Nanomechanical mechanism for lipid bilayer damage induced by carbon nanotubes confined in intracellular vesicles

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Understanding the behavior of low-dimensional nanomaterials confined in intracellular vesicles has been limited by the resolution of bioimaging techniques and the complex nature of the problem. Recent studies report that long, stiff carbon nanotubes are more cytotoxic than flexible varieties, but the mechanistic link between stiffness and cytotoxicity is not understood. Here we combine analytical modeling, molecular dynamics simulations, and in vitro intracellular imaging methods to reveal 1D carbon nanotube behavior within intracellular vesicles. We show that stiff nanotubes beyond a critical length are compressed by lysosomal membranes causing persistent tip contact with the inner membrane leaflet, leading to lipid extraction, lysosomal permeabilization, and cell death. The precise material parameters needed to activate this unique mechanical pathway of nanomaterials interaction with intracellular vesicles were identified through coupled modeling, simulation, and experimental studies on carbon nanomaterials. One-dimensional nanomaterials are among the known determinants of toxicity (13, 21). The technique of coarse-grained molecular dynamics (MD), demonstrated to be effective in the study of complex biomolecular systems (22, 23), has been applied to whole lipid-bilayer patches to reveal a biophysical mechanism for carbon nanotube interaction with the plasma membranes leading to tip entry and uptake (4, 19, 20). The same technique may also provide insight relevant to internal membrane interactions, although whole vesicle MD is a significant challenge. Here we use a complement of techniques including coarse-grained MD, all-atom MD, in vitro bioimaging, and carbon nanotube length modification to reveal the behavior of vesicle-encapsulated carbon nanotubes and identify the conditions and carbon nanotube (CNT) types that lead to mechanical stress and membrane damage following cellular uptake and packaging in lysosomes (8).

CNTs Confined in Vesicles: Nanomechanics and Coarse-Grained Molecular Dynamics

We began by exploring the basic nanomechanics of CNTs confined in lipid-bilayer vesicles, focusing on possible physical and system is virtually unexplored, yet may be critical for understanding the cellular response to nanotubes/fibers, where shape and stiffness are among the known determinants of toxicity (13, 21). The technique of coarse-grained molecular dynamics (MD), demonstrated to be effective in the study of complex biomolecular systems (22, 23), has been applied to whole lipid-bilayer patches to reveal a biophysical mechanism for carbon nanotube interaction with the plasma membranes leading to tip entry and uptake (4, 19, 20). The same technique may also provide insight relevant to internal membrane interactions, although whole vesicle MD is a significant challenge. Here we use a complement of techniques including coarse-grained MD, all-atom MD, in vitro bioimaging, and carbon nanotube length modification to reveal the behavior of vesicle-encapsulated carbon nanotubes and identify the conditions and carbon nanotube (CNT) types that lead to mechanical stress and membrane damage following cellular uptake and packaging in lysosomes (8).

Significance

Recent experimental studies report correlations between carbon nanotube toxicity and tube length and stiffness. Very little is known, however, about the actual behavior of these fibrous nanomaterials inside living cells following uptake, and the fundamental mechanistic link between stiffness and toxicity is unclear. Here we reveal a nanomechanical mechanism by which sufficiently long and stiff carbon nanotubes damage lysosomes, a class of membrane-enclosed organelles found inside cells that are responsible for breaking down diverse biomolecules and debris. The precise material parameters needed to activate this unique mechanical toxicity pathway are identified through coupled theoretical modeling, molecular dynamics simulations, and experimental studies, leading to a predictive pathogenicity classification diagram that distinguishes toxic from biocompatible nanomaterials based on their geometry and stiffness.


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two dimensionless parameters, \( \Delta p \) and \( \Delta \), the maximum noninterrupted contact time between an MWCNT and a lysosome (Fig. 1 B–E) and top (C and E) views. (B and C) A CNT interacting with a membrane. (D and E) An MLG interacting with a membrane, representing a slice of the near-tip region of the CNT. mechanical mechanisms of membrane damage and permeabilization. One potential damage mechanism is mechanical burst, as membrane tension increases in response to lysosomal enlargement forced by encapsulation of long, stiff tubes. We performed a theoretical analysis of a rigid CNT of length \( L \) trapped in the interior of a lysosome with typical diameter \( D = 1 \) m, osmotic pressure \( \Delta p = 300 \) Pa, and membrane bending stiffness \( \kappa = 20 \) kT (1 kT = 4.2 pN · nm) (SI Appendix, Figs. S1 and S2). A sufficiently long nanotube causes the formation of a membrane tether, and the lysosome exerts a compressive force around \( f = 20 \) pN on the nanotube (SI Appendix, Fig. S2A). Depending on the nanotube length, the maximum membrane tension can reach \( \kappa = 0.08 \) mN/m (SI Appendix, Fig. S2B), which is still much lower than the experimental value of 4–10 mN/m required for instantaneous vesicle lysis (24). These results suggest that lysosomal rupture through CNT-induced membrane tension is unlikely to be the major mechanism of membrane permeabilization.

We then pursued the alternative hypothesis that nanotube confinement leads to local membrane damage due to sustained, direct, forced contact between multiwalled CNT (MWCNT) tips and the inner membrane leaflet. To assess this hypothesis, coarse-grained MD (CGMD) simulations were performed to investigate the maximum noninterrupted contact time \( t_{\text{max}} \) between a confined tube and the lysosomal membrane at the nontether end (Fig. L4 and SI Appendix, section 5 and Fig. S6). Our theoretical relations \( \sigma D^3/\kappa = F_1(\Delta p D^3/\kappa, L/D) \) and \( fD/\kappa = F_2(\Delta p D^3/\kappa, L/D) \) suggest that two dimensionless parameters \( \Delta p D^3/\kappa \) and \( L/D \) govern the problem under consideration (SI Appendix, section 1). Guided by this insight, we were able to substantially reduce computational costs by considering a lysosome with diameter of 100 nm under an osmotic pressure of 40 kPa, which would have dimensionless parameters \( \Delta p D^3/\kappa \) and \( L/D \) very similar to those of typical lysosomes with diameter from 0.1 to 1.2 \( \mu \)m (25) under osmotic pressure of 300 Pa (SI Appendix, section 4). A sufficiently long MWCNT leads to a cherry-shaped structure with one end of the nanotube encapsulated in a membrane tether and the other non-tethered end directly indenting the lipid bilayer (Fig. L4, insets). Similar structures have been observed in our theoretical calculations (SI Appendix, Fig. S1) and extraction of membrane tubes by optical tweezers (26). Note that this asymmetric configuration has lower energy than the symmetric configuration tethered at both ends (SI Appendix, Figs. S3D and S4). If the tube length \( L \) is smaller than the lysosome diameter \( D \), thermal fluctuations tend to prevent sustained contact. However, if the encapsulated tube is long and stiff, fluctuation is limited by confinement and long-term noninterrupted contact becomes possible. Fig. L4 shows that larger \( L/D \) values lead to longer maximum noninterrupted contact time \( t_{\text{max}} \) which increases exponentially with \( L/D \) in the range \( L/D > 1 \), regardless of the membrane composition and tube diameter (SI Appendix, section 7 and Figs. S8–S10). This exponential dependence is consistent with the prediction by Bell's model extending the classical rate theory for chemical reactions to account for the effect of an external force (27) (SI Appendix, section 1). By extrapolating the simulation results to experimental timescales, the maximum noninterrupted contact time goes up to 1 h as \( L/D \) reaches 3.62 (SI Appendix, section 6 and Fig. S7). Therefore, the length of an encapsulated nanotube, despite having minor influence on membrane tension and compressive force, plays a critical role in determining the maximum noninterrupted contact time between the nanotube and inner membrane leaflet. Understanding the consequences of this prolonged local contact requires all-atom MD simulations.

All-Atom MD Shows Phospholipid Extraction by Long MWCNTs

We used all-atom MD methods to assess our hypothesis that local lysosomal membrane damage is caused by the above-predicted mechanical contact between a nanotube tip and the inner membrane leaflet. To limit the size of the simulation, we considered a multilayered graphene (MLG) sheet as a model for a cross-sectional slice of the near end section of an MWCNT interacting with a lipid bilayer (Fig. 1 B–E and SI Appendix, section 8). This local view allowed us to focus on the effect of a vertical graphic surface near the tip of a nanotube on the lipid bilayer. A constant force \( F_2 \) was applied to mimic the compressive force on the nanotube associated with confinement. To expedite the local membrane damage within the timescale of all-atom simulations, a larger compressive force is assumed in simulating the critical damage time, which is then extrapolated to the time- and force scales in the experiments. Fig. 2A–D represents representative configurations of a three-layer graphene sheet interacting with a dipalmitoyl phosphatidylcholine (DPPC) lipid bilayer under a compressive force of 500 pN. At the onset of contact, the membrane was able to endure the compressive force...
from the vertical graphene surface without disruption, but lipid extraction by graphenic surface that is energetically favorable (SI Appendix, section 15 and Fig. S15) was observed in the snapshot at 148 ns. The early lipid extraction events were slow and steady despite some loss of membrane integrity. However, at 264 ns, the lipid extraction process suddenly became faster and disruptive with clusters of lipids climbing the vertical graphene surface. The instability of lipid extraction results in a permeable membrane that would allow leakage of lysosomal contents into the cytoplasm. The critical damage time $t_c$ for the membrane to become permeable is defined as the onset of the burst of lipid extraction, corresponding to the inflection points in the curves of center-of-mass distances in SI Appendix, section 10, Fig. S11. Similar lipid extraction was shown by Tu et al. in modeling the interaction between graphene nanosheets and bacterial membranes in the absence of a compressive force (1). Note that the interaction between finite-sized graphenic nanosheets and lipid bilayers could be strongly facilitated by the corners of the nanosheets (2). In our present simulations, the MLGs represent a slice of an MWCNT and hence have no corners. In this case, as spontaneous lipid extraction is hindered by a high energy barrier (2), a compressive force on the nanotube is required and its magnitude can be correlated to the critical damage time $t_c$. Fig. 2E shows that the critical condition to induce lysosomal permeabilization can be expressed as a power-law relationship between the contact force and the critical damage time, with lower contact force postponing membrane damage, which is insensitive to membrane composition (SI Appendix, section 14 and Fig. S14). Interestingly, $t_c$ only has a weak dependence on the number of graphene layers (Fig. 2E), corresponding to the number of nanotube walls. Further investigation revealed that this weak dependence is due to the localization of contact force distribution at the inner and outer layers of the MLG (SI Appendix, section 11 and Fig. S12). Note that similar lipid extraction processes have been observed on a small MWCNT (SI Appendix, Fig. S16A) and on an MLG–membrane system of smaller lateral size (SI Appendix, Fig. S16B and C). The geometrical effects of the nanotube tip are shown in Fig. 2F, which enables us to calculate $t_c$ by an MWCNT (see details in SI Appendix, sections 12 and 13 and Fig. S13).

Combining the results of CGMD and all-atom simulations allows a prediction of the link between material properties and lysosomal damage. The CGMD simulations show that the maximum noninterrupted contact time between the nanotube and lysosome increases exponentially with the tube length. When the maximum noninterrupted contact time exceeds the critical damage time, membrane permeability is induced through the lipid extraction instability observed in our all-atom simulations. It can thus be predicted that, at the same radius, longer nanotubes induce longer contact time and thus lysosomal membrane permeabilization leading to cell toxicity, in agreement with our experimental results described next.

Model Validation—Imaging of Nanotube Uptake and Vesicle Interactions

A variety of bioimaging modalities were used to test the nanomechanical model predictions and reveal their implications for cell response and toxicity. A panel of carbon nanomaterials of widely varying geometry was created by a combination of acquisition, oxidative shortening, and detailed characterization of the as-received and processed materials (SI Appendix, Table S3 and Figs. S19–S22). From that panel, one long, stiff nanotube sample (MWCNT-7) and a zero-dimensional isometric particle reference sample (carbon black) were delivered to hepatocytes (Fig. 3 and SI Appendix, Fig. S26) or lung epithelial cells (SI Appendix, Figs. S23 and S24) in culture. Important target cells for CNT toxicity include lung epithelial cells and hepatocytes, and these parenchymal cells are known to internalize nanoparticles by active cellular uptake via endocytosis (28, 29) (Fig. 3 A and B). Spherical carbon black nanoparticles colocalize with punctate red fluorescent vesicles, indicating intact vesicle membranes (Fig. 3C and SI Appendix, Fig. S23C), and
nanoparticle localization in membrane-bound cytoplasmic vesicles was confirmed using transmission electron microscopy (TEM) (Fig. 3E and SI Appendix, Fig. S30). The MWCNTs were partially colocalized with punctate cytoplasmic vesicles, and longer CNTs were seen penetrating through the vesicle membrane into the cytoplasm by TEM (Fig. 3F). Immunogold labeling confirmed that these membrane-bound vesicles are lysosomes (SI Appendix, Fig. S30). Lysosomal membrane permeabilization was assessed using a fluorescence assay for a lysosomal protease, cathepsin B, which retains activity following release into the cytoplasm (30). Confocal fluorescence imaging revealed that exposure to CNTs induced low-intensity, diffuse fluorescence reflecting focal release of cathepsin B into the cytoplasm (Fig. 3D). Image analysis was used to further distinguish intact lysosomes from permeable ones based on object identification and sizing. Intact lysosomes show narrow size distribution and distinct edges (SI Appendix, Fig. S31A; high threshold in SI Appendix, Fig. S31B), whereas permeable lysosomes show weaker, diffuse fluorescence over larger areas in the cytoplasm (SI Appendix, Fig. S31B (Right)) and low threshold size distributions] associated with cathepsin B leakage following exposure to MWCNT-7. Additional verification of cathepsin B release and lysosomal damage due to long, stiff MWCNT-7 was obtained using confocal fluorescence microscopy and quantitative high content imaging (Fig. 3G). The release of cathepsin B initiates a proteolytic cascade culminating in activation of caspases leading to cell death via apoptosis (31) (shown in Fig. 4A and SI Appendix, Figs. S24A, S24 C–E, and S27 C, E, and F). Colocalization of cathepsin B release and caspase activation was demonstrated in lung epithelial cells 24 h after exposure to MWCNT-7 (SI Appendix, Fig. S24E), suggesting a link between lysosomal damage induced by exposure to long, rigid MWCNT-7 and cell death. The causal link between cathepsin B release and cell toxicity was assessed using a selective cathepsin B inhibitor, methyl ester CA-074. Coexposure of hepatocytes to long, rigid MWCNT-7 and the cathepsin B inhibitor methyl ester CA-074 partially prevented cell death as well as caspase activation (SI Appendix, Fig. S18 B and C). These observations confirm the basic molecular dynamics predictions (vide supra) that rigid MWCNTs disrupt lysosomal membrane integrity in a length-dependent manner.

**Cellular Response to Carbon Nanomaterials of Diverse Geometry**

The nanomechanical model described above offers a testable prediction of the length threshold above which CNTs will mechanically activate this lysosomal damage pathway. The proposed mechanism requires a tube length greater than the lysosome diameter to force the tips in contact with the inner leaflet by compression forces exerted by the confining membrane, together with sufficient stiffness to resist tube buckling. In contrast, short or flexible nanotubes cannot be mechanically forced into contact with the membrane, and should contact the membrane only through Brownian motion or under the influence of electrostatic colloidal forces. Because the samples studied here all have negative surface charge in cell culture medium (SI Appendix, Table S3), these electrostatic forces are repulsive and we would not expect persistent tip-membrane contact or lysosomal damage to occur without the mechanical forces associated with the confinement of long, stiff 1D structures. We undertook to test these predictions and validate the model through additional experiments on shortened nanotubes, small-diameter (flexible) nanotubes, and carbon nano- horns (SI Appendix, Table S3).

![Fig. 4. Cellular interactions with other carbon nanoforms of diverse geometry.](image-url)
Uptake and Lysosomal Interactions of Shortened CNTs

The MWCNT-7 sample was progressively shortened using oxidative treatment and ultrasonication followed by thermal annealing to restore the original graphenic surfaces (SI Appendix, Table S3 and section 17). The original MWCNT-7 sample (mean length 11.7 μm) induced cathepsin B release (Fig. 3 D and G and SI Appendix, Fig. S31B), while the same sample shortened to 0.5 μm was successfully compartmentalized in cytoplasmic vesicles (Fig. 4C) and did not induce cathepsin B release from hepatocytes (Fig. 3G) or toxicity (Fig. 4B). MWCNTs longer than ~1 μm were observed in contact with, or piercing membranes of cytoplasmic vesicles (Fig. 4D) and this sample induced intermediate toxicity (Fig. 4B and SI Appendix, Fig. S25). The MWCNT length threshold for the onset of toxicity (0.5–1 μm) is in the range of the measured mean lysosome diameter of ~0.5–1 μm (SI Appendix, Fig. S31), which is in agreement with the predictions of the model.

Uptake and Lysosomal Interactions of Carbon Nanohorns and Flexible CNTs

We next investigated whether thin (more flexible) MWCNTs could initiate lysosomal membrane permeabilization and cathepsin B release into the cytoplasm. Two samples of thinner MWCNTs, MWCNT-flex1 and MWCNT-flex2 (SI Appendix, Table S3), were compartmentalized into cytoplasmic vesicles in hepatocytes and did not induce cathepsin B release (Figs. 3 G and 4F and SI Appendix, Fig. S27 H and I). Finally, nanomaterials with ultrasharp features have been reported to pierce lysosomal membranes (9). To investigate the role of ultrafine-scale surface roughness in this sample set, hepatocytes were also exposed to carbon nanohorns, which do not have an overall 1D geometry, but rather are aggregated single-walled nanotube cones with outward protruding high-curvature tips (~1–3 nm). Agglomerates of carbon nanohorns were observed compartmentalized in cytoplasmic vesicles (Fig. 4E) with no release of cathepsin B (SI Appendix, Fig. S27G). Both the carbon nanohorns and the thinner CNTs have higher surface area than the long, rigid MWCNTs or the carbon black nanoparticles; however, they did not induce cell death at equivalent mass (Fig. 4B) or surface area doses (SI Appendix, Fig. S25) after 24 or 48 h. Lysosomal damage in this system is not related to high surface area or sharp nanoscale surface features, but rather to length and stiffness.

Pathogenicity Classification Diagram for 1D Nanocarbons

Our combined results can be used to propose a general criterion for the ability of a carbon nanomaterial to induce lysosomal damage by this nanomechanical mechanism. For sustained contact with the lysosome membrane, the encapsulated fibrous material must be able to withstand the confining compressive force without buckling. We hypothesize that pathogenicity is only triggered when the encapsulated fibrous material reaches a critical length/width boundary where it is able to buckle and induce mechanical stress in the lysosome (Fig. 4). The criterion for buckling relies only on the tube length \( L \) and effective diameter \( d \) (see details in SI Appendix, section 16). Fig. 5 presents the criterion in the form of a classification diagram, where the thick blue curve is the calculated threshold for CNT buckling under a typical compressive force exerted by the lysosomal membrane (20 pN). This buckling curve together with the characteristic lysosome size divides a general set of nanotubes into two categories: “biologically stiff” or “biologically soft.” Nanotubes falling in the pink shaded region are predicted to have sufficient stiffness and length to mechanically induce lysosomal permeability leading to cathepsin B release as well as inflammasome activation (17). This nanomechanical theory is compared with data from the present study (square symbols) and the literature (diamonds) in Fig. 5. The red symbols represent samples reported to activate this pathway, and blue symbols represent samples reported to be inactive. The theory correctly identifies pathogenic from nonpathogenic nanocarbons in the broad sample set, although more data will eventually be required to assess the accuracy of the buckling criterion in the borderline region. Note that this theory is for carbons with as-produced graphenic surfaces, and not for functionalized materials, which may involve a wide range of grafted or adsorbed molecular segments that can interact in chemically specific ways with biological membranes.

High aspect ratio is often cited as a key fiber property associated with pathogenicity. To better understand the role of aspect ratio (AR), Fig. 5 includes dashed lines of constant AR from 1 to 1,000. Isometric nanomaterials (AR=1) cannot reach the critical lysosome size while remaining at the nanoscale, and are nonpathogenic by this pathway. In contrast, nanomaterials with AR≈100 often fall in the pathogenic regime. Interestingly, many materials with very high AR (∼1,000) are also nonpathogenic, as they are too easily buckled to behave as rigid fibers within the lysosome. Buckling is favored by a combination of long length and small diameter (i.e., high AR), and the high AR is therefore unreliable as a simple, monotonic indicator of the potential of a fibrous material to induce pathology.

Our proposed classification diagram can be readily generalized to other 1D materials by choosing an appropriate material-dependent elastic modulus to use in the Euler buckling theory (SI Appendix, Fig. S18). For a wide variety of other 1D material classes (metal, oxide, polymer) this generalized classification diagram shows a critical threshold in length/width space that represents a transition from biologically soft to stiff, and thus identifies the important subset of all 1D materials with the potential to induce lysosomal permeability by this nanomechanical mechanism. As an example, the diagram correctly predicts that many thin nanofibrous materials of intrinsic low stiffness [e.g., polymers, worm-like micelles (32)] would be nonpathogenic by this pathway.

Conclusions

The confinement of 1D nanomaterials in intracellular lipid-bilayer vesicles produces conformations and behaviors that are revealed...
by MD and in vitro bioimaging. Cellul ar attempts to package long 1D nanomaterials in spherical vesicles leads to material compression that forces persistent mechanical contact between the tube tip and inner membrane leaflet, which for CNTs causes lipid extraction, membrane permeabilization, release of cathepsin B, and cell death by apoptosis. In contrast, this mechanism predicts intact lysosomes and lower toxicity for nanotubes that are short (<1 μm), or of very high L/D that easily buckle in the presence of the lysosomal compression force (~20 pN), or for other materials with similar graphenic chemistry but isometric shape (e.g., carbon black, carbon nanohorns). These predictions are validated in vitro experiments using hepatocytes as well as lung epithelial cells exposed to a diverse panel of synthetic nanocarbons. A quantitative material classification diagram distinguishes pathogenic from biocompatible nanotube varieties based on a buckling criterion that relies only on minimum and maximum dimension. The present results also suggest that the observed low pathogenicity of tangled tubes relative to straight tubes (3, 21) may be usefully understood as an effect of intrinsic biological softness, defined as the resistance to buckling under lysosomal compressive forces. Unlike tangling, which is an agglomeration or deformation state subject to change through material handling or processing, biological softness is a fundamental nanomaterial property directly related to diameter and length. This mechanism of understanding provides guidance for safe design and material selection of 1D nanomaterials for both biomedical and nonbiomedical applications.

Methods

CGMD Simulations. CGMD simulations of a CNT encapsulated inside a lysosome were performed to investigate the maximum noninterrupted contact between the CNT and a membrane patch built from solvent-free CG lipids (4, 22). Further details of the CG models of the membrane and CNT can be found in SI Appendix, sections 2 and 3.

All-Atom MD Simulations. All-Atom MD simulations were performed to investigate how an MLG, corresponding to a near-tip slice of an MWCNT, or an MWCNT interacts with a membrane patch. The membranes were constructed from bilayers of the Berger lipids. The Berger lipid force field was used for lipids combined with an Optimized Potentials for Liquid Simulations representation of MLGs and MWCNTs. DPPC was adopted to build the lipid bilayer. Further details are provided in SI Appendix, section 9.

Nanomaterial Panel and Characterization. A panel of carbon nanomaterials of diverse geometry and stiffness was assembled and characterized, including commercial MWCNTs from Mitsui & Co. (MWCNT-7) and NanoLab, Inc., which were synthesized using catalytic chemical vapor deposition. The process of shortening and surface restoration used to create variants of the MWCNT-7 sample is described in SI Appendix, section 17. The panel of carbon nanomaterials included zero-dimensional reference materials including carbon black M120 (Cabot Corporation) and carbon nanohorns, courtesy of David Geoghean, Oak Ridge National Laboratory, Oak Ridge, TN, as isometric (low-AR) reference carbon materials.

Cell Culture and Exposure Conditions. Experiments were conducted on two cell types: hepatocytes (AML12 cells; American Type Culture Collection; CRL-2254) and lung epithelial cells (H460 cells; American Type Culture Collection; HTB-177). The cells were exposed to commercial MWCNTs, carbon nanohorns, and carbon black and assessed for viability as described in SI Appendix, Figs. 519 and 520.

Lysosomal Permeabilization. After exposure of cells to carbon nanomaterials, the integrity of lysosomes is determined using a cethespin B target peptide sequence conjugated to a red fluorophore, which is cleaved by active cathepsin B enzyme. Diffuse red cytoplasmic fluorescence indicates lysosomal membrane permeabilization whereas punctate cytoplasmic fluorescence indicates intact lysosomes. Lysosomal permeabilization was quantified using cell segmentation and analysis and quantitative high content fluorescence imaging as described in SI Appendix, section 17.

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**Supporting Information (SI)**

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**SI Appendix 1 | Theoretical Analysis of a Lysosome with an Encapsulated Nanotube**

Lysosomes are ubiquitous intracellular organelles serving as the primary degradative compartments of eukaryotic cells. They are bound by a lipid bilayer membrane with specific composition of phospholipids, sterols and membrane proteins, which together determine the mechanical response of lysosomes. For example, molecular dynamics simulations have demonstrated that the membrane proteins approximated as adhesive Janus particles can lead to substantial changes in membrane shape [1]. From a theoretical perspective, a lysosome can be approximated by a vesicle of a certain spontaneous curvature. The bending stiffness of the vesicle membrane depends on the composition of phospholipids and sterols of the lysosome, and the spontaneous curvature is determined by the lysosomal membrane proteins. The most abundant phospholipids in lysosomal membrane are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM) [2]. Recent work on measuring the bending stiffness of lipid vesicles of similar membrane compositions suggests that $\kappa = 20 \text{ kgT}$ is a representative value of the bending stiffness of lysosomes [3]. To illustrate the effect of spontaneous curvature $c_0$ on the mechanical response of lysosomes to the encapsulated tube, we have performed case studies at both zero spontaneous curvature and finite spontaneous curvatures $c_0 = \pm \delta/L$, where $L$ is the lysosome diameter. The following theoretical results indicate that different spontaneous curvatures would not qualitatively modify the mechanical response of a lysosome to an encapsulated tube. Therefore, in the main text and most parts of the numerical simulations in SI, we will focus on the case of zero spontaneous curvature ($c_0 = 0$).

To analyze the response of a lysosome to an encapsulated nanotube, we construct a theoretical model in which the lysosome is modeled as a deformed vesicle with fixed area $A$ at a certain osmotic pressure $\Delta p$ with a protrusion induced by a rigid nanotube of length $L$ (Fig. S1a). The deformed vesicle is assumed to retain an axisymmetric configuration. The free energy of the system is described by the Helfrich functional as

$$E = \pi \kappa \int_0^l \left( \frac{d\theta}{ds} + \frac{\sin \theta}{r} \right)^2 r ds + \sigma A - \Delta p(V - V_0) - f L,$$

where $\sigma$ is a Lagrange multiplier interpreted as the membrane tension conjugated to area $A$; $V_0 = A^{3/2}/(6\sqrt{\pi})$ is the volume of a spherical lysosome, $V$ is the volume of the lysosome associated with the osmotic pressure $\Delta p$; $f$ is the compressive force between the nanotube and lysosome membrane, stretches the lysosome along the $z$-axis; $\theta$, $s$, and $\kappa$ are the tangent angles, arclength, and bending stiffness of the lysosome membrane; and $l$ is the undetermined total arclength of the shape profile. All lengths are scaled by the effective diameter of the lysosome, $D = D/\sqrt{\pi}$. Other dimensionless parameters are the normalized pressure $\bar{\sigma} \equiv \sigma D^3/\kappa$, normalized membrane tension $\bar{\sigma} \equiv \sigma D^3/\kappa$, and normalized compressive force $\bar{f} \equiv f D/\kappa$. With these dimensionless parameters, the free energy $E$ can be expressed as

$$\frac{E}{\kappa} = \pi \int_0^l \left( \frac{d\theta}{ds} + \frac{\sin \theta}{r} \right)^2 r ds + \bar{\sigma} \frac{A}{\pi} - \bar{p} \frac{V - V_0}{D^3} - \bar{f} L,$$

suggesting dimensionless relations $\bar{\sigma} = F_1(\bar{p}, L/D)$ and $\bar{f} = F_2(\bar{p}, L/D)$. Here we focus on the case of $L > D$, in which the lysosome is deformed due to the geometric mismatch. In the case of $L < D$, strong thermal fluctuation is expected to prevent close contact between the nanotube and lysosome membrane.

With geometric relations $dr/ds = \cos \theta$ and $dz/ds = \sin \theta$, the elastic deformation energy $E$ at given $\Delta p$ and $L$ can be represented as a function of the unknown tangent angle $\theta(s)$ which is approximated by a Fourier series as [4–6]

$$\theta(s) = \theta_0 + (\theta_1 - \theta_0) s^2 + \sum_{i=1}^N a_i \sin \frac{\pi i s}{l}. \tag{1}$$

Here $N$ is the number of Fourier modes chosen as $N = 80$; $a_i$ is the Fourier amplitudes, $s$ is the arclength, and $l$ is the total arclength of the shape profile; and $\theta_0$ and $\theta_1$ are the tangent angle angles at $s = 0$ and $l$, respectively. In our case, $\theta_0 = 0$ and $\theta_1 = \pi$. Here we adopt the coordinate with origin at the bottom of the vesicle. We employ interior point optimization method to determine the minimum energy state. The nanotube length $L$ and the lysosome area $A$ serve as equality constraints during energy minimization. The elastic energy as a function of $\theta(s)$ under these constraints is minimized with respect to the Fourier amplitudes $a_i$ and total arclength $l$. Upon energy minimization, $a_i$, $l$, $\sigma$ and $f$ are determined. With the knowledge of $a_i$ and $l$, the tangent angle $\theta(s)$ becomes known, and then the
elast terminated deformation energy and corresponding configuration of the lysosome can be obtained. As the length of the encapsulated nanotube increases, the deformed lysosome evolves from a lemon-like shape to a cherry-like shape with a membrane tether covering the nanotube (Fig. S1b).

It has been reported that lysosomes become unstable as they grow beyond a critical diameter around $D_c = 5\ \mu m$ [7] due to internal osmotic pressure associated with the acidic lysosomal environment. Taking a typical value of membrane tension for long-term stability as $\sigma_c = 0.4\ \text{mN/m}$ [8], a typical osmotic pressure $\Delta p$ of the lysosome is determined as $\Delta p = 4\sigma_c/D_c \sim 300\ \text{Pa}$. With the knowledge of typical lysosome diameter $D = 1\ \mu m$, osmotic pressure $\Delta p = 300\ \text{Pa}$ and membrane bending stiffness $\kappa = 20\ k_B T$, the compressive force on the nanotube is $f = 20\ \text{pN}$ as a membrane tether forms (Fig. S2a) and the membrane tension is found to reach an asymptotic maximum $\sigma = 0.08\ \text{mN/m}$ (Fig. S2b).

Besides the case of typical osmotic pressure $\Delta p \sim 300\ \text{Pa}$, we performed numerical studies at other pressures up to $\Delta p \sim 500\ \text{Pa}$ (Fig. S3a and b). Similar profiles of compressive force and membrane tension are observed. As indicated in Fig. S3a, the force $f$ between the nanotube and the deformed membrane increases rapidly as the length $L$ increases and then saturates to a nearly constant value upon the formation of a membrane tether. As shown in Fig. S3b, at a certain $\bar{p}$ the membrane tension undergoes a slight increase as $L/D$ increases and then decreases and saturates at a nearly constant value with tether formation.

In the above analysis, the shape of the deformed lysosome is

![Fig. S1. Conformation of a lysosome containing a rigid nanotube. (a) Schematic of a lysosome (blue) deformed by a rigid nanotube (thick black) at osmotic pressure $\Delta p$ in the adopted cylindrical coordinate $(r, \phi, z)$. $\theta$ is the tangent angle of the lysosome membrane surface. The arclength $s$ is defined along the lysosome membrane, measured from the bottom pole ($s = 0$). (b) Selective lysosome configurations at $\bar{p} = 3570$ (serving as a correspondence to the case of $\Delta p = 300, D = 1\ \mu m$ and $\kappa = 20\ k_B T$) for different nanotube lengths ($L/D = 1.3, 2, 2.5, \text{and} 3$).](image1)

![Fig. S2. Theoretical calculations of lysosomal membrane disruption induced by encapsulation of a stiff CNT. Mechanical response of a lysosome with diameter $D$ of $1\ \mu m$, osmotic pressure $\Delta p$ of 300 Pa and membrane bending stiffness $\kappa$ of $20\ k_B T$ to an encapsulated rigid nanotube of length $L$. Compressive force $f$ (a) and membrane tension $\sigma$ (b) as functions of the length ratio $L/D$.](image2)
Fig. S3. Mechanical response of a lysosome to an encapsulated rigid nanotube at selected osmotic pressures for different length ratios $L/D$. (a) Normalized compressive force $\bar{f}$, membrane tension $\bar{\sigma}$ as a function of the length ratio $L/D$. The peak value of $\bar{\sigma}$ in (b) at $\bar{p} = 6400$ is $\bar{\sigma} \sim 1600$, corresponding to a membrane tension of $0.13 \text{ mN/m}$. The results at $\bar{p} = 6400$ correspond to the case of $\Delta \bar{p} \sim 500$, $D = 1 \mu\text{m}$ and $\kappa = 20 \text{k}_B T$. (c) $\bar{f}$ at $\bar{p} = 3570$ for different nanotube diameters. (d) Free energy of the $\phi$-shaped and cherry-shaped lysosomal structures at $\bar{p} = 3570$.

obtained from energy minimization of Eq. (1) with an implicit assumption that the nanotube diameter is smaller than that of the formed membrane tether given approximately as $\sqrt{2\kappa/\sigma}$. However, this assumption is not valid for a nanotube whose diameter is larger than $\sqrt{2\kappa/\sigma}$ but still orders of magnitude smaller than the effective diameter $D$ of the lysosome. To evaluate the size effect of the nanotube diameter on the force $f$, we performed numerical optimizations with inequality constraints preventing penetration between the membrane tether and encapsulated nanotube. We considered nanotubes with two diameters: $0.09D$ and $0.18D$, both larger than $\sqrt{2\kappa/\sigma}$ in the case of $\bar{p} = 3570$ but orders of magnitude smaller than $D$. Our results showed that the interaction force $f$ is insensitive to the nanotube diameter (Fig. S3c).

As indicated by the selected lysosome configurations, the lysosome evolves from a shape of up-down symmetry to a cherry-shaped configuration as the length ratio $L/D$ exceeds a certain value. To demonstrate that this is an energy-driven process, we compare the free energy of these two types of configurations in Fig. S3d. In the case of $\bar{p} = 3570$ and $\kappa = 20 \text{k}_B T$, the lysosome adopts a symmetry breaking structure as $L/D > 1.45$, whose free energy $E$ is about $10 \text{k}_B T$ smaller than that of the $\phi$-shaped structure. This energy-driven process is confirmed by experiments where the spherical portion of the vesicle moves from the middle to one end of the encapsulated one-dimensional microtubule [9].

A similar process in structure transformation is also observed in our coarse-grained molecular dynamics simulations (Fig. S4). To obtain the $\phi$-shaped structure initially, the center-of-mass of vesicle is restrained overlapping the center of the MWCNT. After releasing the constraints, the $\phi$-shaped vesicle (Fig. S4a) spontaneously transformed to the cherry-shaped configuration within 1 µs (Fig. S4b), with a system energy decrease of $-312.6 \text{ kcal/mol}$ (Fig. S4c). The theoretical analysis and CGMD simulations indicate that the cherry-shaped configuration is the most favorable for a vesicle encapsulating a MWCNT at a high length ratio $L/D$.

To illustrate the effect of spontaneous curvature $c_0$ on the mechanical response of lysosomes to the encapsulated tube, we compare the lysosome membrane tension and compressive force in the case of zero spontaneous curvature with these at $c_0 = \pm \frac{4}{D}$ (Fig. S5). The free energy of the system at a finite $c_0$ is given by

$$E = \pi \kappa \int_0^L \left( \frac{d\theta}{ds} + \frac{\sin \theta}{r} - c_0 \right)^2 r ds + \sigma A - \Delta \bar{p} (V - V_0) - fL.$$

As shown in Fig. S5, the compressive force $\bar{f}$ and membrane tension $\bar{\sigma}$ as functions of $L/D$ at $c_0 = \pm \frac{4}{D}$ exhibit similar trends as these at zero spontaneous curvature. These results suggest that lysosomal membrane proteins that lead to a spontaneous curvature does not qualitatively modify the mechanical response of lysosomes to encapsulated tubes.
SI Appendix 2 | Coarse-grained (CG) Molecular Dynamics (MD) Simulations: Methodology

CGMD simulations of a CNT encapsulated inside a lysosome were performed based on the LAMMPS package [10] (http://lammps.sandia.gov) to investigate the maximum non-interrupted contact time between the CNT and a membrane patch built from solvent-free CG lipids [11]. Each solvent-free CG lipid molecule was approximated by three connected beads with one hydrophilic head bead and two hydrophobic tail beads [1, 12]. The bead diameter was set at 1 nm, in order to construct a lipid bilayer with appropriate membrane thickness and areal density (area per lipid). To ensure that the constructed lipid bilayer is thermodynamically stable with mechanical properties (expansion modulus and bending stiffness) inside the experimentally reported range [13, 14], we chose the depth of the energy well as 0.56 kcal mol$^{-1}$ in our potentials (see SI Appendix, section 3). The coarse-grained model of MWCNT contains three walls of CG beads with all the nearest and second-nearest beads connected by harmonic springs of stiffness 224 kcal mol$^{-1}$ nm$^{-2}$. The nearest-neighbor distance of beads on the outermost surface of the CNTs was 2 nm. Further details of the CG models of the membrane and CNT can be found in SI Appendix, section 3. The CGMD simulations can be found in SI Appendix, section 3. The CGMD simulations were performed under a constant ambient temperature of 310 K by the Nose-Hoover thermostat [15, 16], with time step fixed at 100 fs. A lysosome with a confined CNT was placed in the middle of the simulation box. The translational and angular momentums of the CNT were set to zero to fix its center of mass, while the membrane was allowed to move freely during the simulations. The lysosome was initially constructed with a spherical diameter of 100 nm, and the encapsulated MWCNTs had lengths from 50 nm to 170 nm. To mimic osmotic pressure, outward normal forces were imposed on lipid heads in the inner layer of the membrane. Three levels of osmotic pressures in the model lysosome, 2 kPa, 10 kPa and 40 kPa, were considered. The maximum non-interrupted contact time between the non-tethered nanotube end and lysosome is calculated at different osmotic pressures and tube lengths.

SI Appendix 3 | Interaction Potentials in CGMD Simulations

In our CGMD simulations, the bead-bead interactions are described by the following potentials:

\[
U_{\text{WCA}}(r) = 4\varepsilon \left[ \left( \frac{\sigma'}{r} \right)^{12} - \left( \frac{\sigma'}{r} \right)^{6} + \frac{1}{4} \right] \left( 0 < r < r_{\text{cut}} \right),
\]

\[
U_{\text{COS}}(r) = \left\{ \begin{array}{ll}
-\varepsilon + U_{\text{WCA}}(r) & (r < r_{\text{cut}}), \\
-\varepsilon \cos^{2} \left( \frac{\pi(r - r_{\text{cut}})}{2w} \right) & (r_{\text{cut}} \leq r \leq r_{\text{cut}} + w), \\
\end{array} \right.
\]

\[
U_{\text{FENE}}(r) = -\frac{1}{2}k_{\text{FENE}}r_{\text{cut}}^{2} \ln \left( 1 - \frac{r^{2}}{r_{\text{cut}}^{2}} \right) \left( 0 < r < r_{\text{infty}} \right),
\]

where $r_{\text{cut}} = 2^{1/6}\sigma'$, and $\varepsilon$ and $\sigma' (\sigma' = \alpha\sigma)$ are the energy well depth and bead diameter, respectively. Note that $\sigma$ is used
here to denote bead diameter and should not be confused with membrane tension used earlier. To construct a lipid bilayer with appropriate thickness and properties, the bead diameter $\sigma$ was set at 1 nm, while the energy depth $\varepsilon$ was taken as 0.56 kcal mol$^{-1}$. The parameter $\varepsilon$ can be deduced from temperature, which is set at $k_BT = 1.1\varepsilon$ ($T = 310$ K) [1, 11, 12]. For lipids, the nearest neighbor beads are connected by FENE bonds with $k_{\text{FENE}} = 30\varepsilon/\sigma^2 = 16.8$ kcal mol$^{-1}$ nm$^{-2}$ and $r_{\infty} = 1.5\sigma = 1.5$ nm. The head beads were also connected to the second tail by harmonic bonds with rest length $r_0 = 4\sigma = 4$ nm and force constant $k_{\text{harmonic}} = 10\varepsilon = 2.8$ kcal mol$^{-1}$ nm$^{-2}$. The non-bonded interaction parameters between each two beads of CNT, lipid heads and tails are detailed in Table S1.

Table S1. Parameters of non-bonded interactions.

<table>
<thead>
<tr>
<th>Bead type I</th>
<th>Bead type II</th>
<th>Interactions</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid head</td>
<td>Lipid head</td>
<td>WCA</td>
<td>$\alpha = 0.95$</td>
</tr>
<tr>
<td>Lipid head</td>
<td>Lipid tail</td>
<td>WCA</td>
<td>$\alpha = 0.95$</td>
</tr>
<tr>
<td>Lipid tail</td>
<td>Lipid tail</td>
<td>COS</td>
<td>$\alpha = 0.95, w = 1.6\sigma$</td>
</tr>
<tr>
<td>Lipid head</td>
<td>CNT</td>
<td>WCA</td>
<td>$\alpha = 0.95$</td>
</tr>
<tr>
<td>Lipid tail</td>
<td>CNT</td>
<td>WCA</td>
<td>$\alpha = 0.95$</td>
</tr>
</tbody>
</table>

SI Appendix 4 | Osmotic Pressure in CGMD Simulations

Osmotic pressure is essential to maintain the integrity of lysosomes. To mimic the osmotic pressure in solvent-free CG simulations, normal forces were added outwards on lipid heads in the inner layer of the membrane. For each lipid head in the inner layer, we first searched for its nearest neighbors within a specified cutoff distance (2 nm). The normal vector of this lipid head was defined by averaging the normal vectors of planes formed by every two neighbors and the lipid head itself. The area per lipid (APL) in our CG model was 67.91 Å$^2$, and the normal forces on each lipid head in the inner layer were taken to be $1.96 \times 10^{-5}$ kcal mol$^{-1}$ Å$^{-1}$, $9.78 \times 10^{-5}$ kcal mol$^{-1}$ Å$^{-1}$ and $3.91 \times 10^{-4}$ kcal mol$^{-1}$ Å$^{-1}$, corresponding to osmotic pressures of 2 kPa, 10 kPa and 40 kPa, respectively. Since the theoretical relations $\sigma D^2/\kappa = F_1(\Delta p D^3/\kappa, L/D)$ and $f D/\kappa = F_2(\Delta p D^3/\kappa, L/D)$ suggest that there exist two dimensionless parameters $\Delta p D^3/\kappa$ and $L/D$ that govern the problem under consideration, we decided to consider a lysosome with diameter of 100 nm under an osmotic pressure of 40 kPa. Since we would have dimensionless parameters $\Delta p D^3/\kappa$ and $L/D$ very similar to those of typical lysosomes with diameter varying from 0.1 $\mu$m to 1.2 $\mu$m [17] under an osmotic pressure of 300 Pa. Additional calculations were made under osmotic pressures of 2 kPa and 10 kPa, corresponding to $\sim 20$ Pa and 80 Pa, respectively, in a typical lysosome with diameter of 0.5 $\mu$m.

SI Appendix 5 | Contact Between CNT and Vesicle in CGMD Simulations

A monotonically decreasing and non-negative Weeks-Chandler-Andersen (WCA) potential [11] as defined in the Eq. (2) was used to describe the repulsion between hydrophobic CNT and hydrophilic lipid heads. It also enabled us to probe the contact between a lysosome and an encapsulated CNT. The WCA potential energies were calculated at every time step (100 fs) for both ends of the CNT during our CGMD simulations. The curves of the WCA energy versus the simulation time were made up of a series of non-zero signals which indicate contact between the CNT and lysosome within the cutoff ($r_{\text{cutoff}} = 2^{1/6}\sigma$). The contact time is defined as the time span of each non-zero WCA signal. The maximum non-interrupted contact time was calculated at different osmotic pressures and CNT lengths. Based on our CGMD and theoretical studies, the lysosome formed a tether structure at one end of a long CNT (Fig. 1A). Note that we only considered the maximum non-interrupted contact time on the non-tether end of the CNT. Based on the observation that the extraction of lipids only occurs on the outer wall of the MWCNT, it was assumed that the tethered end of the CNT, which is fully covered with hydrophilic lipid heads, will not contribute to lipid extraction. To further assess this assumption, we have also simulated a three-layer graphene sheet in parallel contact with a membrane patch under a compressive contact force of 500 pN, as shown in Fig. S6. In this case, no lipid extraction was observed for the whole duration of a 500 ns simulation. Therefore, it can be concluded that the lysosomal membrane permeabilization induced by lipid extraction occurs on the non-tethered end.

SI Appendix 6 | Fitting of Maximum Non-interrupted Contact Times $t_{\text{max}}$ as a Function of $L/D$

According to Bell’s model extending the classical rate theory for chemical reactions to account for the effect of an external force [18], the contact time $t$ and the compressive force $f$ should obey an exponential relation as $t \sim t_0 \exp(\Delta f/(k_BT))$, where $t_0$ is the contact time in the absence of $f$, and $\Delta f$ is the effective cutoff distance between the tube tip and surrounding lysosome membrane, beyond which the contact is lost as the tube tip and membrane become separated. The term $f \Delta f$ represents an enhancement in the activation energy barrier for breaking the contact. As demonstrated in Fig. S2a, $f$ and $L/D$ obey an approximately linear relation at small perturbation, i.e. $t \sim \exp(L/D)$. This is consistent with our CGMD simulations in Fig. 1A showing that a larger $L/D$ leads to longer maximum non-interrupted contact time $t_{\max}$, which increase exponentially with $L/D$ in the range $L/D > 1$. By fitting the data, the following
empirical expression is obtained for \( t_{\text{max}} \) (ns) as

\[
\log_{10}(t_{\text{max}}) = mL/D + b,
\]

where \( m = 3.94 \) and \( b = -1.72 \) are found under the osmotic pressure of 40 kPa corresponding to the typical normalized pressure in the theoretical analysis. Fig. S7 also includes the results under osmotic pressures of 2 kPa and 10 kPa. The non-interrupted contact time at 2 kPa and 10 kPa follows the same exponential relation with \( L/D \) in the range \( L/D > 1 \).

**SI Appendix 7 | Maximum Non-interrupted Contact Times**

CGMD simulations have been performed to investigate the effects of lipid types, coating proteins and tube diameters on the calculated maximum non-interrupted contact times \( t_{\text{max}} \) as follows.

**Lipid Types.** In the solvent-free coarse-grained lipid model used in our calculation of the contact time, the membrane properties (bending stiffness, area per lipid, etc.) can be tuned by the parameter \( w \) in the WCA potential [11]. To construct membrane composed of lipids of different types, we use three different values of \( w \) (1.6, 1.5 and 1.4), corresponding to bending stiffness of 30 \( k_{\text{B}}T \), 20 \( k_{\text{B}}T \) and 15 \( k_{\text{B}}T \), respectively [11]. A non-homogeneous membrane of mixed lipid types is also considered, in which half of the lipids have \( w \) equal to 1.6 and the other half 1.4. Fig. S8 a-d shows the equilibrium configurations of the lysosome (with a diameter of 100 nm) encapsulating a MWCNT (with a length \( L \) of 170 nm) at different parameter values of \( w \). Similar cherry-shaped structures are observed with one end of the nanotube encapsulated in a membrane tether and the other non-tethered end directly indenting the lipid bilayer. Fig. S8e shows similar results for the maximum non-interrupted contact time between a MWCNT and a lysosome for different lipid types. In all cases, larger \( L/D \) values lead to longer maximum non-interrupted contact time, which increases exponentially with \( L/D \) in the range \( L/D > 1 \). These results suggest that the contact time is insensitive to the lipid type/composition.

**Coating Proteins.** To study the effect of coating proteins on contact time, we employ a new type of beads (CP). This bead repels the MWCNT via the WCA potential and attracts lipid heads via the COS potential. The non-bonded interactions between protein beads and other beads are detailed in Table S2.

**Table S2. Non-bonded interactions between protein beads (CP) and other beads.**

<table>
<thead>
<tr>
<th>Bead type I</th>
<th>Bead type II</th>
<th>Interactions</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP</td>
<td>CP</td>
<td>COS</td>
<td>( \alpha = 0.95, w = 1.6 )</td>
</tr>
<tr>
<td>CP</td>
<td>Lipid head</td>
<td>COS</td>
<td>( \alpha = 0.95, w = 1.6 )</td>
</tr>
<tr>
<td>CP</td>
<td>Lipid tail</td>
<td>WCA</td>
<td>( \alpha = 0.95 )</td>
</tr>
<tr>
<td>CP</td>
<td>CNT</td>
<td>WCA</td>
<td>( \alpha = 0.95 )</td>
</tr>
</tbody>
</table>

The coating proteins are constructed as clusters of CP beads. The number of CP beads in each cluster is controlled to limit the spread area of coating proteins within the range of biological relevance [19]. To mimic the embedded coating proteins, one bead in each cluster is linked to a lipid head through a harmonic bond. Fig. S9a shows the equilibrium configuration of the lysosome with coating proteins. The cherry-shaped structure is still observed. The MWCNT is seen to penetrate through the gaps between coating proteins and directly contact with the lipid bilayer. The maximum non-interrupted time of contact between the MWCNT and the lysosome is calculated and compared with the case without coating proteins (Fig. S9b). The result shows that the maximum non-interrupted contact time \( t_{\text{max}} \) still follows an exponential law when \( L/D > 1 \) and is slightly larger in the presence of the coating proteins. Based on this result, we conclude that the exponential scaling law of the contact time is insensitive to the coating proteins on the lysosomal membrane.

**Tube Diameter.** To investigate the effect of tube diameter on the predicted contact time, we have studied additional cases where the outer diameters of the MWCNTs are taken as 6 nm and 8 nm, and compare with our previous CGMD simulations. Fig. S10a and b shows the equilibrium configurations of a lysosome (with diameter \( D \) of 100 nm) encapsulating a MWCNT with diameter of 10 nm and 6 nm, respectively. The maximum non-interrupted contact time between the MWCNT and the lysosome at different values of the CNT diameter is calculated and shown in Fig. S10c. These results suggest that the predicted contact time is insensitive to the diameter of the encapsulated CNT.

In summary, CGMD simulations indicate that the maximum non-interrupted contact time \( t_{\text{max}} \) between a lysosome and an encapsulated MWCNT is insensitive to lipid types, coating proteins and tube diameter.

**SI Appendix 8 | Modeling at Different Scales**

Fig. 1 A-E shows different scales of approaches in our modeling. The insets in Fig. 1A indicates the schematic of a vesicle with a CNT inside. It is the model we used in theoretical calculations and CGMD simulations. As the length of the encapsulated nanotube was beyond the diameter of the vesicle, the vesicle deformed to be a cherry-like configuration with a membrane tether covering the nanotube. The membrane tension and the contact force were calculated as a function of the ratio between the tube length and vesicle diameter. The instantaneous lysis tension, the membrane tension required to rupture a vesicle, is usually in the range 4 mN/m-10 mN/m [8], which is much larger than the values we obtained for a lysosome with a CNT inside by theoretical calculations. In our work, the long-time contact between the non-tether end of CNT and the vesicle was regarded as the cause of lysosome/endosome instability under low membrane tension.
Fig. S8. CGMD simulations of the maximum non-interrupted time of contact between a MWCNT and a lysosome for different lipid types. (a-d) The equilibrium configurations of the lysosome at \( w = 1.6 \) (a), 1.5 (b), 1.4 (c) and mixed 1.4/1.6 (d). (e) Maximum non-interrupted contact time \( t_{\text{max}} \) at different \( w, L/D \) and \( \Delta p = 40 \) kPa.

Fig. S9. CGMD simulations of the maximum non-interrupted contact time between a MWCNT and a lysosome coated by proteins. (a) The equilibrium configuration of the lysosome with coating proteins. (b) Maximum non-interrupted contact time \( t_{\text{max}} \) at different length ratios of \( L/D \) and \( \Delta p = 40 \) kPa.

Fig. S10. CGMD simulations of the maximum non-interrupted time of contact between a lysosome and an encapsulated MWCNT of different diameters. (a and b) The equilibrium configurations of a lysosome encapsulating a MWCNT with diameter of 10 nm (a) and 6 nm (b). (c) The maximum non-interrupted contact time \( t_{\text{max}} \) for different tube diameters \( L/D \) and \( \Delta p = 40 \) kPa.
The same configuration was used in CGMD simulations to study the maximum contact time between the MWCNT and the vesicle. To uncover the vesicle damage mechanism due to long-time contact, the all-atom MD simulations were carried out for MWCNT (Fig. 1 B and C) and MLG (Fig. 1 D and E) interacting with membranes. The MWCNT simulations (Fig. 1 B and C) by which we could focus on the interaction between the non-tether end of CNT and the vesicle membrane were performed to investigate the dependence of critical damage forces on the size and shape of the tube end. The MLG simulations (Fig. 1 D and E) enabled us to further focus on one section of circumference on the CNT end. Constant forces were placed on the MLGs to mimic the contact forces derived from theoretical calculations. The critical damage conditions were studied to obtain the relation between the contact force and the critical damage time of membrane.

**SI Appendix 9 | All-atom MD Simulations: Methodology**

All-atom MD simulations based on the GROMACS package (version 4.6.5) [20] were performed to investigate how a multi-layered graphene (MLG), corresponding to a near-tip slice of a MWCNT, or a MWCNT interacts with a membrane patch. The MLG simulations were necessary to reduce the system size and extract the results to large sized nanotubes. The membranes were constructed from bilayers of the Berger lipids [21], which is the all-atom lipid model most commonly used in membrane-protein simulations [22, 23]. The Berger lipid force field, as an extensively validated membrane force field, was employed in our all-atom simulations combined with an OPLS [24] representation of MLGs and MWCNTs. The majority of phospholipids assembled in the lysosomal membrane is phosphatidylcholine (PtdCho; PC) [25]. The dipalmitoyl phosphatidylcholine (DPPtdCho, DPPC), one of the typical and most widely used phosphatidylcholine, was adopted in our all-atom simulations to build a lipid bi-layer with dimensions of 12.9 nm x 12.3 nm (512 lipids). For high computational efficiency, water molecules were represented by a simple point-charge SPC/E model [26] with a polarization correction. Periodic boundary conditions were applied in all three directions. The all-atom simulations were performed under a constant temperature of 310 K controlled by velocity-rescale thermostat with a coupling time constant of 0.1 ps. To maintain a constant surface tension in the lipid bilayer, semi-isotropic pressure coupling (1 bar), which was isotropic in the plane of the membrane but independent in the out-of-plane direction, was used with a coupling time constant 1 ps. The fast smooth particle-mesh Ewald (SPME) [27] was used to calculate the long-rang electrostatic interactions with a cutoff of 1.2 nm. The time step was fixed at 2 fs. After a 100 ns initial equilibration of solvated lipid systems, MLGs or MWCNTs were introduced into the all-atom simulations by replacing overlapping water molecules. Position restraints were applied to orient the MLGs or MWCNTs vertically with respect to the membrane. The lowermost edge of the MLGs or MWCNTs was initially 2 nm above the membrane surface (Fig. 1 D and E). After 40 ns of re-equilibration, the MLGs or MWCNTs were released in the vertical direction and pulled down with a velocity of 0.1 nm ns$^{-1}$, until they reached the membrane surface. To reduce dynamic effects, the system was relaxed for another 60 ns before production runs. The production includes two types of simulations (MLGs and MWCNTs). In the type I simulations, MLGs were considered and the system was periodic in the out-of-plane direction to mimic a slice of the CNT edge contacting the membrane. Constant forces were placed on the MLGs to investigate the critical contact time for membrane damage, while the center-of-mass of membrane was fixed. In the type II simulations, MWCNTs were considered and steered molecular dynamics [28] simulations were performed to study how the critical damage force depends on the tube radius (or circumference). An uncapped or capped CNT was inserted into the membrane with a constant rate of 0.01 nm ns$^{-1}$ using virtual springs by which the center-of-mass distance between CNTs and membrane was constricted. The force profiles as a function of the simulation time were calculated during the CNT insertion, and the peak force was defined as the critical damage force.

**SI Appendix 10 | Critical Contact Time of Membrane Damage in MLG Simulations**

![Fig. S11. The center-of-mass (COM) profiles. Time evolutions of the COM distances between the three-layer graphene and the membrane along the vertical direction. Each curve corresponds to a contact force.](image-url)

The lipid extraction is uncovered as the primary damage mechanism of membrane under long-time contact by MLGs or MWCNTs. The simulation results have shown that the membrane disruption clearly depends on the time scale of contact application. The critical contact time $t_c$ of membrane damage is defined as the onset of unstable lipid extraction to represent the time tolerance of membrane under a sustained contact force by MLGs. In the all-atom simulations of MLGs interacting with membrane, the center-of-mass (COM) distances between the MLGs and membrane are calculated as a function of the simulation time and the sustained contact force. Fig. S11 shows the example of the three-layer case. All the curves of COM distances start with gentle slopes decreasing at mild rates. The slight decreases are caused by shallow MLG penetration and a few extracted lipids (Fig. S11). These early lipid extractions that are stable can be sustained safely by membrane. After inflection points, the curves of COM distances fall off cliffs, suggesting the instability of lipid extraction. Such inflection points that indicate the unstable lipid extractions are considered as the moment of membrane failure as shown in Fig. 2 A-D. The critical contact time of membrane damage, $t_c$, is protracted as the sustained time of membrane under contact force $F$. The lower contact force proves to be sustainable for longer critical contact time. In Fig. S11, the critical contact times under the contact forces of 500 pN, 600 pN, 650 pN, 700 pN, 800 pN and 1000 pN are 264.1 ns, 118.1 ns, 75.1 ns, 43.1 ns, 22.4 ns and 5.7 ns, respectively, corresponding to
the data points of the three-layer case in the double logarithmic diagram of Fig. 2E.

SI Appendix 11 | Distribution of Reaction Force on Different Layers of MLGs

Fig. 2E shows the critical damage time profiles as a function of contact force and layer number in MLG simulations. The lower contact force and higher layer number can postpone the critical time to membrane permeabilization. However, when the layer number is more than two, the critical damage time increases insignificantly with the layer number of MLG. The reason for which the curves of the layer number over two tend to converge is considered as the contact force localizing on the outer two layers. To verify our hypothesis, we perform an all-atom MD simulation of four-layer graphene inserting into the membrane to investigate the force distribution on each layer. Following the approaching processes described in the all-atom MD simulation details, the four-layer nanosheet was pulled down 0.5 nm deeper after it reached the membrane (Fig. S12a). A virtual spring was used to constrain the center-of-mass (COM) distance between the nanosheet and the membrane. After 10 ns equilibration, the tension of the virtual spring was stabilized at 208 pN. For another 40 ns relaxation, forces on each layer supported by membrane were calculated and normalized by the tension of virtual spring. Fig. S12b shows the normalized forces on each layer as a function of simulation time. As we expected, the support reaction was observed to localize on the outer layers (Layers #1 and #4). The inner layers hardly contribute to the contact force. Meanwhile, the lipid extraction as the long-time failure mode is only able to take place on the surface of the nanosheet. The localizations of contact force and lipid extraction make the critical damage time insensitive to the layer number over two. For MLG with more than two layers in Fig. 2E, a linear equation between \( t_c \) (ns) and compressive force \( F_c \) (pN) fits all data:

\[
\log_{10} t_c = -m' \log_{10} F_c + b',
\]

where \( m' = 5.116 \) and \( b' = 16.40 \).

SI Appendix 12 | Dependence of Critical Membrane Damage Time on the Geometry of CNT Tip

The fitting Eq. (3) enables us to calculate the critical membrane damage time under a typical compressive force (\( \sim 20 \) pN) exerted by the lysosomal membrane. However in reality, the compressive force distribution and lipid extraction may not be uniform around the CNT tip and the localized distribution of contact force may expedite membrane damage. To study this effect, steered molecular dynamics (SMD) [28] simulations of a CNT inserting into the membrane at a constant rate were performed to investigate how the critical damage force depends on the tube circumference. Fig. 2F shows that the critical damage force is not proportional to the circumference of the CNT edge, but follows another power law. The larger the CNT radius, the lower the critical damage pressure is required at the same insertion rate (0.01 nm ns\(^{-1}\)). The results from both uncapped and capped CNTs indicate that the damage force is independent of the tip shape when the radius is large enough. The fitting equation for the uncapped CNT is chosen to describe the relation between the critical damage force \( F \) and the tube circumference \( C \) as

\[
F = aC^k,
\]

where \( a = 286.7 \) and \( k = 0.448 \). This implies the following relation between damage forces at two different circumferences,

\[
\frac{F_1}{F_2} = \left( \frac{C_1}{C_2} \right)^k.
\]

Note that the out-of-plane dimension \( L_C \) in the MLG simulations was 12.3 nm, translating into a contact force of \( f = 20 \) pN obtained from the theoretical calculations. Considering typical diameter \( d \) of the Mitsui CNTs used in our experiments as 80 nm, the circumference is \( c = \pi d \) and the contact force can be converted to the MLG in all-atom MD simulations as

\[
F_C = f \left( \frac{L_C}{c} \right)^k = f \left( \frac{L_C}{\pi d} \right)^k = 5.22 \text{ pN}.
\]
Therefore, the critical membrane damage time for the CNT with a diameter of 80 nm can be extrapolated from the power law in Eq. (3) as $t_{c} \mid F_{c} = 5.22 \text{ pN} \approx 5391 \text{ s} \approx 1.5 \text{ h}$.

**SI Appendix 13 | Critical Damage Force in MWCNT Simulations**

The MLG simulations that mimic a segment of MWCNT edge interacting membrane provided us the scale law between critical damage time and contact forces. The lipid extraction inducing membrane damage is a localized failure starting from a random contact point along the MLG edge. Therefore, the critical condition of membrane damage may depend on the length of the segment we used in the MLG simulations. To study the length dependence, the inserting simulations of MWCNTs with different radii were performed to obtain the relation between the CNT circumferences and their corresponding critical damage forces (Fig. 2F). Here in this section, it is explained how we defined the critical damage force. Uncapped or capped CNTs inserted the membrane with a constant rate of 0.01 nm ns$^{-1}$. Fig. S13 shows the resistance force from membrane as a function of simulation time when three-walled CNTs of different mean circumferences inserted. All the curves start with plains and then increase linearly after the MWCNTs approach the membrane. They dropped after reaching their peaks, corresponding to membrane damage. The maximum forces were defined as the critical damage forces for MWCNTs of different circumferences.

**Fig. S13.** The interaction force profiles in the SMD simulations of MWCNTs. The reaction forces on the three-walled CNTs by the membrane are calculated as a function of simulation time when the MWCNTs insert at a constant rate. Each curve corresponds to a mean circumference of a three-walled CNT.

**SI Appendix 14 | Dependence of Critical Membrane Damage Time on Membrane Composition**

To study the effect of membrane composition on lipid extractions and the critical damage time leading to lysosomal permeability, we constructed a more realistic subcellular membrane by replacing 30\% of DPPC lipids with cholesterol [29]. Lipid extractions were still observed under forced contact between the MLG and the membrane (Fig. S14 a-c). Fig. S14 d-f show that the binding of cholesterol on the graphenic surface only occurs after the instability of lipid extraction, and thus has little influence on the critical damage time leading to lysosomal permeability in Fig. S14g. The results of POPC lipid bilayer also indicate that the lipid extractions and critical damage time are insensitive to lipid types.

**Fig. S14.** The effect of membrane composition on lipid extractions and the critical damage time leading to lysosomal permeability. (a-c) Representative configurations of a one-layer graphene sheet interacting with a DPPC/cholesterols membrane under a compressive force of 50 pN at 120 ns (a), 180 ns (b) and 200 ns (c). (d-f) The same snapshots of (a-c) without visualization of DPPC lipids. (g) The critical damage time as a function of the contact force induced by the MLG for DPPC, POPC and DPPC/cholesterols membrane.

**SI Appendix 15 | Robustness of Lipid Extractions by MWCNTs**

To verify the robustness of lipid extractions by MWCNTs and MLGs, the binding energies between DPPC lipids and the membrane, DPPC lipids and the graphenic surface were calculated by using steered MD simulations [28], umbrella sampling and the weighted histogram analysis method [30, 31], respectively. The energy required to extract a single DPPC lipid from the lipid bilayer or the infinite graphenic surface is 52.47 $k_{B}T$ or 60.21 $k_{B}T$, respectively (Fig. S15). Therefore, the lipid extractions from the membrane to the surface of MWCNT are energetically favorable and robust, rather than an artifact of the MD simulations.
To correlate the tubular form of MWCNT with the flat morphology of MLG, we have performed all-atom MD simulations of a MWCNT with diameter of 8 nm in contact with the membrane patch. In this simulation, similar lipid extraction was observed on the tube surface (Fig. S16a), suggesting that the tubular form will not qualitatively affect the lipid extraction mechanism. We used the MLG to model a slice of much larger MWCNTs (with diameter around 8 nm) used in the experiments. This local view allowed us to focus on the effect of a slice of the graphenic surface near the tip of the nanotube on the lipid bilayer. The tubular curvature of such large tubes is much smaller than that of the 8 nm tube and has negligible effect on the lipid extraction process. Therefore, we conclude that the tubular curvature does not play a significant role in lipid extraction, as long as the tube diameter is larger than the size of a lipid molecule.

We also investigated the effect of the lateral size of the MLG-membrane system on lipid extraction. In most simulations, the lateral size of the periodic MLG, which is the same as the width of the membrane patch, was fixed at 12.3 nm. We performed an additional simulation of a one-layer graphene sheet (with a length of 8.67 nm) interacting a membrane patch of smaller size (8.97 nm × 8.67 nm; 340 lipids), and similar lipid extractions were observed, as shown in Fig. S16b and c. At its early stage, lipid extraction is localized within a region with a width of 3 nm, comparable to the length of a lipid molecule (Fig. S16c). This indicates that lipid extraction is insensitive to the lateral size of the MLG-membrane system, as long as it is larger than the length of a lipid molecule.

**SI Note 16 | Buckling Criterion of Confined CNTs and Other 1D Nanomaterials**

To explain the experimental results, the impact of diameter or wall number on bending or flexibility of MWCNTs was calculated to determine the crucial dimensions in relationship to bending or folding (Fig. 5). The concentric tubes are modeled as the equivalent continuum structure of MWCNT. The mechanical properties are given from the previous atomistic studies [32–34]. The Young’s modulus $E$ is 5.12 TPa, the thickness of each tube $t$ is taken as 0.066 nm, and the diameter spacing $s$ is fixed at 0.68 nm.

Consider the buckling instability of a MWCNT with $n$ walls as shown in Fig. S17a. For the $i$-th tube, the wall thickness $t$ is usually much smaller than the mean diameter $d_i$, and the second area moment can be written as (neglect the higher order of $t$)

$$I_i = \int y^2 dA = \frac{\pi}{8} td_i^3.$$  

[4]

The second area moment of the whole MWCNT can be obtained by summing up $I_i$ from all the concentric walls as

$$I_{MWCNT} = \sum_i I_i = \sum_i \frac{\pi}{8} td_i^3.$$  

[5]

For a solid MWCNT with the innermost wall diameter $d_0 = s = 0.68$ nm and outermost wall diameter $d_n = d$, the $i$-th wall diameter is $d_i = d_0 + i \times s$ and the second moment of area is

$$I_{solid} = \sum \frac{\pi}{8} td_i^3 = \frac{\pi}{32s} (d + s)^2 (d + 2s)^2.$$  

[6]

Based on the Euler buckling theory, the critical buckling force $F_{cr}$ is

$$F_{cr} = \frac{\pi^2 E I}{I^2},$$  

[7]
where $l$ is the length of the MWCNT. If the critical buckling force $F_{cr}$ is known (e.g. $\sim 20$ pN from theoretical calculations), the critical condition for buckling can be expressed in terms of a critical tube length as

$$l = \pi \sqrt{\frac{EI}{F_{cr}}}.$$  \[8\]

Fig. S17b shows this relation (black solid line). A CNT with dimensions above the curve would buckle under the compressive contact force of $20$ pN induced by the confining lysosome.

More generally, a hollow MWCNT with inner ($d_1$) and outer ($d_2$) diameters can be made equivalent to a solid MWCNT with the same second area moment $I_{solid}$. Since the wall spacing $s$ is usually much smaller than diameter $d$, $I_{solid}$ may be simplified as

$$I_{solid} \approx \pi t d^4.$$  \[9\]

Thus, for a hollow MWCNT with inner diameter $d_1$ and outer diameter $d_2$ (Fig. S17 a and b), the second moment of area is

$$I_{hollow} = I_{solid}(d_2) - I_{solid}(d_1) \approx \pi t(d_2^4 - d_1^4) / 32s.$$  \[10\]

Setting these two equations equal,

$$d_2^4 - d_1^4 = d^4$$  \[11\]

leads to an effective diameter $d$ of a hollow MWCNT. As shown in Fig. S17b, under the compressive force of $20$ pN, the smaller the $d$ and the longer the $l$, the more flexible and susceptible to buckling the MWCNT is.

The same model can be extended to other 1D nanomaterials by writing the moment of inertia for solid and hollow cylinders as

$$I'_{solid} = \pi d^4/64 \quad \text{and} \quad I'_{hollow} = \pi (d_2^4 - d_1^4)/64,$$  \[12\]

where $d$ is the outer diameter of a solid cylinder, $d_1$ and $d_2$ are the inner and outer diameters of a hollow cylinder. Similarly, setting $d_2^4 - d_1^4 = d^4$ leads to an equivalent diameter $d$ in the buckling phase diagram.

Based on the Euler buckling Eq. (8), Fig. S18 illustrates the buckling criteria under the same compressive force of $F_{cr}$($\sim 20$ pN) for typical 1D nanomaterials including Ag, Ni, titanium dioxide (TiO$_2$), cerium oxide (CeO$_2$) nanowires, polymer fibers, worm-like micelles, as well as MWCNTs. The critical curves of buckling that distinguish biologically soft from biologically stiff materials are compared to the typical size range of lysosome. Nanomaterials falling in the region between their buckling thresholds and the lysosome size are predicted to have sufficient stiffness and length to mechanically induce lysosomal permeability. The diagram can also correctly predict that many thin nanofibrous materials of intrinsic low stiffness (e.g. polymers, worm-like micelles [35]) would be non-pathogenic by this pathway.

### SI Appendix 17 | Experimental Methods

**Carbon nanotubes** (MWCNT-7) tubes were oxidatively shortened by treating $50$ mg of MWCNTs with concentrated H$_2$SO$_4$/HNO$_3$ (3:1) in a 100-W ultrasound bath for $20$ h or $100$ h with bath temperature maintained below $40$ °C. After acid treatment, the MWCNTs were washed three times using DI water and again with nanopure water to neutral pH. After overnight drying at $60$ °C, the MWCNTs were annealed in argon flow at $1100$ °C for $10$ h to remove many of the oxygen-containing functional groups introduced by the acid treatment. The resulting materials have a nominal length of $1$ µm (1 µm MWCNT-7, 20-h treatment) or $0.5$ µm (0.5 µm MWCNT-7, 100-h treatment).

The length and diameter distributions of all nanotube samples were obtained by image analysis of SEM and TEM micrographs. For HRTEM, carbon materials were dispersed in ethanol followed by sonication for $15$ min at room temperature, and the dispersed samples were imaged on a JEOL JEM-2010. Means and standard deviations were computed from at least one hundred single tube measurements using NIH ImageJ software. Mean diameters for the MWCNT hollow cores were also measured and used to calculate an effective diameter of an equivalent fully dense cylinder for the mechanical stiffness modeling and pathogenicity classification diagram (Fig. 5).

Hydrodynamic particle/agglomerate sizes and ζ-potentials were also measured in cell culture medium after dispersion in DPPC and albumin by dynamic light scattering using a Zetasizer Nano ZS dynamic light scattering system (Malvern Instruments) after dispersing the materials in DPPC/BSA solution and DMEM/F12 cell culture media (Invitrogen). Surface areas were determined by nitrogen vapor adsorption (Quantachrome Autosorb-1) analyzed using Brunauer–Emmett–Teller (BET) theory, and Raman spectra were obtained using a Witec alpha 300M+ Confocal Raman Microscope. The iron content of flexible MWCNTs were determined by filed emission SEM (LEO 1530 VP) with energy dispersive X-ray analysis.

Carbon nanomaterials were characterized by transmission electron microscopy (TEM); surface area, ζ-potential, and dynamic light scattering determinations; and Raman spectroscopy (Table S3 and Figs. S19-S22). These samples had a negative ζ-potential and formed agglomerates in cell culture medium ranging
from 269 nm to 1370 nm with a narrow polydispersity index.

Cell Culture and Exposure Conditions. Carbon nanoparticles were dispersed using 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (Avanti Polar lipids) and albumin (Sigma; A9576-50ml) before addition of cell culture medium. To 1000 μg carbon nanoparticles 20 μl of a 10 mg/ml DPPC/BSA solution and 40 μl of a 30% albumin solution (Sigma; A9576-50ml) were added and sonicated in a Branson 2510 for 5 min. To each vial 400 μl DMEM/F12 (Invitrogen) cell culture medium was added and the dispersion was sonicated for additional 30 min. Following an additional dilution to produce a 500 μg/ml stock solution the dispersion was sonicated for additional 45 min. For hepatocytes the carbon nanoparticle stock solution was diluted from 500 μg/ml to 40 μg/ml in DMEM/F12 cell culture medium containing 10% FCS, whereas RPMI 1640 served as a dilution medium for lung epithelial cells.

Cell Viability. A colorimetric assay (CCK-8; Dojindo; CK04-05), was used to assess viability by determination of dehydrogenase activity in cells using the water-soluble tetrazolium salt (WST-8) after exposure of cells to carbon nanoparticles. 5,000 cells/well hepatocytes or 10,000 lung epithelial cells were seeded into a 96 well plate and exposed after overnight incubation to carbon nanoparticles for 24 h, respectively. The cells were washed once with cell culture medium, then exposed to 100 μl/well phenol-red-free medium containing WST-8 substrate at a concentration of 1:10 for 3 h. 90 μl of the supernatant was transferred into another 96 well plate and the absorbance was quantified at 450 nm using Spectramax M2 ( Molecular Devices). Cell viability was confirmed using Quant-iT™ PicoGreen®dsDNA Assay Kit (Invitrogen; P11496) that quantitates DNA content [42]. To visualize apoptotic hepatocytes, cells were exposed to carbon nanoparticles for 24 h, fixed and permeabilized. Apoptotic pototocytic cells with fragmented DNA were labeled with fluorescein-12-dUTP (TUNEL assay) and the nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were visualized using spinning disk Olympus confocal fluorescence (Model IX81) motorized inverted research microscope.

Confocal Fluorescence Microscopy. Hepatocytes were exposed to carbon nanomaterials for 24 h. The cells were washed and stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were visualized using a spinning-disk Olympus confocal fluorescence (Model IX81) motorized inverted research microscope with transmitted light illuminator to assess uptake. Hepatocytes were washed and incubated with anti-rabbit Rab5 (Cell Signaling #3547S) or anti-rabbit LAMP-1 (Abcam; ab24170) antibody and stained with Alexa Fluor 488 donkey anti rabbit IgG (Molecular Probes; A21206) conjugated with fluorescein isothiocyanate (FITC) or Alexa Fluor 555 donkey anti rabbit IgG (Molecular probes A31572) and 2′,5′-Bi-1H-benzimidazole, 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-23491-52-3 solution (Hoechst 33342) to visualize the cell nuclei.

Internalization of multiwalled carbon nanotubes (MWCNTs) or spherical carbon black nanoparticles (Table S3) by lung cells or hepatocytes in monolayer culture was visualized using brightfield and confocal fluorescence imaging (Fig. 3 A and B) and confirmed using TEM (Figs. 3 E and F and 4 C-F). Both zero-dimensional and one-dimensional nanoparticles were initially co-localized with endolysosomal vesicles as identified using the late endosomal marker, Rab5, and the lysosomal marker, LAMP1 (Fig. S29). LAMP-1 expression was confirmed using immunogold labeling and TEM (Fig. S30) and the intraluminal pH was acidic as indicated by acridine orange fluorescence (Fig. S29).

Lysosomal Permeabilization. CellProfiler (version 2.1.1, Broad Institute) was utilized for cell segmentation and analysis. First, fluorescently labeled lysosomes were segmented as primary objects using a manual threshold strategy (threshold set at 0.1 or 0.3), and aggregated objects were distinguished using the “Intensity method”. The minimum and the maximum object diameter was set to 4 pixels and 100 pixels, respectively, and all objects outside the range were discarded. Then, the “Measure-ObjectSizeShape” module was employed to extract the area of the detected objects, which is the actual number of pixels in the region followed by conversion to average diameter of detected objects micron units. Fluorescent features from the same experiment, whereas n here stands for lysosomes (n ≥ 200) were imaged and analyzed using identical settings. The statistical significance of differences between mean feature sizes for the long, stiff MWCNT-7 sample relative to all other samples at the same threshold value were evaluated using a standard t-test and Anova.

Single cell quantitative high content imaging (Opera Phenix; Perkin Elmer) was utilized for automated fluorescence imaging to determine the percentage of cells with punctate or diffuse cytoplasmatic fluorescence.

Lysosomal Integrity and Cathepsin B Release. Hepatocytes and lung epithelial cells were exposed to 10 μg/ml 20 μg/ml or 40 μg/ml carbon nanomaterials for 24 h. Cells were stained with Magic Red Cathepsin B detection reagent as suggested by the supplier (Immunohistochemistry Technologies; #937) for 60 min, followed by 2′,5′-Bi-1H-benzimidazole, 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-23491-52-3 (10 mg/ml Hoechst 33342) staining for 5 min to visualize cell nuclei.

Acridine Orange Staining. To verify the location of MWCNTs and carbon black in lysosomes, acridine orange (AO; Invitrogen), a cationic, lipophilic fluorochrome, was employed. Acridine orange enters acidic compartments such as lysosomes where it becomes protonated and sequestered due to the low pH value resulting in red emission of the dye. Hepatocytes were exposed to 10 μg/ml MWCNTs or carbon black for 24 h, then incubated with 1:10000 μl/ml of 33.2 mM acridine orange and 1:2000 μl of a 16.2 mM 2′,5′-Bi-1H-benzimidazole, 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-23491-52-3 solution (Hoechst 33342) in cell culture medium for 30 min.

Caspase Activation. A red SR FLICA®Poly Caspase Assay Kit (Immunohistochemistry; cat.no: 917) was used to assess caspase activation. This in vitro, whole cell caspase assay employs the red fluorescent caspase inhibitor probe SR-VAD-FMK to label activated caspases in living cells. The percentage of cells (red) with caspase activation (red) was quantified using SlideBook 6 (Olympus) image analysis program or Harmony analysis program (Perkin Elmer). Co-localization of cathepsin B release and caspase activation was performed using single cell quantitative high content imaging (Opera Phenix, Perkin Elmer) and visualized using spinning disc confocal microscopy (Olympus).

Transmission Electron Microscopy. Two different methods were used to verify that carbon nanomaterials are internalized by hepatocytes. Cells were fixed in Karnovsky’s fixative [5% (vol/vol) glutaraldehyde, 4% (vol/vol) formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4] (Electron Microscopy Sciences; 11650) at 4 °C and then rinsed three times with 0.1 M sodium cacodylate buffer. After pelleting the cells were postfixed in 2% (vol/vol) aqueous osmium tetroxide (Electron Microscopy Sciences; RT 19152). Samples were dehydrated in ice-cold, graded
ethanol solutions and then in ice-cold anhydrous acetone. Infiltration of specimens was achieved using Durcupan ACM resin (Electron Microscopy Sciences) with decreasing proportions of acetone and finally Durcupan alone. Cells were embedded in Durcupan and polymerized at 60 °C for 48 h. Cell blocks were then sectioned at 80 nm or 120 nm on a Reichert Ultracut Ultramicrotome with a diamond knife. Sections were placed on copper grids and viewed on a Philips 410 transmission electron microscope equipped with an Advantage HR CCD camera. Images were acquired with Advanced Microscopy Techniques imaging software. The intracellular localization of MWCNTs, carbon black and nanohorns was also confirmed by growing cells in monolayer on coverslips, which were then fixed and dehydrated as mentioned above. Cover slips were then infiltrated with Durcupan embedding media cell face down on a Thompson mold and polymerized above. Cover slips were then infiltrated with Ducrupan embedment media cell face down on a Thompson mold and polymerized at 60 °C. Following polymerization, blocks were placed on dry ice and cover slips were snapped off leaving cell monolayer on blocks that were sectioned as described above.

LAMP1-Immunogold Localization. Hepatocytes were cultured in 12 well plates containing coverslips and exposed to 10 µg/ml carbon black or MWCNTs. After fixation in paraformaldehyde and glutaraldehyde; cells were permeabilized and exposed after 30 min blocking 0.3 M glycine to anti-rabbit LAMP-1 (Abcam; ab24170) antibody; diluted 1:1000 in 1% BSA-PBS solution over night. Cells were washed with PBS and incubated with goat anti-rabbit conjugated with ultrasmall gold nanoparticles (EMS #25100) overnight. Following PBS washing, cells were fixed in 2.5% glutaraldehyde and washed with phosphate buffer and water. After silver enhancement (EMS #25521) cells were washed and osmicated following dehydration and embedding in Spurr resin. Cell blocks were then sectioned at 80 nm or 120 nm on a Reichert Ultracut Ultramicrotome with a diamond knife. Sections were placed on copper grids and viewed on a Philips 410 transmission electron microscope equipped with an Advantage HR CCD camera. Images were acquired with Advanced Microscopy Techniques imaging software.

Statistics. Statistical significance was determined using an unpaired t-test and one way ANOVA to compare the differences between the means of untreated and treated cells in triplicate cultures. *P of value smaller than 0.05 was considered to be statistically significant.

SI Appendix 18 | Supplementary Table and Experimental Figures

Table S3 listing physicochemical characterization of carbon nanomaterials we used and supplementary experimental figures S19-S31 are presented at the end of the SI.

Table S3. Carbon nanomaterials panel and physicochemical characterization.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Origin</th>
<th>Iron content</th>
<th>Surface area (m$^2$/g)</th>
<th>Length (µm)</th>
<th>Diameter (nm)</th>
<th>ζ-potential in cell culture medium (mV)</th>
<th>Hydrodynamic size in cell culture medium (nm)</th>
<th>Polydispersity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWCNT-1 MER Corp</td>
<td>&lt; 0.1% Fe</td>
<td>10</td>
<td>6.1 ± 2.5</td>
<td>163 ± 49</td>
<td>-11.9 ± 0.5</td>
<td>1157</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>MWCNT-7 Mitsui &amp; Co</td>
<td>&lt; 0.2 wt.% Fe</td>
<td>20</td>
<td>11.7 ± 3.6</td>
<td>80 ± 18</td>
<td>-11.4 ± 0.3</td>
<td>1370</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>M120 Carbon Black Cabot Corp</td>
<td>Not detectable</td>
<td>35</td>
<td>N/A</td>
<td>75</td>
<td>-11.3 ± 0.1</td>
<td>273</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>Carbon nanohorns Oakridge National Laboratory</td>
<td>Not detectable</td>
<td>122</td>
<td>N/A</td>
<td>40 ~ 60</td>
<td>-12.1 ± 0.7</td>
<td>269</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>1.0 µm MWCNT-7 Mitsui / Brown</td>
<td>- -</td>
<td>- -</td>
<td>0.85 ± 0.49</td>
<td>47 ± 19</td>
<td>-10.5 ± 1.0</td>
<td>600</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>0.5 µm MWCNT-7 Mitsui / Brown</td>
<td>- -</td>
<td>- -</td>
<td>0.51 ± 0.30</td>
<td>34 ± 13</td>
<td>-10.3 ± 0.2</td>
<td>342</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>MWCNT- flex1 Nanolab</td>
<td>&lt; 1 wt.% Fe</td>
<td>217</td>
<td>1 ~ 5</td>
<td>11 ± 4.6</td>
<td>-11.5 ± 1.5</td>
<td>289</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>MWCNT- flex2 Nanolab</td>
<td>&lt; 1 wt.% Fe</td>
<td>190</td>
<td>5 ~ 20</td>
<td>14 ± 8.5</td>
<td>-11.5 ± 0.5</td>
<td>300</td>
<td>0.60</td>
<td></td>
</tr>
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</table>

Fig. S19. Morphologies and dimensions of the various types of multi-walled carbon nanotubes in the sample panel. HRTEM images and histograms of length and diameter distributions of (a) MWCNT-1, (b) MWCNT-7 [42], and two samples produced by oxidative shortening. (c) 1 µm MWCNT-7 and (d) 0.5 µm MWCNT-7.
Fig. S20. Morphologies and diameters of flexible carbon nanotube samples in the material panel. HRTEM images and histograms of diameter distribution for flexible MWCNTs (NanoLab, Inc.) (a) MWCNT-flex1 and (b) MWCNT-flex2.

Fig. S21. HRTEM images of reference carbon materials. (a) Carbon black (M120 from Cabot Corporation). (b) Carbon nanohorns (courtesy of D. Geohegan, Oakridge National Laboratory, TN).
Fig. S22. Raman spectra of the carbon nanomaterial panel used in this research. The disorder-induced D band around 1330 cm⁻¹ and graphitic G band around 1580 cm⁻¹ are characteristic bands for sp² carbon materials. The D to G intensity ratio (D/G ratio) is a measure of the extent of crystalline defects and disorder. The band around 2650 cm⁻¹ is the G' peak, which is an overtone of the D peak. The degree of crystalline order in this sample set varies from carbon black and nanohorns (lowest) to the large-diameter CNTs (highest). The shortening procedure does not have a major effect on crystalline order.

Fig. S23. Lysosomal membrane permeabilization and transmission electron micrographs of lung epithelial cells exposed to carbon nanomaterials (a) MWCNT-7, (b) MWCNT-flex2, (c) carbon black particles and (d) carbon nanohorns. MWCNT-flex2, carbon black and carbon nanohorns are localized within the lysosomes, whereas MWCNT-7 penetrates through the lysosomes into the cytoplasm. Lysosomal membrane permeabilization was induced only after exposure of cells to MWCNT-7 as indicated by diffuse cytoplasmic fluorescence (a) reflecting release of cathepsin B into the cytoplasm.
Fig. S24. Quantitative evaluation of viability and cellular co-localization of cathepsin B and caspase activation after exposure of lung epithelial cells to carbon black or MWCNTs. (a) Quantitative determination of viability using WST-8 assay after exposure of lung epithelial cells to carbon nanomaterials; (b) quantitative determination of cathepsin B release into the cytoplasm (cathepsin B positive cells) co-localized with caspase activation; * $P < 0.05$. Cells were exposed to 40 µg/ml MWCNT-7 for 24 h and cathepsin B release was quantitated using Magic Red Cathepsin B detection kit and caspase activation using Flica Caspase Assay. (c-e) Confocal imaging to visualize the release of cathepsin B into the cytoplasm (red; c) and caspase-positive cells (green; d) after exposure to MWCNT-7. Cells were exposed for 24 h to 40 µg/ml MWCNT-7. The cells were imaged using an Olympus confocal microscope to visualize cathepsin B release and caspase activation. The overlaid image (e) showed the co-localization of cathepsin B release and caspase activity in lung epithelial cells exposed to MWCNT-7 for 24 h.
Fig. S25. Cell viability and cathepsin B release based on surface area dose and time. (a) Following exposure to equal surface area doses of carbon nanoparticles, viability of hepatocytes was assessed using the dehydrogenase activity assay WST-8. Carbon black, flexible MWCNTs and carbon nanohorns did not significantly decrease viability, whereas MWCNT-7 and 1 µm MWCNT-7 significantly decreased viability (* P < 0.05). (b and c) Time course of cathepsin B release and cell death induced in hepatocytes exposed to carbon black or carbon nanotubes (MWCNT-7). Cell viability (b) and cathepsin B positive cells (c) were quantified using confocal imaging (Perkin Elmer; Harmony software analysis program). The percentage of viable cells and cathepsin B positive cells were evaluated after 8 h, 21 h and 23 h. Cathepsin B positive cells and cell death were not significantly detected within the first 8 h. A significant increase in cathepsin B positive cells and toxicity was detected after 21 h (* P < 0.05).

Fig. S26. Cathepsin B localization in hepatocytes after exposure to carbon nanomaterials. (a) Carbon black, (b) carbon nanohorns, (c) shortened nanotubes (0.5 µm MWCNT-7) and (d) flexible MWCNTs after 24 h, lysosomal integrity was assessed using cathepsin B substrate (red) and nuclear stain Hoechst (blue fluorescence). Exposure to carbon black, carbon nanohorns, shortened nanotubes or flexible MWCNTs does not lead to the release of cathepsin B as demonstrated by punctate red fluorescent cytoplasmic vesicles.
Fig. S27. Visualization of apoptotic cells induced by exposure of hepatocytes to carbon nanomaterials. (a) Unexposed cells, (b) carbon black, (c) MWCNT-1, (d) 0.5 μm MWCNT-7, (e) 1 μm MWCNT-7, (f) MWCNT-7, (g) carbon nanohorns, (h) MWCNT-flex1 and (i) MWCNT-flex2 at 20 ppm. Cells exposed to 0.5 μm MWCNT-7 (d), MWCNT-flex1 (h) and (i) or carbon nanohorns (g) show no caspase activation as indicated by weak red cytoplasmic fluorescence compared to cells exposed to 1 μm MWCNT-7 (e), MWCNT-1 (c) and MWCNT-7 (f) showing strong red cytoplasmic fluorescence indicating caspase activation.

Fig. S28. Cellular interactions with other carbon nanoforms of diverse geometry. (a) Caspase and TUNEL assays to visualize caspase activation (red fluorescence) and induction of apoptosis (green fluorescence) in hepatocytes exposed to 20 μg/ml MWCNT-7 for 24 h. The size bar in (a) is 20 μm. (b) Determination of caspase activation using fluorescence substrate after exposure of cells to MWCNT-7 alone or in combination with cathepsin B inhibitor methyl ester CA 074. Cells after co-exposure showed a significantly decreased number of caspase positive cells compared to cells exposed to MWCNT-7 alone (* P < 0.05). Cathepsin B inhibition prevents cell death induced by exposure to MWCNT-7. (c) After exposure of hepatocytes to MWCNT-7 or MWCNT-7 and the cathepsin B inhibitor, methyl ester CA-074 for 24 h, cell viability was quantified using the WST-8 assay. Cells co-exposed to the cathepsin B inhibitor and MWCNT-7 showed significantly increased viability (* P < 0.05) compared to cells exposed to MWCNT-7 alone.
Fig. S29. Colocalization of internalized carbon nanoparticles with endosomal and lysosomal markers in hepatocytes. (a) Visualization of carbon nanoparticles in endosomes using Rab5 as marker. After exposure to carbon nanomaterials endosomes were visualized with Rab5 antibody and FITC-labeled secondary antibody (green fluorescence) and DAPI as a nuclear stain (blue fluorescence). Carbon black particles co-localizes with endosomes, whereas MWCNT-7 only partially co-localizes with the endosomes (yellow arrows). (b) After exposure to carbon nanomaterials lysosomes were visualized using LAMP-1 antibody and TRITC-labeled secondary antibody (red fluorescence) and DAPI as a nuclear stain (blue fluorescence). Partial co-localization of MWCNT-1 and MWCNT-7 with lysosomes can be visualized, whereas carbon black particles co-localize with lysosomes. (c) Visualization of carbon nanomaterials in lysosomes using acridine orange. After exposure of cells to carbon nanomaterials lysosomes were visualized using acridine orange (red fluorescence) and Hoechst (blue fluorescence) as a nuclear stain. Carbon black particles can be visualized within lysosomes, whereas MWCNT-7 only partially co-localizes within lysosomes. (d) Visualization of carbon black particles and MWCNT-7 in lysosomes using cathepsin B substrate (red fluorescence); after exposure of cells to carbon black particles and MWCNT-7, lysosomes were visualized using cathepsin B substrate and Hoechst (blue fluorescence) as a nuclear stain. Cathepsin B substrate in lysosomes co-localizes with carbon black, whereas MWCNT-7 only partially co-localizes with cathepsin B. (e-h) Confocal fluorescence imaging of carbon nanotube uptake and lysosomal permeabilization. Hepatocytes exposed to 20 µg/ml MWCNT-7 for 8 h. Lysosomal integrity was assessed using cathepsin B substrate (red) and nuclear stain Hoechst (blue fluorescence). The corresponding images (e and g) (brightfield) to (f and h) (confocal fluorescence) illustrate the localization of the carbon nanotubes and piercing through the cell membrane. Incomplete uptake of carbon nanotubes (yellow arrows) after 8 h does not lead to cathepsin B release as demonstrated by punctate red fluorescent cytoplasm in the vicinity of carbon nanotube uptake.
Fig. S30. Transmission electron micrographs of LAMP-1 immunogold labeled lysosomes of hepatocytes. Carbon black particles are localized within lysosomes, whereas MWCNT-7 penetrates through the lysosomes into the cytoplasm (red arrows indicate carbon nanomaterials; yellow arrows indicate LAMP1 immunogold particles).

Fig. S31. Image analysis for semi-quantitative assessment of lysosomal permeabilization. Panel c containing mean values of fluorescent feature sizes after exposure to stiff MWCNTs, flexible MWCNTs, and carbon black isometric particle reference material. (a and b) Computer generated images following cathepsin B staining and histograms of the sizes of cathepsin B-rich regions for: untreated control (a), and MWCNT 7-treated cells (b). Use of high fluorescence threshold identifies primarily intact lysosomes, while the low fluorescence threshold also captures low-intensity diffuse reflectance from lysosomal leakage. The image insets show line profiles that illustrate the sharp peaks marking intact lysosomes with black background (a) in contrast to the peaks with inter-peak shoulders (b) representing diffuse reflectance in the cytoplasm. (c) Mean diameter and standard deviation of fluorescent features for various samples showing the significant increase in feature size at low threshold due to lysosomal leakage following exposure to long stiff MWCNT-7. The asterisk denotes statistically significant differences ($P < 0.005$) between mean feature sizes for the long, stiff MWCNT-7 sample relative to all other samples at the same threshold value: untreated controls, carbon black and the MWCNT-flex2 samples. The image analysis shown here reveals a statistically significant increase in the lysosome size (high threshold data) upon exposure to long, stiff MWCNT-7 samples relative to untreated and carbon black-exposed cells, and a larger increase in the mean size of all fluorescent features due to the appearance of diffuse fluorescence associated with lysosomal leakage.