Graphene microsheets enter cells through spontaneous membrane penetration at edge asperities and corner sites

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Understanding and controlling the interaction of graphene-based materials with cell membranes is key to the development of graphene-enabled biomedical technologies and to the management of graphene health and safety issues. Very little is known about the fundamental behavior of cell membranes exposed to ultrathin 2D synthetic materials. Here we investigate the interactions of graphene and few-layer graphene (FLG) microsheets with three cell types and with model lipid bilayers by combining coarse-grained molecular dynamics (MD), all-atom MD, analytical modeling, confocal fluorescence imaging, and electron microscopic imaging. The imaging experiments show edge-first uptake and complete internalization for a range of FLG samples of 0.5- to 10-μm lateral dimension. In contrast, the simulations show large energy barriers relative to $k_BT$ for membrane penetration by model graphene or FLG microsheets of similar size. More detailed simulations resolve this paradox by showing that entry is initiated at corners or asperities that are abundant along the irregular edges of fabricated graphene materials. Local piercing by these sharp protrusions initiates membrane propagation along the extended graphene edge and thus avoids the high energy barrier calculated in simple idealized MD simulations. We propose that this mechanism allows cellular uptake of even large multilayer sheets of micrometer-scale lateral dimension, which is consistent with our multimodal bioimaging results for primary human keratinocytes, human lung epithelial cells, and murine macrophages.

molecular dynamics simulation | graphene-cell interaction | lipid membrane | edge cutting | corner penetration

Graphene is a 2D plate-like material consisting of a single layer of hexagonally arranged carbon atoms with extraordinary electrical (1), mechanical (2), and thermal properties (3). Bilayer, trilayer (4), few-layer graphene, and multilayer structures with thickness <100 nm are closely related nanomaterials that are often manufactured by thermal exfoliation of graphite and often exist as dry powders during processing (5), which increases the probability of workplace exposure. For the purposes of this article, we refer to this set of structures as “graphene familia nanomaterials” (GFNs) (6). Many GFNs have aerodynamic diameters less than 5 μm and thus fall into the category of potentially respirable materials. Even GFNs with large lateral dimensions (>20 μm) typically fall in the potentially respirable range, and for these the large lateral dimension presents challenges for macrophage-mediated clearance, raising the possibility of long residence times in the lung (7). Workplace measurements and laboratory simulations have documented the potential for human inhalation exposure to engineered nanomaterials during several steps in manufacturing processes (8). In addition to inhalation of dry powders, carbon-based nanomaterials can become airborne during sonication of particles in suspension (9) and during cutting or drilling of composites. In addition to occupational exposures, GFNs may be deliberately implanted or injected for biomedical applications that include biosensors (10), tissue scaffolds (11, 12), carriers for drug delivery (13, 14) and gene therapy (15), antibacterial agents (16), and bioimaging probes (17, 18). The large specific surface area of graphene allows high-density biofunctionalization or drug loading (19, 20) and more efficient tumor targeting capability (13), and graphene may also offer lower toxicity and better manufacturing reproducibility than some other material platforms (18, 21).

The lateral dimensions of GFNs span orders of magnitude, from 10 nm to >100 μm (larger than most target cells), and the maximum dimensions of a nanomaterial are important for cell uptake, renal clearance, blood–brain barrier transport, and many other biological phenomena. Graphene-based sheets of small lateral dimension (<100 nm) are being developed for drug delivery and diagnostic applications. These “nanosheets” have been observed to enter cells (6, 13–15, 17, 18) and their membrane translocation has been studied experimentally (22) and through simulation (23, 24). Titov and coworkers conducted coarse-grained molecular dynamics simulations to study the interaction of small graphene and few-layer graphene (FLG) nanosheets with a lipid bilayer and reported stable graphene–lipid hybrid structures (23). Guo et al. studied the translocation of small graphene nanosheets across lipid bilayers and their effects on membrane deformation (24). Much less is known about the fundamental cellular interactions of graphene materials with micrometer-scale lateral dimension (graphene microsheets) that are a main thrust in current graphene materials development. The urgency to develop guidelines for manufacturing safer biomedical diagnostics and therapies (6, 18), as well as regulating occupational and environmental exposure (25, 26), justifies significant efforts to clarify the mechanisms of cell membrane interaction with carbon nanomaterials and with graphene microsheets in particular.

Previous studies of carbon nanotubes entering the cell membrane have suggested that there exists a critical structural dimension on the order of the bilayer thickness (around 4 nm), below which direct penetration into the bilayer becomes possible and above which receptor-mediated endocytosis is needed for the uptake (27–29). Graphene is atomically thin in one dimension, but typically large in two other dimensions, and our preliminary modeling work showed large energy barriers for membrane penetration even when monolayer sheets encounter the lipid bilayer edge-first. The entry barriers were found to...
be even larger for multilayer graphene materials, which are of particular interest in our study due to large-scale manufacturing by exfoliation. It was unclear at the preliminary stage which types of graphene materials (what range of layer numbers and lateral dimensions) would be capable of cell entry and by what biophysical mechanism. We therefore conducted a coupled experimental and computational study of graphene–membrane interactions, using molecular dynamics and in vitro cell imaging with emphasis on the geometry and dynamics of cell penetration and uptake. The simulations use similar coarse-graining methods to those in our previous study for the tip entry of carbon nanotubes and related 1D materials (30), but require higher spatial resolution due to the atomic thickness of graphene. We investigate the atomic-scale mechanisms of cell interaction with few-layer graphene microsheets via coarse-grained molecular dynamics (CGMD) and all-atom steered molecular dynamics (SMD) simulations. The CGMD simulations illustrate the dynamic process of graphene–bilayer interaction, whereas the SMD simulations allow us to determine the energy barriers associated with initial graphene penetration. SMD simulations predict that idealized graphene sheets with smooth edges will not penetrate lipid bilayers at room temperature due to high energy barriers, even in cases where the initial encounter is strictly edge-on. Examination of actual fabricated graphene microsheets reveals highly irregular edge topographies, and we therefore develop a separate theoretical treatment of graphene–bilayer interaction initiated at sharp corners or protrusions. This latter model predicts that cell uptake can be initiated by a spontaneous localized piercing of the membrane at corners or asperities followed by spontaneous propagation along the graphene edge to achieve full penetration. These new model predictions are validated by confocal fluorescence bioimaging and electron microscopy, which show edge-first or corner-first membrane penetration and complete cellular internalization for a range of few-layer graphene microsheets and three cell types.

Results and Discussion

Coarse-Grained Molecular Dynamics. Fig. 1 shows the CGMD results for graphene and few-layer graphene interacting with lipid bilayers. In the first simulation set, a small, rhombic, monolayer graphene flake with edge length of 6.4 nm is placed initially at a distance about 4 nm above and parallel to a square patch of lipid bilayer with 992 lipid molecules and 67,817 water molecules in a cubic box with edge dimension 24 nm (Fig. 1A). Periodic boundary conditions are imposed in all three dimensions. Under thermal fluctuations, the graphene flake undergoes Brownian motion, including rapid vibration, rotation, and migration in the vicinity of the bilayer. Spontaneous piercing into the bilayer is observed to begin as soon as the flake finds a configuration with one of its sharpest corners oriented nearly orthogonally to the membrane (Fig. 1B and C). Piercing is facilitated by the attractive interactions between graphene and the tail groups of lipids, but occurs only after the tip of the penetrating corner touches the hydrophobic core of the bilayer. In our CG simulations, the lipid membrane can be fully penetrated through by the graphene flake, accompanied by the tilt of the graphene flake to maximize its coverage with the membrane interior. The small graphene flake in our simulation eventually ends up embedded in the bilayer due to its small dimensions (Movie S1). The simulation of a rhombic graphene flake with two different corner angles (30° and 60°) demonstrates that orthogonal piercing of the sharpest corner of graphene has the lowest energy barrier and is the most preferred entry pathway. The edge planes of graphene have a complex chemistry and are typically decorated with hydrophilic oxygen functional groups, whereas in cell culture medium graphene may exhibit adsorbed proteins that can also reduce apparent hydrophobicity. The set of GFN samples used in our bioimaging studies has C/O ratios ranging from 10 to 32 by X-ray photoelectron spectroscopy (31) with the oxygen atoms presumed to be on edge and defect sites. Also, the amount of adsorbed protein was measured to contribute 3–46% of the GFN surface area, depending on conditions and sample (SI Text). To investigate these effects, further CGMD simulations of bilayer interaction with graphene flakes of different shapes and surface chemistry (different corner/edge functionalizations and hydrophilic/hydrophobic properties) confirm that small graphene sheets tend to penetrate into the cell via spontaneous piercing at their sharpest hydrophobic corner. A series of calculations with different interaction parameters between graphene and lipid molecules are also performed to determine that the corner-first entry mode is robust over a range of dissipative particle dynamics (DPD) parameters (SI Text). We will show shortly that the nearly orthogonal orientation of a sharp graphene corner with respect to the bilayer minimizes their interactive free energy and is the thermodynamically preferred configuration even before penetration begins.

We also performed CGMD simulations of large few-layer graphene sheets interacting with lipid bilayers (Fig. 1 F–H). We began by studying an ideal, atomically smooth, infinite graphene edge interacting with lipid bilayers, but did not observe penetration (SI Text, Fig. S6A, and Discussion). We realized that the edges of experimentally fabricated graphene exhibit atomic-scale roughness (Fig. 1E) as seen by atomically resolved scanning tunneling microscopy (32–34), and most few-layer graphene sheets also show very rough edges (see Fig. 4), as well as terraced or beveled edge structures that become successively thinner toward one of the two faces (35, 36). Fig. 1F shows a model terraced edge structure created on a five-layer FLG flake interacting with a bilayer. The simulation system consists of a patch of bilayer with 2,016 lipid molecules and 133,052 water molecules in a cubic box with dimensions of 24 nm × 48 nm × 24 nm. The plate-like FLG, which has a ragged edge topography mimicking those observed in experiments, is composed of five atomic layers with an equilibrium interlayer distance of 0.34 nm and placed initially at a distance about 3 nm above and orthogonal to the bilayer. Each graphene layer is assigned a different color. The first two and last two layers are set to be symmetrical with respect to the midlayer. To mimic part of a much larger structure for which Brownian motion is limited, the top edge of the FLG is clamped and periodic boundary conditions are imposed in all three dimensions of the simulation box. The lipid bilayer undergoes Brownian motion in the vicinity of the large graphene edge for 2.19 µs under the confinement of a harmonic potential. The latter is then removed and the bilayer membrane is set free to interact with the ragged graphene edge.
It is seen that the FLG penetrates the bilayer despite its size, starting with localized piercing at sharp protrusions along the edge. The penetrated portion of the membrane then propagates along the whole edge, resulting in full penetration (Movie S2). In this process, the energy barrier to penetration is overcome by local piercing at sharp corners along the nominally flat edge, and the full penetration is driven by the attractive interaction between the graphene and the tail groups of lipids once initial piercing is successful. We have tested the robustness of this entry mode by carrying out further CGMD simulations on monolayer or few-layer graphene flakes with an isolated protrusion or a terrace or by initiating contact near a corner or a locally folded edge (SI Text and Figs. S2–S7). The simulation results all show similar pathways that are initiated by localized piercing at an atomically thin graphene feature, which requires only thermal energy to overcome the small barrier, followed by spreading and complete penetration driven by hydrophobic forces between the graphene and the bilayer core. We believe this entry mechanism may be generic for cell uptake of all 2D hydrophobic nanomaterials with atomic-scale thickness. The importance of localized corner piercing for entry initiation can also be demonstrated by carrying out simulations on ideal, atomically smooth, infinite graphene edges without the irregular features observed in real samples. In this case, our simulations show that the lipid barrier is expelled away from the graphene edge due to a combination of a strong energy barrier and entropic interactions between the bilayer and an atomically smooth graphene edge (SI Text and Fig. S6A). This last simulation was done to further demonstrate the importance of local piercing as the initiating event, but does not correspond to any known biological exposure scenario, because it would require cell contact with a uniform, atomically smooth, horizontally aligned, long-length graphene edge structure that is difficult to achieve in practice.

Our simulations demonstrate local orthogonal piercing at a sharp corner or asperity that initiates graphene penetration of lipid bilayers. To better understand this behavior, we use the thermodynamic integration (TI) technique (37) to calculate the free energy of the system as a function of two orientation angles (θ, φ) of a rhombic graphene flake when one of the sharp corners of the flake is fixed at a distance of 0.4 nm above the bilayer (the detailed implementation of the TI method is given in SI Text). Here θ is the angle between the long diagonal axis of the flake and the bilayer within the graphene plane and φ is the angle between the vectors normal to the graphene plane and the membrane plane (Fig. 1A). The free energy associated with orthogonal corner piercing (90°, 90°) is set to 0 as a reference value. Not surprisingly, the orientation (30°, 0°), which corresponds to the graphene flake parallel to the bilayer plane, shows the highest free energy because it induces the most severe confinement of thermal motion in this configuration. We further normalized the calculated free energy by its peak value at the parallel configuration (30°, 0°). The plotted normalized free energy in Fig. 1A demonstrates that the orthogonal orientation (90°, 90°) exhibits the lowest free energy due to its weakest confinement on the thermal motions of both membrane and graphene (thereby maximizing the entropy of the system).

**All-Atom Simulations.** Our CGMD simulations suggest that localized corner piercing plays a critical role during the initial stage of cell uptake of graphene. To determine the energy evolution associated with such initial piercing events, further simulations are conducted on an all-atom model of corner piercing of a monolayer graphene flake across a bilayer patch of 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC) lipid in a box of water molecules. The all-atom simulations are of two types. Type I simulations are designed to test whether there exists a positive driving force for piercing in an all-atom MD simulation. As shown in Fig. 2A, a triangular graphene flake initially placed in a corner-piercing configuration across the bilayer was observed to spontaneously move downward, penetrating further into the bilayer (Movie S3). In type II simulations, the energy barrier associated with corner piercing is calculated by SMD simulations (38), in which a graphene corner is pulled across the bilayer by a virtual spring (see SI Text for more details). Fig. 2B shows the graphene–bilayer interaction energy calculated from the SMD simulations as a function of the penetration distance. The SMD calculations confirm that the energy barrier is small, only ～Sk_BT, for the graphene corner to pierce through the top hydrophilic head region of the bilayer. Shortly after this point, the total interaction energy starts to decrease due to favorable interactions between the lipid tails in the core of the bilayer and an ever-increasing area of immersed graphene. Thus, both our CGMD and all-atom simulations reveal that corner piercing involves a small energy barrier comparable to thermal energy and is essentially a spontaneous process.

Both CGMD and all-atom simulations indicate that the lipid bilayer structure remains essentially intact upon graphene insertion except that the hydrophobic tails of lipids are somewhat straightened due to strong adhesion onto the side surfaces of graphene. In a continuum description, the energy change accompanying piercing can be expressed in terms of four variables: h_H and h_T (thicknesses of the head and tail groups in the lipid monolayer, as shown in Fig. 2C) and γ_H and γ_T (interaction energy densities between one side surface of graphene and head and tail groups of lipids relative to that between solvent and graphene). For a graphene corner with an internal angle of 2θ, the rate of energy change during piercing can then be written as a piecewise function of the penetration depth h as (SI Text)

\[
\frac{dE}{dh} = \begin{cases} 
4h_0\gamma_H \tan \alpha & 0 < h \leq h_H \\
4(h - h_H)\gamma_H \tan \alpha & h_H < h \leq h_H + 2h_T \\
4(h - 2h_T)\gamma_H + 2h_T\gamma_T \tan \alpha & h_H + 2h_T < h \leq 2h_H + 2h_T \\
8(\gamma_H h_H + \gamma_T h_T)\tan \alpha & 2h_H + 2h_T < h.
\end{cases}
\]

Our all-atom simulation shown in Fig. 2A indicates that there is a positive driving force for the last regime of piercing, i.e., \( \gamma_H h_H + \gamma_T h_T < 0 \). Under this condition, it can be seen that the energy increases in the first regime, 0 < h < h_H, but decreases in...
both regimes \( h_{B} + 2 h < h \leq 2 h_{H} + 2 h_{T} \) and \( 2 h_{H} + 2 h < \). The energy peak occurs when the graphene tip lies in the hydrophobic core, \( h_{B} < h \leq h_{H} + 2 h_{T} \), at a point defined by \( \Delta E/dh = 0 \). This gives the energy barrier for piercing as \( E_{\text{barrier}} = 2(1 - \tan\gamma_H) h_{H}T_{H} \tan \alpha \) at a critical penetration depth of \( h_{cr} = (1 - \tan\gamma_H) h_{H} \). The surface interaction energy densities \( \gamma_H \) and \( \gamma_{H} \) may be estimated as \( \gamma_H = 7k_{B}T \text{ nm}^{-2} \). \( \gamma_{H} = 7k_{B}T \text{ nm}^{-2} \) if they are considered to be of the same order of magnitude as those between the hydrocarbon tail groups and water (39). Taking \( h_{H} = 0.5 \text{ nm} \) and \( \alpha = 45^\circ \) shows that the energy barrier for corner piercing is \( E_{\text{barrier}} = 2(1 - \tan\gamma_H) h_{H}T_{H} \tan \alpha \approx 7k_{B}T \) at a critical penetration depth of \( h_{cr} = (1 - \tan\gamma_H) h_{H} \approx 1.0 \text{ nm} \). These numbers are in excellent agreement with the direct SMD simulation results shown in Fig. 2B.

It may be tempting to make an analogy between graphene corner piercing into a lipid bilayer described here and mechanical piercing by stress concentration at sharp corners. However, we emphasize that the former is an essentially spontaneous process, whereas the latter is driven by an applied force.

Bioimaging Experiments. Figs. 3 and 4 present confocal fluorescent and ex situ electron micrographs that confirm the MD predictions of edge/corner-first penetration and cell entry of few-layer graphene microsheets. Lung epithelial cells and keratinocytes are representative of the epithelial lining of the respiratory tract and the skin, respectively (40), and form flat, single-cell monolayers in vitro (Fig. 3). Polarized epithelial cells have a well-organized microtubular cytoskeletal network whereas macrophages have a subcortical distribution of actin filaments that can be visualized using indirect immunofluorescence confocal microscopy (Fig. 3 A–C, Insets). Plate-like graphene microsheets are internalized by human lung epithelial cells (Fig. 3 A and B) and macrophages (Fig. 3C) and can be visualized within the cytoplasm using confocal imaging. It is interesting that the graphene basal planes show preferential orientation parallel to the basolateral cell surface attached to the substrate. Plate-like graphene microsheets physically disrupt the cytoskeletal organization of both lung epithelial cells (Fig. 3 A and B) and macrophages (Fig. 3C). In thin sections using transmission electron microscopy (TEM), some graphene microsheets can be visualized within cytoplasmic vacuoles inside macrophages (Fig. 3D) and lung epithelial cells (Fig. 3E). The overall structure and integrity of subcellular melanosomes preserved as revealed by TEM (Fig. 3 D and E), confirming our in vitro assays that preserved cell viability (SI Text and Fig. S8).

The imaging protocols used in Fig. 3 are useful to show graphene internalization and orientation, but do not reveal the entry mode. We therefore carried out shorter time exposures to capture the uptake process, using ex situ field-emission SEM of target cells with the outer membrane enhanced by osmium tetroxide postfixation. This imaging was carried out both with and without critical point drying to check for drying artifacts. Fig. 4 shows high-resolution cross-sections of cells exposed to graphene after 5 or 24 h. Edge-first or corner-first penetration of the membrane is seen in all cases and for all three target cell types. Fig. 4 C and D shows particularly clear cases of membrane penetration that appears to have initiated at an asperity or protrusion on the graphene edge (Fig. 4C) or initiated at a graphene corner (Fig. 4D). Note that the edges of these sheets are observed to be highly irregular (Fig. 4A, C, and D) and provide numerous sites for initial penetration as described in the modeling (SI Text and Fig. S9). Internalization of these plate-like graphene microsheets ranging in lateral dimension from 0.5 nm up to 500 nm (Table S1) did not compromise cell viability at the doses and time points used in these in vitro studies. More generally, however, inhaled nanoparticles can be associated with adverse health effects (41) and Schinwald et al. (7) reported that graphene nanoplatelets induced granuloma formation and lung inflammation following pharyngeal aspiration in mice. Intracellular uptake and cytoplasmic localization of plate-like graphene nanomaterials may interfere with cytoskeleton organization (Fig. 3 A–C) and normal physiological functions including protein secretion (40), barrier function (42), and cell migration during differentiation and repair (43). Internalization of nanoparticles by macrophages has been shown to disrupt phagocytosis and clearance of particles and microbes from the lungs (44, 45). Schinwald et al. (7) provide evidence that graphene nanoplatelets are not readily cleared from the lungs and induce release of proinflammatory mediators from macrophages. Other studies report that graphene nanomaterials are biocompatible (46, 47), and much more work is needed before graphene material health risks can be fully assessed.

In summary, coarse-grained molecular dynamics, all-atom steered MD, analytical modeling, and live-cell and ex situ bioimaging were used to investigate the fundamental mechanisms of graphene interactions with lipid bilayers. The simulations reveal direct bilayer penetration that begins with localized piercing at sharp corners or at protrusions along graphene edges followed by propagation along the edge to achieve full penetration. For a small graphene flake, Brownian motion and entropic driving forces in the near-membrane region first position the flake orthogonally to the bilayer plane, which then leads to spontaneous corner piercing. All-atom steered molecular dynamics simulations track the free energy evolution during corner piercing and reveal only a small energy barrier, comparable to \( k_{B}T \). Interestingly, in the absence of sharp corners or edge protrusions, the cell membrane has a high intrinsic energy barrier against penetration by long graphene edge segments even though they are atomically thin. Such uniform, atomically smooth, horizontally

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Fig. 3. Cellular uptake and internalization of few-layer graphene microsheets. (A–C) Confocal images of human lung epithelial cells (A and B) and mouse macrophages (C) exposed to graphene microsheets (0.5- to 25- μm lateral dimension) after 24 h and 5 h, respectively. The nuclei in A and B are visualized (blue fluorescence) with 4′,6-diamidino-2-phenylindole (DAPI). The microtubules of the lung epithelial cells (A and B) are visualized using antitubulin beta antibodies conjugated with FITC (green fluorescence), whereas the actin cytoskeleton of macrophages shown in C is visualized using rhodamine-phalloidin (red fluorescence). In unexposed lung epithelial cells (A and B, Inset), cytoplasmic microtubules (MT) form a linear network spanning across the cytoplasm. Internalized graphene flakes (yellow arrows, A and B) physically displace the linear microtubular network. In unexposed macrophages (C, Inset), filamentous actin (F) is organized into aggregates beneath the plasma membrane. Internalized graphene flakes with large lateral dimension (yellow arrow, C) induce dense aggregates of actin filaments whereas submicron graphene sheets (yellow arrowhead, C) do not disrupt the actin cytoskeleton. Transmission electron micrographs of macrophages (D) and lung epithelial cells (E) exposed to 10 ppm FLG sheets (~800 nm in lateral dimension) for 5 h and 24 h show localization in the cytoplasm within membrane-bound vacuoles (blue Insets). Graphene microsheets inside vacuoles appear as electron-dense linear sections (D, Inset) or irregular flakes (E, Inset).
aligned, long-length graphene edges are rare, however, so in practice cell penetration is spontaneous due to the presence of atomic- or nano-scale edge roughness that essentially eliminates the energy barrier. Experimental imaging studies confirm graphene penetration of cell membranes in a dominant edge-first or corner-first mode for each of three cell types studied: lung epithelial cells, keratinocytes, and macrophages. The experiments also show penetration and successful uptake of GFN flakes as large as 5–10 μm in lateral dimension, which supports the model prediction that penetration activation barriers are not intrinsically length dependent, because of initiation at local sharp features. Once the initial energy barrier for spontaneous membrane penetration has been overcome, we hypothesize that interaction between the hydrophobic basal surfaces of graphene microsheets with the inner hydrophobic region of the plasma membrane promotes cellular uptake. Hydrophobic surfaces are considered to represent damage-associated molecular patterns (DAMPs) that nonspecifically activate the innate immune response (48). Hydrophobic cellular surfaces (49) and surface functionalized nanoparticles (50) are more readily internalized and initiate more potent innate immune responses than weakly charged, hydrophilic surfaces. By this mechanism, we hypothesize that graphene microsheets that penetrate into hydrophobic lipid domains may be recognized as DAMPs by target cells that are the primary human keratinocytes, the graphene stock solution was diluted to 10^−3 g/mL graphene in 1-mg/mL ethanolic stock solution with a range of lateral dimensions (0.5–10 μm) and layer numbers (4–25). Scale bars, 2 μm.)

Materials and Methods

Coarse-Grained MD Simulations. The coarse-grained simulations in this paper were based on DPD, which is a Lagrangian method derived from coarse graining of molecular dynamics (51) widely used as a mesoscopic simulation method for biomembrane systems (52–55). The lipid bilayer membrane is represented by the H2(T12)2 coarse-grained model (SI Text and Fig. S1) (51). The hydrophilic lipid heads and hydrophobic lipid tails are shown as red and yellow beads, respectively. A unit cell of CG graphene consists of three beads with the nearest-neighbor distance of 0.4 nm and internal angle of 60°. The interaction among the beads is chosen to keep elastic properties of CG graphene consistent with experimental results (2, 56). The interaction parameters between graphene and lipid molecules are calibrated against parameters from all-atom MD simulations and also varied in a range to test the robustness of key observations. The simulations are performed in the number-volume-temperature (NVT) ensembles with the time step taken as Δt = 0.003. and carried out by using the software package LAMMPS (57). More details of the method are given in SI Text.

All-Atom MD Simulations. The all-atom MD simulations were performed in NAMO (58) and visualized in VMD (59). The graphene and the POPC lipid bilayer membrane were generated using VMD Graphene Builder and Membrane Builder, respectively. The graphene and lipid bilayer system was then fully hydrated (adding water molecules), using VMD. The CHARMM36 force field (60) with added parameters for the graphene and TIP3 water molecules was adopted in this study. The system was equilibrated for 500 ns and production runs were performed as the third step. Because the lipid membranes generated by the membrane plug-in of VMD were far from the equilibrium state, an equilibrium run of 0.5 ns was carried out in which everything except lipid tails was fixed. This first step allowed the lipid tails to melt into a fluid-like configuration. In the second step, the systems were equilibrated for about 2 ns in the number-pressure-temperature (NPT) ensemble at temperature of 310 K and pressure of 1 atm. Next, 500-ns production runs were performed as the third step. In all three steps of simulation, the out-of-plane degrees of freedom of the carbon atoms of the graphene flakes were restricted using harmonic constraints. Further details of the all-atom simulations are given in SI Text.

Bioimaging Experiments. Experiments were conducted on three cell types: murine macrophages, lung epithelial cells, and primary human keratinocytes. The cells were exposed to commercial few-layer graphene microsheet samples with a range of lateral dimensions (0.5–10 μm) and layer numbers (4–25). The FLG samples were dispersed using 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (Avanti Polar lipids)/albumin before addition into cell culture medium (Fig. S10). One hundred microliters of graphene flakes in a 5-mg/mL ethanolic stock solution were added to 62 μL of a 40-mg/mL DPPC/ethanol solution and vortexed for 5 s and followed by addition of 838 μL PBS containing 2% (vol/vol) albumin to produce a 500 μg/mL (ppm) grapheneflakes in PBS stock solution containing 2.5 mg DPPC and 2% albumin/PBS. The amount of adsorbed protein that results from application of this protocol was measured in separate control experiments and found to comprise 3–46% of the GFP surface area, depending on conditions and sample (SI Text). For keratinocytes, the graphene stock solution was diluted to 10 μg/mL graphene in 1-mg/mL ethanolic stock solution with a range of lateral dimensions (0.5–10 μm) and layer numbers (4–25). Scale bars, 2 μm.)

Confocal fluorescence microscopy. Lung epithelial cells were exposed to graphene flakes for 24 h. The cells were washed and stained with antitubulin antibody conjugated with fluorescein isothiocyanate (FITC) (Cell Signaling; 36239) and 4,6-diamidino-2-phenylindole (DAPI). Macrophages were exposed to graphene for 3 h as described above. The cells were washed and stained with rhodamine-conjugated phalloidin and DAPI. Images were visualized using a spinning-disk Olympus confocal fluorescence microscope (Model IX81) motorized inverted research microscope to assess uptake and cytoskeletal organization.

Electron microscopy. For TEM sample preparation two different methods were used to verify that graphene flakes are taken up by the cells. Cells were fixed in Karnovsky’s fixative (5% K), postfixed in 1% osmium tetroxide and dehydrated through graded acetone series. The samples were then infiltrated with araldite resin. Ultrathin sections were stained with uranyl acetate and lead citrate. The sections were observed on a transmission electron microscope.


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tioning cells grown in monolayer on glass coverslips. For scanning electron microscopy, cells were postfixed in 2% (vol/vol) aqueous osmium tetroxide for 30 min followed by dehydration in 25% (vol/vol), 50% (vol/vol), 70% (vol/vol), 2 x 95% (vol/vol), and 3 x 100% (vol/vol) ethanol. After critical point drying according to the manufacturer’s directions (Ladd Research), the coverslips were sputter coated with gold and viewed using field-emission SEM (LEO 1530-VP). More details of the method are given in SI Text.

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Supporting Information

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SI Text

Coarse-Grained Molecular Dynamics Simulations. In our coarse-grained molecular dynamics (CGMD) simulations, a cluster of atoms is represented as a single bead located at the center of mass. The simulations are based on the method of dissipative particle dynamics (DPD) (1, 2). Beads i and j interact with each other via a pairwise additive force consisting of three contributions: (i) a conservative force, \( F_{ij}^{c} \); (ii) a dissipative force, \( F_{ij}^{d} \); and (iii) a random force, \( F_{ij}^{r} \). The total force on bead \( i \) is given by

\[
F_i = \sum_{j \neq i} (F_{ij}^{c} + F_{ij}^{d} + F_{ij}^{r}),
\]

where the sum acts over all particles within a cutoff radius \( r_c \). Specifically, in our simulations,

\[
F_i = \sum_{j \neq i} a_{ij} \omega(r_{ij}) \hat{r}_{ij} - \gamma \omega^2(r_{ij}) \dot{r}_{ij} + \sigma \omega(r_{ij}) \xi \gamma \Delta t^{-1/2} \dot{r}_{ij},
\]

where \( a_{ij} \) is the maximum repulsive force, \( r_{ij} \) the distance, \( \hat{r}_{ij} \) the unit vector, and \( v_{ij} \) the relative velocity between beads \( i \) and \( j \). \( \xi \) denotes a random number with zero mean and unit variance, and \( \omega(r_{ij}) \) is a normalized distribution function given by

\[
\omega(r_{ij}) = \begin{cases} 1 - r_{ij}/r_c & r_{ij} < r_c, \\ 0 & r_{ij} \geq r_c; \end{cases}
\]

\( \gamma \) and \( \sigma \) are parameters related to each other as \( \sigma^2 = 2k_B T \), where \( k_B \) is Boltzmann’s constant and \( T \) is the temperature.

The mass, length, and timescales are all normalized in the DPD simulations. Here, the unit of length is taken to be the cutoff radius \( r_c \), the unit of mass to be that of the solvent beads, and the unit of energy to be \( k_B T \). All other quantities are expressed in terms of these basic units. The reduced DPD units can be converted to SI units by examining the membrane thickness and the lipid diffusion coefficient.

The displacement of bilayer thickness is 3.5 \( r_c \) and the effective timescale of the simulation can be determined from the simulated lateral diffusion constants of lipid bilayer (3). By comparison with typical experimental values that the distance, \( 1 \sim 2 \) \( k_B T \) nm and \( 1 \sim 2 \) \( k_B T \) ns, it can be shown that one DPD length unit corresponds to \( \sim 0.8 \) nm in physical units and the time unit to \( \tau \sim 24 \) ps. In terms of the normalized units, the standard parameter values of \( \sigma = 3.0 \) and \( \gamma = 4.5 \) are used in our study.

The simulation box for the uptake of single-layer graphene flake is a cube of size 30 \( \times \) 30 \( \times \) 30 subject to periodic boundary conditions, with 289 graphene beads, 12,896 lipid beads, and 67,817 water beads at a particle density of approximately 3 \( \times \) 10\(^{-6}\) (5). The system for large few-layer graphene sheets contains a total of 163,185 particles, with 26,208 of them representing lipids. The lipid bilayer membrane is represented by the \( H_2S(T_2) \) coarse-grained model proposed by Groot and Rabone (2), as shown in Fig. S1. The hydrophilic lipid heads and hydrophobic lipid tails are shown as red and yellow beads, respectively. A harmonic spring force

\[
F_{ij}^{s} = k_s (1 - r_{ij}/r_c) \hat{r}_{ij}
\]

is applied on all neighboring beads in the lipid chain, with the spring constant set as \( k_s = 100.0 \) and equilibrium bond length set as \( r_c = 0.70 \) \( \text{nm} \) (6). The bending resistance of lipid molecules is considered by applying a harmonic constraint on the adjacent three beads,

\[
F^b_{ij} = -\nabla V_{\text{bend}}.
\]

\[
V_{\text{bend}} = k_B(\theta - \theta_0)^2/2,
\]

where \( k_B, \theta \), and \( \theta_0 \) are the bending constant, the inclination angle, and its equilibrium value between two consecutive bonds, respectively. Following Groot and Rabone (2), the parameters for the bending constant and the equilibrium angle among three consecutive lipid tail beads or three consecutive lipid head beads in a lipid molecule are \( k_B = 6 \) and \( \theta = 180^\circ \); for the head bead connected to the lipid tails and the first beads in the tails (beads 3, 4, and 9 in Fig. S1), \( k_B = 3.0 \) and \( \theta = 120^\circ \); for the two consecutive head beads and the first beads in each tail (beads 2, 3, and 4 or beads 2, 3, and 5 in Fig. S1), \( k_B = 4.5 \) and \( \theta = 120^\circ \).

Each unit cell of CG graphene consists of three beads with the nearest-neighbor distance of 0.4 \( \text{nm} \) and internal angle of 60\(^\circ\). By applying a suite of standard mechanical tests on a 6.4 \( \times \) 6.4-\( \text{nm} \) square coarse-grained graphene model (7, 8), we calibrate Young’s modulus, shear modulus, and bending stiffness in our CGMD model to the experimental values for the elastic properties of graphene (9, 10). The bond and angular constants used are \( k_B = 111.4 \text{kcal mol}^{-1} \text{Å}^{-2} \) and \( k_B = 715 \text{kcal mol}^{-1} \text{rad}^{-2} \). The dihedral angle force constants, multiplicity, and potential minima are defined as \( k_B = 42.3 \text{kcal mol}^{-1} \text{Å}^{-1} \), and \( \eta_0 = 180^\circ \), respectively. For the large few-layer graphene sheets in Fig. 1, the bilayer is initially confined by a harmonic potential in the vicinity of the graphene edge with parameters \( k_B = 15k_B T/\tau_c \) and an equilibrium \( z \) position that is \( 6 \tau_c \) below the edge of graphene sheets.

To denote the hydrophilic/hydrophobic property of beads in the DPD system, the repulsive interaction parameters for the same types of beads are \( a_{ij} = 25 \), the interaction parameters for two beads of different types are set as \( a_{GHT} = a_{HT} = a_{GS} = 100 \) and \( a_{HTS} = 25 \) (11), where \( S \) represents the solvent bead, \( G \) represents the graphene bead, and \( H \) and \( T \) stand for the lipid head and the lipid tail, respectively. The interaction parameters between graphene and lipid molecules are taken to be \( a_{GHT} = 155 \) and \( a_{GTS} = 45 \), which were derived from matching with the correct graphene–membrane interaction parameters \( r_H = 15k_B T/\tau_c \) and \( r_T \) from our all-atom MD simulations. The equilibrium interlayer distance of few-layer graphene (FLG) is 0.34 nm by specifying bead pairs from different layers to interact via a hybrid of DPD and standard 12–6 Lennard–Jones potential, with interaction parameters \( a_{DPD} = 5, a_T = 1, a_S = 0.25, \) and \( r_G = 1 \).

To test whether the corner-entry mode of graphene flake is robust within a broad range of DPD parameters, the same simulations described in Fig. 1 were repeated under varying interaction parameters \( a_{GHT} \) and \( a_{GTS} \). Two groups of simulations are carried out, one group with fixed \( a_{GTS} = 45 \) while \( a_{GHT} \) varies in the range \( 75 \sim 250 \) and the other group with fixed \( a_{GHT} = 155 \) while \( a_{GTS} \) varies in the range \( 5 \sim 125 \). It was observed that in all simulations the graphene flake adopted the corner-entry mode during the initial stage of interaction. However, it seems that the final positions of graphene inside the membrane can be affected by the force-field parameters, especially the interaction parameter \( a_{GTS} \) between graphene and lipid core. When \( a_{GTS} \) is small, in which case the affinity between graphene and lipid core is strong, the graphene flake enters the
membrane interior to seek maximum contact with the hydrophobic core. As $a_{GT}$ grows, the graphene flake is seen to pierce through and tilt across the bilayer or simply attach to the bilayer surface under sufficiently large $a_{GT}$.

**Corner Piercing of Graphene Flakes with Different Shapes into a Liped Bilayer.** We have performed CGMD simulations of graphene flakes of different shapes interacting with a lipid bilayer. Besides the rhombic graphene flake described in the main text, we have also carried out simulations for graphene flakes of other shapes, including equilateral triangle, square, and hexagon (Fig. S2). The interaction parameters are identical in these simulations. In all cases, the graphene flakes are able to penetrate into the lipid bilayer by nearly orthogonal piercing at a sharpest corner. These CGMD simulations show that corner piercing is the preferred entry mode during the initial stage of graphene uptake.

**Corner Piercing of Graphene with Functionalized Corners and Edges into a Liped Bilayer.** The hydrophilic/hydrophobic surface properties of graphene may be chemically modified (12–15). For example, graphene oxide surfaces are partially hydrophilic with a typical water contact angle of 40°–50° (16). A question of interest is whether and how corner chemical functionalization might change the ways with which graphene flakes interact with membrane. For this question, we have performed CGMD simulations of square graphene flakes with mixed hydrophilic and hydrophobic corners interacting with a patch of lipid bilayer. In our CGMD simulations, the interaction parameters of functionalized hydrophilic graphene corners with the surrounding solvent and lipid molecules are $a_{DH} = 100$, $a_{DS} = 45$, $a_{DS} = 45$, and $a_{EF} = 155$, where $G$ represents the functionalized graphene beads. The same simulations described in Fig. 1A–D in the main text are repeated under these modified parameters for functionalized corners. The hydrophilic beads are shown in purple and the remaining hydrophobic part of graphene is displayed in gray. Figs. S3–S5 show the interaction patterns between a bilayer and graphene flakes with various numbers of functionalized hydrophilic corners, edges, or mixed hydrophilic edges and corners. It can be seen that corner piercing occurs at a hydrophobic corner in all cases. As a graphene flake undergoes Brownian motion in the vicinity of the bilayer, membrane piercing occurs as soon as the flake finds a configuration with one of the sharpest hydrophobic corners oriented nearly orthogonal to the bilayer (Figs. S3B, S4B, and S5B). Aided by the attractive interactions between hydrophobic graphene and tails of lipid molecules, the graphene flakes spontaneously pierce into the bilayer (Figs. S3C, S4C, and S5C). Fig. S3 shows that the piercing corner ends up embedded inside the bilayer core, whereas the hydrophilic corners remain adhered to the membrane surface due to the affinity between lipid heads and the functionalized corners (Fig. S3D). In the cases of hydrophilic edges, the graphene can induce a pore in the membrane due to the repulsive interactions between functionalized edge segments and the membrane core.

Our simulations also indicate that cell uptake of small graphene flakes can be inhibited when all of the corners are functionalized to be hydrophilic (4C in Fig. S3). In this case, the graphene flakes in Brownian motion still tend to point one of the corners at the bilayer (4C in Fig. S3B), in support of our calculation of configurational free energy in Fig. 1I. However, in the absence of any hydrophobic corner, due to the repulsive interaction between lipid tails and functionalized graphene corners, the graphene just tilts toward the membrane surface instead of piercing into the bilayer (4C in Fig. S3C). Some of the lipid heads underneath the hydrophobic part of graphene are squeezed out of the region and some are indented into the bilayer core. The graphene becomes attached to the membrane surface under the affinity between lipid heads and the functionalized graphene corners (4C in Fig. S3D). In this case, it is possible that some endocytic or phagocytic uptake process can still be activated, as observed during cell uptake of protein-coated nanosheets (17).

**Penetration of Graphene Sheets with Roughened Edges into a Bilayer.** Atomically resolved scanning tunneling microscopy investigations have shown that the graphene edges typically exhibit roughness at atomic scales (18–20). Here, we repeat the same simulations described in Fig. 1 F–H for the cutting process of a monolayer graphene or a FLG with different configurations of edge roughness, including a sharp protrusion, a terrace step, contact near a corner, or contact with a finite strip with two corners (Fig. S6). The lipid bilayer undergoes Brownian motion in the vicinity of the graphene edge for 2.19 μs, confined by the same harmonic potential as described in the main text related to Fig. 1. The confinement is then removed and the bilayer membrane is set free to interact with the ragged graphene edge.

In the case of an ideal, atomically smooth, infinite graphene edge interacting with a bilayer in the absence of any sharp corners, our simulations show that the lipid bilayer is repelled away from the graphene edge due to a combination of entropic force and entry barrier provided by the hydrophilic heads of the lipids (Fig. S6 A–D). For a stacked FLG with a sharp extrusion, a similar localized-piercing-followed-by-edge-cutting entry pathway as in the case of the ragged edge described in Fig. 1E is observed (Figs. S6 E–H). For a monolayer graphene with a 120° terrace step shown in Fig. S6f, although the smooth segments of graphene edge provide a strong energy barrier, the bilayer membrane is observed to be pierced through at the corner of the terrace step, which serves as a low barrier entry point. In the process, the bilayer exhibits obvious fluctuations under repulsion from the smooth edge segments (Fig. S6f). Aided by the attractive interactions between the lipid tails and hydrophobic graphene, the invasive part of the membrane then spreads along the whole edge, resulting in full penetration (Fig. S6 K and L). For a stacked FLG strip with two corners (Fig. S6M), the initial cutting process is somewhat similar to that of the graphene monolayer with a terrace step. The corner pierces into the bilayer accompanied by the deformation of the bilayer due to repulsion from the smooth edge segment (Fig. S6n). The invasive part of the bilayer then spreads along the edge. After the penetration of the lowest edge of FLG (Fig. S6O), the lipid bilayer climbs along the terraced edges aided by the attractive interaction between the graphene and the tail groups and achieves full penetration of the FLG (Fig. S6P).

**Penetration of Graphene with Closed Edges into a Bilayer.** In addition to various types of edge roughness, closed edges that result from self-folding of graphene have been observed to sometimes coexist with open edges under transmission electron microscopy (TEM) (21, 22). Can the invasive bilayer membrane following local piercing at a sharp corner propagate along a closed graphene edge? This question is addressed by further CGMD simulations. As Fig. S7 A and E shows, the folded graphene edge has a closed, tube-like edge compared with the open edge of unfolded graphene. The purple beads represent the fixed boundary of graphene. Similar to the case of an ideally smooth open edge, no penetration is observed for an ideal, infinite smooth closed edge, as shown in Fig. S7 A–D. The folded graphene edge cannot directly cut through the bilayer due to the repulsive interaction from the lipid heads. For a closed graphene edge coexisting with a flap of open edge protrusion shown in Fig. S7E, lipid penetration is seen to start with corner piercing at the flap and then is able to spread along the closed edge, leading to full penetration of the whole edge through the bilayer. Fig. S7 F–H shows three typical stages of graphene penetration: corner piercing into the bilayer through the open edge flap, penetration through the top leaflet of the bilayer, spreading of the invasive part along the closed edge, and full penetration through the bilayer.
**Thermodynamic Integration Technique.** The evolution of free energy as a rhombic graphene flake rotates from the reference orientation of orthogonal piercing (90°, 90°) to an arbitrary orientation \((\theta, \varphi)\) is calculated following the standard thermodynamic integration (TI) scheme. The free energy change is expressed as (23)

\[
\Delta F = \Delta F_1 + \Delta F_2 = \int_0^\lambda \frac{\partial F(\lambda)}{\partial \lambda} d\lambda + \int_0^\xi \frac{\partial F(\xi)}{\partial \xi} d\xi, \quad [S7]
\]

where \(\lambda\) and \(\xi\) are orientation parameters. We adopt a two-step process in the calculations. In the first step, we change the in-plane angle \(\theta\) from 90° to a preselected angle

\[
\theta(\lambda) = \theta(\lambda = 0) = \lambda(\theta(\lambda = 1) - \theta(\lambda = 0)). \quad [S8]
\]

In the second step, the graphene plane is rotated by changing \(\varphi\) from 90° to a preselected value (in which process the angle \(\varphi\) remains constant)

\[
\varphi(\xi) = \varphi(\xi = 0) + \xi(\varphi(\xi = 1) - \varphi(\xi = 0)). \quad [S9]
\]

We let \((\lambda, \xi) = (0, 0)\) correspond to the initial orthogonal piercing orientation, i.e., \((\theta, \varphi) = (90°, 90°)\), and let \((\lambda, \xi) = (1, 1)\) be the orientation when graphene becomes parallel to the bilayer. All intermediate orientations can be represented by the two parameters \((\lambda, \xi)\) with \(0 < \lambda, \xi < 1\).

In determining the free energy change as the graphene flake is rotated from the reference orientation of orthogonal piercing to arbitrary \(\theta\) and \(\varphi\) with \(\lambda = e\) and \(\xi = \delta\), we discretize the rotation path by a series of \(\lambda, \xi\) values in the range \(0 \leq \lambda, \xi \leq 1\). In the first step, only \(\lambda\) is increased stepwise from \(\lambda = 0\) to \(\lambda = e\). A pair of diagonally opposed forces \(f = 1000k_BT/r_s\) is applied at the two sharpest corners (marked as purple and green) along the selected orientation \(\theta(\lambda)\) while \(\varphi\) is kept constant by constraining the other two corners, resulting in an effective confining potential,

\[
U_R = 2\sqrt{3}L \sin \left( \frac{\theta - \theta(\lambda = 0)}{2} \right), \quad [10]
\]

which constrains the rotational motion of the graphene flake. Here, \(L\) is the edge length of graphene. \(\Theta\) is the angle between the long axis of the flake and the bilayer surface within the graphene plane. Under the confinement of this potential, the rhombic graphene oscillates around an equilibrium orientation \((\Theta)\) in the vicinity of \(\theta(\lambda)\). Note that \((\Theta)\) is the ensemble average of the angle \(\Theta\) over 1 µs. The integrand of Eq. S7 is then expressed as

\[
\frac{\partial F(\lambda)}{\partial \lambda} = \left( \frac{\partial U_R}{\partial \lambda} \right)_{\theta(\lambda)} = 2fL \left( \langle \sin[\theta(\lambda) - \Theta] \rangle | \theta(\lambda = 1) - \theta(\lambda = 0) \rangle \right). \quad [S11]
\]

After \(\lambda\) is increased to \(\lambda = e\), we keep \(\theta\) constant and increase \(\xi\) stepwise from \(\xi = 0\) to \(\xi = \delta\), rotating the graphene plane with respect to the membrane. Using similar confinement to that of Eq. S10, the rhombic graphene oscillates around an equilibrium orientation \((\Phi)\) in the vicinity of \(\phi(\xi)\) for 1 µs. Thus, the free energy change associated with the rotation of the graphene flake can be integrated as

\[
\Delta F = \sqrt{3}L \left( \int_{\theta(\lambda = 0)}^{\theta(\lambda = e)} \langle \sin[\theta(\lambda) - \Theta] \rangle d\theta \right)_{\xi = 0} + \int_{\phi(\xi = 0)}^{\phi(\xi = \delta)} \langle \sin[\phi(\xi) - \Phi] \rangle d\phi. \quad (0 \leq e, \delta \leq 1). \quad [S12]
\]

**All-Atom MD simulations.** The all-atom MD simulations are performed in NAMD (24) and visualized in VMD (25). The graphene and the POPC lipid bilayer membrane are generated using VMD Graphene Builder and Membrane Builder, respectively. The graphene and lipid bilayer system is then fully hydrated (adding water molecules) using VMD. The CHARMM36 force field (26) with added parameters for the graphene and TIP3 water model is adopted in the simulations. All simulations are composed of two equilibration steps and one production step. Because the lipid membranes generated by the membrane plug-in of VMD are far from the equilibrium state, an equilibrium run of 0.5 ns is carried out for all simulations, in which everything except lipid tails is fixed. This first step allows the lipid tails to melt into a fluid-like configuration. In the second step, systems are equilibrated for about 2 ns in NPT ensemble at a temperature of 310 K and pressure of 1 atm. In the third step, production runs are performed. In all three steps of each simulation, the out-of-plane degrees of freedom of carbon atoms of the graphene flake are restricted using harmonic constraints.

More details of the simulation technique for two types of simulations described in the main text are elaborated. In type I simulations, the bilayer membrane spanning the \(x\)-plane of the simulation box is composed of 256 POPC lipids, and the average simulation box size is about 6 nm × 14 nm × 15 nm (in \(x\)-\(y\) directions). The triangular graphene flake with an internal angle of \(2\alpha = 40°\) composed of 2,764 carbon atoms is placed across the lipid bilayer in the initial configuration. The total number of atoms in the system is 129.867. After the initial two steps of equilibrating the system, MD simulation is performed for 4.2 ns in which the spontaneous piercing is observed.

In type II simulations, the bilayer membrane with an area of \(6 \times 6\) nm² consisted of 104 lipids, and the triangular graphene flake with a corner of \(2\alpha = 90°\) is composed of 926 carbon atoms. Different from the type I simulations, steered MD simulations (27) are performed in the third step of type II simulations to calculate the free energy change during the corner-piercing process. In our steered molecular dynamics (SMD) simulations, a dummy atom is attached to a carbon atom (referred to as the steered atom) of the graphene flake by a virtual spring with a spring constant \(K = 7\) kcal mol⁻¹ Å⁻². The graphene was pulled by moving the dummy atom at a constant velocity of \(v = 0.005\) Å/ps along the normal direction of the lipid membrane. The spring force as a function of time \(f\) is calculated according to the expression \(F = K(h_0 + v(t) - h(t))\), where \(h_0\) and \(h(t)\) are the initial and current projected positions of the steered atom along the pulling direction, respectively. To prevent the bilayer membrane from drifting downward during the piercing process, a small number of water molecules underneath the bilayer are fixed in space. To make graphene piercing as close as possible to a quasi-static process, we adopt the smallest dummy atom moving velocity \((v = 0.005\) Å/ps) that has been used in previous SMD simulations (28, 29). The free energy change \(E(h)\) is then obtained by integrating the spring force \(F\) along the path \(h\). Note that even though the friction force acting on the graphene flake is small because of the small moving velocity used, it is still subtracted from the spring force \(F\) when the free energy is calculated.

**Analytical Model of Energy Change During Piercing of a Graphene Corner into Lipid Bilayer.** Referring to Fig. 2C in the main text, the energy change during piercing can be expressed in terms of the following parameters: \(h_0\) and \(h(t)\) (thicknesses of the head and tail groups in the lipid monolayer) and \(\gamma_H\) and \(\gamma_T\) (interaction energy densities between one side surface of graphene and head and tail groups of lipids relative to that between solvent and graphene). As the penetration depth increases, we have four different regimes, corresponding to the graphene tip lying \((i)\) in the upper hydrophilic region \((0 < h \leq h_H)\), \((ii)\) in the hydrophobic region  

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core \((h_H < h \leq h_H + 2h_T)\), (iii) in the lower hydrophilic region \((h_H + 2h_T < h \leq 2h_H + 2h_T)\), and (iv) beyond the bilayer \((2h_H + 2h_T < h)\). Simple geometric relations show that the energy change can be written as a piecewise function:

\[
E = \begin{cases} 
2h^2 \gamma_H \tan \alpha & 0 < h \leq h_H \\
\frac{4h_H \gamma_H \tan \alpha}{4h_H + (h - h_H)} & h_H < h < h_H + 2h_T \\
\frac{4h_H \gamma_H \tan \alpha}{4(h - 2h_T) \gamma_H + 2h_T \gamma_T \tan \alpha} & h_H + 2h_T < h \leq 2h_H + 2h_T \\
8(h_H \gamma_H + h_T \gamma_T \tan \alpha) & 2h_H + 2h_T < h.
\end{cases}
\]

[S13]

We expect \(\gamma_H > 0\) (repulsion) and \(\gamma_T < 0\) (attraction). It is most convenient to analyze the slope of the energy change with \(h\):

\[
\frac{dE}{dh} = \begin{cases} 
4h_H \gamma_H \tan \alpha & 0 < h \leq h_H \\
4(h - h_H) \gamma_H + 2h_T \gamma_T \tan \alpha & h_H < h < h_H + 2h_T \\
4(h - 2h_T) \gamma_H + 2h_T \gamma_T \tan \alpha & h_H + 2h_T < h \leq 2h_H + 2h_T \\
8(h_H \gamma_H + h_T \gamma_T \tan \alpha) & 2h_H + 2h_T < h.
\end{cases}
\]

[S14]

The slope of energy change is clearly positive throughout the first regime, \(0 < h \leq h_H\). Our all-atom simulation shows that there is a positive driving force for piercing, \(\gamma_H h_H + \gamma_T h_T < 0\), once graphene has completely penetrated the bilayer, i.e., regime iv. The expressions above show that the slope of the energy change is negative throughout both regimes ii and iii, \(h_H + 2h_T < h \leq 2h_H + 2h_T\) and \(2h_H + 2h_T < h\), respectively. Therefore, the energy peak occurs in regime ii, which lies in the hydrophobic core \(h_H < h \leq h_H + 2h_T\), at a point defined by \(dE/ dh = 0\):

\[
h_H \gamma_H + (h - h_H) \gamma_T = 0 \quad [S15]
\]

or

\[
h = \left(1 - \frac{\gamma_H}{\gamma_T}\right) h_H. \quad [S16]
\]

Inserting this into the energy expression gives the energy barrier for corner piercing as

\[
E_{\text{barrier}} = 2 \left(1 - \frac{\gamma_H}{\gamma_T}\right) h_H \gamma_H \tan \alpha. \quad [S17]
\]

The Cultivation of Three Cell Types. J774 murine macrophages (ATCC; TIB-67) were cultured in RPMI medium 1640 (Invitrogen; 11875) containing 10% (vol/vol) FCS in ulralow-attachment culture dishes (Corning; 3262) at 37 °C. The cells were pelleted for 5 min and fresh medium was added to the cells followed by seeding the cells on coverslips in six-well plates (diameter per well, 35 mm) to reach a cell density of 70% after 24 h. The cells were allowed to attach overnight at 37 °C and exposed to 10 μg/mL test materials for an additional 24 h.

Human primary keratinocytes (ATCC; PCS-200-011) were cultured in dermal cell basaT medium supplemented with 0.4% bovine pituitary extract (BPE), 0.5 mg/mL recombinant human TGF-α, 5 mM L-glutamine, 100 ng/mL hydrocortisone, 5 μg/mL insulin, 1.0 μg/mL epinephrine, and 5 μg/mL apotransferrin (PCS-200-011) in cell culture flasks at 37 °C. After removing the cell culture medium, keratinocytes were briefly rinsed with PBS (Invitrogen; 10010) and incubated for 5 min in trypsin/EDTA solution for primary cells (ATCC; PCS-999-003) at 37 °C. Trypsin neutralization solution (ATCC; PCS-999-004) was added and the cells were pelleted for 5 min at 240 × g in a IEC Centra MP4R centrifuge. Complete growth medium was added to the keratinocytes, and cells were plated onto glass coverslips to reach a cell density of 70% after 24 h in six-well plates (diameter per well, 35 mm). The cells were allowed to attach overnight at 37 °C and exposed to 10 μg/mL test materials for an additional 24 h.

Graphene Materials Characterization (30). When immersed in biological fluid, nanoparticles generally develop a dynamic protein corona that may also include ion or small biological molecules in the adsorbed state (31–33). We wanted to investigate to what degree this corona alters the surface of our materials in the conditions relevant to our cellular studies. In our experiments, the main source of protein is from the albumin,1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) treatment protocol used to facilitate dispersion. To estimate the fraction of the graphene-family nanomaterial (GFN) surfaces covered by albumin, we reproduced the dispersion protocol in separate control experiments and measured the amount of adsorbed protein. To make this measurement, the graphene materials were removed from the treatment solution, using centrifugal ultrafiltration (Amicon Ultra centrifugal tube; EMD Millipore), and the amount of albumin remaining in the supernatant was determined using a QuantiChrom bacillus Calmette–Guérin Albumin Assay Kit (BioAssay Systems). The amount of albumin on the surface of the graphene material sample was determined by mass balance, and using an estimate for the size of the albumin protein (34), a fractional surface coverage was determined. This procedure was then repeated after the graphene materials were incubated in RPMI 1640 cell culture medium at 37 °C for 2 h to test for the state of adsorption during the experimental conditions for the cell studies. It was found that after the initial coating, the fractional coverage of albumin on the graphene surface varied from 0.09 to 0.78. After incubation in cell culture media, 41–57% of the albumin was found to desorb from the
samples, leaving a fractional coverage of 0.03–0.46 as the surface state relevant to the cell interaction experiments.

Target cells were exposed to GFNs at doses between 10 ppm and 40 ppm for 24 h. Cell number was evaluated using pico green dsDNA reagent (Invitrogen) according to the manufacturer’s instructions. Cell numbers ranged from 80% to 110% of controls exposed to dispersion vehicle alone for all three target cells.

**Electron Microscopy.** For TEM sample preparation two different methods were used to verify that graphene flakes are taken up by the cells. 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] (Electro Microscopy Sciences; 11650) at 4 °C and then rinsed three times with 0.1 M sodium cacodylate buffer and postfixed in 2% (vol/vol) aqueous osmium tetroxide (Electro 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 using a Reichert Ultracut Ultramicrotome with either a diamond knife (unreated cells) or a glass 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.

Intracellular localization of graphene nanosheets was also confirmed by sectioning cells grown in a monolayer on glass coverslips. Cells grown on coverslips were fixed with 2% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and then postfixed with 1% (vol/vol) osmium tetroxide in buffer with appropriate rinses between steps. Samples were then en bloc stained with 1% (wt/vol) aqueous uranyl acetate for 1 h at room temperature. Specimens were then dehydrated through a series of graded ethyl alcohols with three changes of 100% (vol/vol) ethanol. Coverslips were then infiltrated with Durcupan embedding media (Electron Microscopy Sciences), embedded cell face down on Thompson molds, and polymerized at 60 °C. Following polymerization, blocks were placed on dry ice and coverslips were snapped off, leaving cell monolayers on blocks to be sectioned. These blocks were trimmed and reembedded in molds to increase surrounding media support to the cell monolayer for sectioning. Samples were sectioned at a thickness of 80 nm for electron microscopy, using a Reichert Ultramicrotome with a diamond knife. The specimens were placed on copper grids stained with Reynolds’s lead citrate to increase contrast and viewed on a Phillips 410 transmission electron microscope equipped with an Advantage HR CCD camera. Images were acquired with Advanced Microscopy Techniques imaging software.

For scanning electron microscopy, cells were postfixed in 2% (vol/vol) aqueous osmium tetroxide for 30 min followed by dehydration in 25% (vol/vol), 50% (vol/vol), 70% (vol/vol), 2 × 95% (vol/vol), and 3 × 100% (vol/vol) ethanol. After critical point drying according to the manufacturer’s directions (Ladd Research), the coverslips were sputter coated with gold and viewed using field-emission SEM (LEO 1530-VP). Alternatively, samples were prepared for scanning electron microscopy without critical point drying to improve preservation of surface morphology. Cells were fixed with 2% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer and then immersed in 0.5% buffered osmium tetroxide, followed by 1% (wt/vol) thiocarbohydrazide and 1% (vol/vol) aqueous osmium tetroxide with appropriate rinses between steps. Specimens were dehydrated in graded ethanol and dried in hexamethyldisilazane. For microscopy, samples were sputter coated with gold palladium and then viewed with a LEO 1530 VSP scanning electron microscope.
Fig. S1. Course-grained molecular dynamics simulation model. Shown are models of POPC lipid molecule and a square graphene monolayer with length of $L = 6.4$ nm. A membrane lipid bilayer spans the simulation box.

Fig. S2. CGMD simulations showing spontaneous orthogonal corner piercing of graphene flakes of different shapes into a patch of lipid bilayer. (A) The initial configurations of graphene flakes lying above and parallel to the bilayer. (B) The graphene flakes are seen to rotate to a corner-first configuration right before piercing starts. (C) Beginning of the corner-piercing process. (D) The small graphene flakes are eventually inserted into the lipid bilayer.

Fig. S3. Interaction between a lipid bilayer and square graphene flakes with various numbers of functionalized hydrophilic corners. (A) The initial configurations of graphene flakes lying above and parallel to the bilayer. (B) The graphene flakes are seen to rotate to a corner-first configuration right before piercing starts. (C) Beginning of the corner-piercing process for 1C, 2C, and 3C, and the graphene flake begins to be attached to the membrane surface along an edge for 4C. (D) The end configurations showing that the functionalized hydrophilic corners adhere to the lipid heads. 1C, one hydrophilic corner; 2C, two adjacent hydrophilic corners; 3C, three hydrophilic corners; 4C, four hydrophilic corners. The hydrophilic corners are shown in purple beads whereas the hydrophobic part of graphene is in cyan.
Fig. S4. Corner piercing of square graphene flakes with various numbers of hydrophilic edges into a lipid bilayer. (A) The initial configurations of graphene flakes lying above and parallel to the bilayer. (B) The graphene flakes are seen to rotate to a corner-first configuration right before piercing starts. (C) Beginning of the corner-piercing process. (D) The end configurations showing that the functionalized hydrophilic edges adhere to the lipid heads and can induce pore formation in the membrane. 1E, one hydrophilic edge; 2E, two adjacent hydrophilic edges; 3E, three hydrophilic edges; 4E, four hydrophilic corners.

Fig. S5. Corner piercing of square graphene flakes with various numbers of mixed hydrophilic edges and corners. (A) The initial configuration of the graphene flake lying above and parallel to the bilayer. (B) The graphene flake is seen to rotate to a corner-first configuration. (C) Beginning of the corner-piercing process. (D) The end configurations showing that the functionalized hydrophilic segments adhere to the lipid heads. 1E&1C, one hydrophilic edge and one hydrophilic corner; 2E&3C, two adjacent hydrophilic edges and three hydrophilic corners; 3E&3C, three hydrophilic edges and three hydrophilic corners; 4E&3C, four hydrophilic corners and three hydrophilic corners.

Fig. S6. CGMD simulation results showing the dynamic interactions of graphene edges with a bilayer. (A–D) An ideal, atomically smooth, infinite graphene edge in the absence of any sharp corners. (E–H) A FLG with a sharp protrusion. (I–L) A graphene edge with 120° terrace step. (M–P) A finite strip of staggered FLG.
Fig. S7. CGMD simulation results showing the dynamic interactions of lipid bilayer with closed graphene edges. (A–D) The bilayer is repelled away from an ideal, smooth infinite closed graphene edge. (E–H) Penetration of the bilayer by a closed graphene edge coexisting with a sharp open edge protrusion. Shown are the concurrent views of cross-section and transverse cutting.

Fig. S8. (A–D) Viability of macrophages (A and B) and human keratinocytes exposed to GFNs (C and D) was assessed using ethidium homodimer/Syto 10 (Molecular Probes). Macrophages (A and B) and human keratinocytes (C and D) were seeded in 12-well plates containing coverslips and exposed to 40 ppm graphene (B and D) for 24 h. The cells were imaged using an Olympus confocal microscope (IX81 motorized inverted research microscope) to visualize live (green fluorescence) and dead (red fluorescence) cells.

Fig. S9. Typical morphologies of graphene-family nanomaterials used in cell imaging studies. SEM micrographs clearly show the ultrathin flake structure, irregular shapes in-plane, and highly irregular edge profiles containing nanoscale roughness that include sharp protrusions or asperities. [Scale bars: (A and B) 0.5 μm and (C and D) 1 μm.] Arrows point to examples of the many irregular protrusions on graphene edges. All images were taken using field-emission SEM (LEO 1530-VP).
Fig. S10. Stability of dispersed GFNs assessed using dynamic light scattering (Zetasizer Nano-2S; Malvern Instruments). No increase in the apparent hydrodynamic diameters indicates that material dispersions were stable and aggregation did not occur.

Table S1. Typical ranges of basic physical and chemical properties of graphene-family nanomaterials used in cell-imaging studies

<table>
<thead>
<tr>
<th>Surface area, m²/g</th>
<th>Typical lateral dimension*</th>
<th>Average layer no. †</th>
<th>Zeta potential with FCS coating, mV</th>
<th>Zeta potential with DPPC and albumin coating, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>26–630</td>
<td>800 nm–5 μm</td>
<td>3–100</td>
<td>−13.2 ± 15.6</td>
<td>−5.5 ± 18.9</td>
</tr>
</tbody>
</table>

Typical oxygen content is shown for all three samples from 3% to 7% by atomic concentration.

* Determined by SEM imaging.
† Provided from vendor, supported by thicknesses seen by electron microscopy and calculation based on surface area (29).

Movie S1. Molecular dynamics simulations of corner piercing of a rhombic graphene flake into a lipid bilayer. The movie corresponds to snapshot sequences shown in Fig. 1 A–D.

Movie S1
Movie S2. Molecular dynamics simulations of a large five-layer graphene sheet with roughened-edge topography and staggered stacking penetrated into a lipid bilayer. The movie corresponds to snapshot sequences shown in Fig. 1 F–H.

Movie S3. All-atom molecular dynamics simulations of a monolayer graphene sheet penetrating across a lipid bilayer. The movie corresponds to the snapshot sequences shown in Fig. 2A.