uptake and OCB Delivery of Micro/Nano Particles into Human Liver Cancer Cells (HepG2).** HepG2 were maintained in Roswell Park Memorial Institute medium (1640 (RPMI 1640 medium) with 2.05 mM of l-glutamine (HyClone Laboratory, Inc., Logan, UT, USA). All of the cells were incubated at 37 °C, 5% CO2 for 24 h. After that, HepG2 were seeded in a 8-well chamber (Lab-Tek II Chambered Coverglass, NUNC, NY, USA) at a density of 5 × 104 cells/well, and then 50 μL of early endosome fluorescent dye reagent (cellLight early endosome-RFP, Bacmam 2.0, Invitrogen, USA) was added. The mixture was incubated overnight at 37 °C, 5% CO2. Then, the test samples (540 ± 29.0 nm PRPs at final concentration of 2.0 μg/mL, and 540 ± 29.0 nm PRPs plus OCBs at final concentration of PRPs and OCBs as 2.0 and 30.0 μg/mL, respectively) were added into each well. The plates were incubated at 37 °C, 5% CO2 for 30 and 60 min. At 30 min before finishing incubation, 50 μL of lysotracker deep red (in anhydrous dimethyl sulfoxide, LysoTracker and Lysosensor probe, Invitrogen, USA) was added. The mixture was incubated overnight at 37 °C, 5% CO2. Then, the test samples (540 ± 29.0 nm PRPs at final concentration of 2.0 μg/mL, and 540 ± 29.0 nm PRPs plus OCBs at final concentration of PRPs and OCBs as 2.0 and 30.0 μg/mL, respectively) were added into each well. The plates were incubated at 37 °C, 5% CO2 for 30 and 60 min. At 30 min before finishing incubation, 50 μL of lysotracker deep red (in anhydrous dimethyl sulfoxide, LysoTracker and Lysosensor probe, Invitrogen, USA) was added. The mixture was incubated overnight at 37 °C, 5% CO2 at a density of 1 × 105 cells/well. The plates were incubated at 37 °C, 5% CO2 for 48 h after incubation, 10 μL of PBS containing 1 mg/mL MTT solution was added to each well and the plates were incubated for 4 h at 37 °C. After that, the medium was removed from the wells and isopropanol (200 μL/well) was added to dissolve formazan crystals. The cells were subjected to absorbance measurement at 540 nm by a microplate reader (Varioskan LUX, Thermo Fisher Scientific Inc., MA, USA). All conditions were tested in triplicate. Cell viability was calculated using the equation below (eq 1).
dye, respectively. Data were processed with FV3000-SW software.

Irritation Test of OCBs and PRPs. EpiSkin (EpiSkin Research Institute, Lyon, France) were transferred into 1 mL of fresh medium and incubated at 37 °C, 5% CO₂ for 24 h. After that, 16 μL of the test substances (30 μg/mL OCBs, 2.0 μg/mL PRPs and the mixture of 2.0 μg/mL OCBs plus 30 μg/mL PRPs) was applied on tissue and then all tissue was covered by nylon mesh and incubated at room temperature for 42 min. For each sample, duplicate independent experiments were performed. Exposure to test substances were followed by rinsing with PBS and mechanically dried. EpiSkin was transferred to the fresh medium and incubated at 37 °C, 5% CO₂ for 42 h then cell viability was measured by the MTT assay. SDS and PBS were used as positive and negative controls, respectively. For each treated tissue, the cell viability was expressed as percentage of the mean negative control tissue. The mean relative tissue cell viability above 50% predicted a nonirritancy potential of test substances.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b00487.

Morphology of starting CB and OCBs; characterization of OCBs; dioleoyl L-α-phosphatidylcholine structure used for the liposome construction; morphology of 1200 nm PRPs after being incubated with OCBs; in vitro cytotoxicity of OCBs in keratinocyte cells; Z-stack image of HepG2 cells after being incubated with PRPs plus OCBs; and size distribution and zeta potential of PRPs and OCBs (PDF)

3D view of the cell's interior with fluorescence signals indicating locations of PRPs in red, nucleus from DAPI in blue, endosomes from the tracker dyes in green, and lysosomes from the tracker dyes in magenta (AVI)

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Notes
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ABBREVIATIONS

OCBs, oxidized carbon black nanoparticles; PRPs, retinal-grafted chitosan particles; HepG2 cells, human liver cancer cells; OCSs, oxidized carbon nanoparticles; CBs, carbon black

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