Intracellular hydrogelation preserves fluid and functional cell membrane interfaces for biological interactions

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Cell membranes are an intricate yet fragile interface that requires substrate support for stabilization. Upon cell death, disassembly of the cytoskeletal network deprives plasma membranes of mechanical support and leads to membrane rupture and disintegration. By assembling a network of synthetic hydrogel polymers inside the intracellular compartment using photo-activated crosslinking chemistry, we show that the fluid cell membrane can be preserved, resulting in intracellularly gelated cells with robust stability. Upon assessing several types of adherent and suspension cells over a range of hydrogel crosslinking densities, we validate retention of surface properties, membrane lipid fluidity, lipid order, and protein mobility on the gelated cells. Preservation of cell surface functions is further demonstrated with gelated antigen presenting cells, which engage with antigen-specific T lymphocytes and effectively promote cell expansion ex vivo and in vivo. The intracellular hydrogelation technique presents a versatile cell fixation approach adaptable for biomembrane studies and biomedical device construction.
The cell membrane is a fluid substrate that harbors a milieu of phospholipids, proteins, and glycans, which dynamically choreograph numerous biological interactions. The long-standing fascination with the various biological functions of cell membranes has inspired model systems and cell-mimetic devices for biological studies1–3, tissue engineering4,5, drug delivery6–8, and immunoeengineering9–12. Toward replicating the cell membrane interface, synthetic bilayer lipid membranes and bioconjugation strategies are commonly adopted in bottom-up engineering of cell membrane mimics13. Alternatively, top-down approaches based on extraction and reconstitution of plasma membranes of living cells are frequently applied to capture the intricate cell-surface chemistries for biomimetic functionalization14–16. As antigen presentation, membrane fluidity, and membrane sidedness are critical factors behind biomembrane functions and can be influenced by membrane translocation processes, methods for harnessing this membranous component continue to emerge with the aim to better study and utilize this complex and delicate biological interface14–16.

To stabilize the fluid and functional plasma membranes and decouple it from the dynamic state of living cells, we envision that a synthetic polymeric network can be constructed in the cytoplasm to replace the cytoskeletal support for stabilizing cellular structures. Unlike endogenous cytoskeletons that are susceptible to reorganization and disintegration upon perturbation and cell death17, a synthetic substrate scaffold can stably support the cell membrane interface for subsequent applications. As the mechanical property of cytoskeletons has drawn comparisons to hydrogels17,18, a cellular fixation approach mediated by intracellular assembly of hydrogel monomers is herein developed. We demonstrate that the intracellular hydrogelation technique effectively preserves cellular morphology, lipid order, membrane protein mobility, and biological functions of the plasma membrane, giving rise to cell-like constructs with extraordinary stability. In addition, a highly functional artificial antigen presenting cell (APC) is prepared with the gelated system to highlight the platform’s utility for biomedical applications.

Results

Intracellular hydrogelation by photoactivated cross-linking.

Three criteria were considered to establish the intracellular hydrogelation technique: (i) Hydrophilic cross-linking monomers with a low-molecular weight were used to facilitate cytoplasmic permeation and minimize membrane partitioning. (ii) Cross-linking chemistry with low-protein reactivity was adopted to facilitate nondisruptive cellular fixation. (iii) Extracellular cross-linking was minimized to prevent cell-surface masking. Based on these considerations, a photoactivated hydrogel system consisting of poly(ethylene glycol) diacrylate monomer (PEG-DA; Mn 700) and 2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropophenone photoinitiator (I2959) was employed. The materials are broadly used in biomedical applications and have little reactivity with biological components19,20. These hydrogel components were introduced into cells through membrane poration with a single freeze–thaw cycle. Following a centrifugal wash to remove extracellular monomers and photoinitiators, the cells were irritated with ultraviolet (UV) light for intracellular hydrogelation (Fig. 1a and Supplementary Fig. 1). To assess the feasibility of intracellular gelation for cellular fixation, HeLa cells were first processed with different PEG-DA cross-linker densities ranging from 4 to 40 wt%. The freeze–thaw treatment allowed PEG-DA monomers to penetrate into the intracellular domain efficiently, and the collected cells had PEG-DA contents equivalent to the input PEG-DA concentrations (Fig. 1b). Following UV irradiation to the PEG-DA infused cells, no alteration to the cellular morphology was observed (Supplementary Fig. 2). An evaluation by atomic force microscopy, however, showed that the gelated cells (GCs) exhibited increasing Young’s moduli that correlated with the PEG-DA concentrations (Fig. 1c). Assessment of GC stability by microscopy showed no observable structural alternation over a 30-day observation period, whereas control cells and non-crosslinked cells exhibited noticeable disintegration within 3 days (Fig. 1d and Supplementary Fig. 3). To further confirm the assembly of hydrogel networks in the intracellular domain, fluorescein-diacylate was added to the cross-linker mixture to covalently imbue the hydrogel network with green fluorescence (Supplementary Fig. 1). Following membrane staining with a lipophilic DiD fluorophore, GCs showed distinctive membranous and hydrogel components (Fig. 1e and Supplementary Fig. 4), displaying a structure reminiscent of substrate-supported lipid membranes13. Solubilization treatment with sodium dodecyl sulfate was applied to examine the integrity of the gelated cytoplasm, and the fluorescent hydrogel matrices in GCs remained intact following membrane dissolution (Supplementary Fig. 4). In a dye-exclusion study, 4 wt% GCs effectively excluded a water-soluble fluorescein isothiocyanate (FITC) dye from entering the cytoplasm (Fig. 1f and Supplementary Fig. 4), thereby confirming the plasma membrane integrity on GCs. We also demonstrated that GCs could be stored by freezing and lyophilization (Supplementary Fig. 5A). In addition, the intracellular gelation process was applied to adherent HeLa cells, effectively preserving the cells’ adherent property and elongated structures (Supplementary Fig. 5B).

Intracellular gelation preserves cellular features.

Examination of the cell membrane interface and the cytoplasmic hydrogel matrix on GCs was performed by transmission electron microscopy (TEM). In comparison to control cells, GCs possessed a perforated, hydrogel-filled interior. Treatment by detergent stripped GCs of their membranous exterior, leaving behind nondissolvable hydrogel matrices (Fig. 2a). To better visualize the membrane interface on GCs, intracellular hydrogelation was applied to avian red blood cells (aRBCs), which are nucleated cells devoid of organelles. As hemoglobins were removed during the gelation process, gelated aRBCs (G-aRBCs) exhibited a clear membrane boundary encircling a perforated nucleus (Fig. 2b and Supplementary Fig. 6). Notably, the addition of a hemagglutinating influenza virus to the G-aRBCs induced direct agglutination (Supplementary Fig. 6), and TEM cryosections showed similar binding patterns between nongelated aRBCs and G-aRBCs (Fig. 2c and Supplementary Fig. 6). These results highlight the intracellular hydrogelation technique enables facile preparation of stable, cell-like constructs without masking cellular surface. Using adherent HeLa cells, we further assessed the influence of intracellular hydrogelation on periplasma components and cellular cytoskeletons. In contrast to live cells that lost membrane ruffles upon incubation in phosphate-buffered saline (PBS) for 4 h, both 4 wt% and 20 wt% GCs effectively excluded a water-soluble fluorescein isothiocyanate (FITC) dye from entering the cytoplasm (Fig. 2d). Confocal microscopy of actin-GFP-transfected HeLa cells also showed that filamentous actin structures were observable in the GCs (Fig. 2e and Supplementary Fig. 7A). Interestingly, in 20 and 40 wt% GCs, actin filaments could be observed 24 h after the gelation process, which suggests that the denser hydrogel matrices could entrap these intracellular components and retard their depolymerization and dissipation (Supplementary Fig. 7A). We also observed that GCs could retain their ruffled exterior over a long period of time (Supplementary Fig. 7B, C), further illustrating that the synthetic hydrogel networks can substitute cytoskeleton in supporting these nanoscale membrane features.
Intracellular gelation preserves lipid order and fluidity. We next examined the influence of intracellular hydrogelation and hydrogel densities on plasma membrane fluidity and membrane lipid order on GCs (Fig. 3a). Assessment of membrane fluidity by fluorescence recovery after photobleaching (FRAP) using a lipophilic DiD dye showed that fluorescence recovery half-times were similar among live cells and GCs of different cross-linking densities (Fig. 3b, c and Supplementary Fig. 8), indicating that the intracellular hydrogel matrices did not influence membrane lipid fluidity regardless of the hydrogel content. Given that membrane order is a critical biophysical parameter that influences the dynamics of membrane proteins, we adopted a Laurdan dye staining approach to quantify membrane order on GCs21. Through multiphoton microscopy followed by image processing to analyze the polarity of the plasma membrane, we were able to distinguish the ordered plasma membrane in live HeLa cells and derive the corresponding generalized polarization (GP) values by tracing the pixel intensities at the cellular periphery (Fig. 3d). Upon applying the technique to GCs and control cells subject to freeze–thaw and UV treatments in the absence of hydrogel, preservation of plasma membrane order by intracellular gelation was confirmed. Whereas nongelated control cells showed noticeable alteration in membrane order and GP values, membrane order in GCs of different hydrogel densities was similar to that of live cells (Fig. 3e, f). These results demonstrate that the gelation process has little influence on the phospholipid bilayer, effectively retaining the membrane fluidity and membrane order in the stabilized GCs.

Membrane proteins retain lateral mobility on GCs. To evaluate the mobility of membrane proteins on GCs, we first chose CD80 as the protein of interest given that CD80’s putative T-cell stimulating functionality is highly dependent on its lateral mobility3. CD80-GFP mobility on GCs was first assessed with total internal reflection fluorescence (TIRF) microscopy, which revealed rapid, random movements of fluorescent punctates. In contrast, fluorescent signals in glutaraldehyde-fixed cells appeared static (Fig. 4a, Supplementary Fig. 9, and Supplementary Movies 1–5). As live cells showed prominent cytoskeleton-directed protein movements owing to actin-mediated CD80 localization22,23, cells treated with a single freeze–thaw cycle were freshly prepared as a control (Supplementary Movies 6 and 7). Calculation of protein diffusivity showed a mean value of 0.118 μm² s⁻¹ for the control cells, which is in accordance with prior studies on the passive
Fig. 2 Structural examination of GCs. a Transmission electron microscopy (TEM) images show the cross-sectional structure of normal HeLa cells (left), 20 wt% gelated HeLa cells (middle), and the hydrogel matrix of 20 wt% gelated HeLa cells following solubilization by 1% SDS (right). (scale bars = 5 μm or 100 nm). Red arrows indicate cell membranes. b TEM cryosection images show the structure of avian erythrocytes and gelated avian erythrocytes (20 wt% PEG-DA) and c binding of hemagglutinating influenza viruses on the surfaces of gelated avian erythrocytes (scale bars = 2 μm or 200 nm). Red arrows indicate cell membranes. Yellow arrows indicate influenza viruses. d Cryogenic scanning electron microscopy (Cryo-SEM) images show the surface features of live HeLa cells, HeLa cells in PBS for 4 h, 4 wt% gelated HeLa cells in PBS, and 20 wt% gelated HeLa cells in PBS. Membrane ruffles were observed on the gelated cells. Scale bars = 10 μm (top row) and 3 μm (bottom row). e HeLa cells transfected with plasmids carrying actin-GFP and the corresponding 4 wt% GCs were imaged under bright-field and fluorescence microscopy. Preservation of actin filament was observed in the GCs. Red arrows indicate membrane ruffles, scale bars = 10 μm.
and 0.0711 μm−1.1

40 wt% GCs, the mean diffusivities were 0.118, 0.0966, 0.0967, actin cytoskeleton (Supplementary Fig. 10D). Given CD80 FRAP on adherent GCs. After photobleaching, rapid recovery further validated the fluency of transmembrane proteins24. For the 4, 10, 20, and 40 wt% GCs, the mean diffusivities were 0.118, 0.0966, 0.0967, and 0.0711 μm2 s−1, respectively (Fig. 4b). No statistical significance, however, was observed among the control cell and the GCs.

Further assessment of protein mobility was performed with FRAP on adherent GCs. After photobleaching, rapid fluorescence recovery further validated the fluidity of CD80-GFP on GCs (Fig. 4c; Supplementary Fig. 10). Curiously, close examination of the GCs by Z-stacked fluorescence microscopy revealed distinctive fluorescent filaments consistent with the patterns of actin cytoskeleton (Supplementary Fig. 10D). Given CD80’s tendency to complex with actin22,23, the observed filamentous patterns can be attributed to the actin/CD80 complexes. To minimize interference by cytoskeleton-directed protein movements and intracellular protein trafficking in comparing protein mobility between GCs and live cells, a first-order kinetics equation was applied to the early time points of the fluorescence recovery curves to derive fluorescence recovery kinetics. Based on the kinetics analysis, it was confirmed that GCs of varying densities possessed similar CD80-GFP recovery rates to those of live cells (Fig. 4c; Supplementary Fig. 10B, C). In contrast, glutaraldehyde-fixed cells showed significantly reduced recovery kinetics, highlighting intracellular hydrogelation as a unique approach for preserving mobile membrane proteins.

To evaluate how the hydrogel density may influence the mobility of different membrane proteins, HeLa cells transfected with glycosylphosphatidylinositol (GPI)-anchored enhanced green fluorescence protein (EGFP-GPI), transferrin receptor (TIR), GFP-tagged tyrosine protein kinase Lyn (Lyn-GFP), and GFP-tagged epidermal growth factor receptor (EGFR-GFP) were separately prepared for FRAP analysis (Fig. 4d). These proteins cover a broad range of differently sized cytoplasmic domains, with EGFP-GPI and TIR possessing no and small cytoplasmic segments and Lyn-GFP and EGFR-GFP having large cytoplasmic regions. Examination with FRAP showed that all the assessed proteins had significantly higher mobile fractions in GCs as compared to glutaraldehyde-fixed cells (Fig. 4e and
T-lymphocyte expansion by DCs hinges on the presence of gelated dendritic cells (G-DCs) and assessed its antigen presenting capability. Effective the potential utility of GCs, we prepared gelated dendritic cells (G-DCs) for antigen presentation demonstrating the construct remained readily detectable (Supplementary Fig. 15), further storage of GCs at 4 °C, the presence of mobile membrane proteins may also entrap proteins with large cytoplasmic domains, resulting in the reduced lateral mobility of Lyn-GFP and EGFR-GFP. For CD80-GFP, EGFP-GPI, and TIR, however, 40% hydrogel core had little influence on their relative mobility. In addition, at gelation densities between 4 and 20 wt%, recovery kinetics for all the examined membrane proteins had similar lateral mobility as on live cells. Error bars represent geometric mean with 95% confidence interval. Fig. 4 Examination of membrane protein lateral mobility on GCs. a Representative trajectories from CD80-GFP fluorescence tracking on different GCs and control cells examined by TIRF microscopy, b Diffusion coefficients of CD80-GFP were calculated from the TIRF fluorescence tracking data. Error bars represent geometric mean with 95% confidence interval. c Representative images showing recovery of CD80-GFP fluorescence in 4 wt% GC following photobleaching. Red rectangles indicate the photobleached area of interest. Scale bars = 2 μm. d A schematic illustration of membrane proteins with different sizes of intracellular domains, including CD80-GFP, EGFP-GPI, TIR, Lyn-GFP, and EGFR-GFP, which were assessed for their lateral mobility on GCs. e Recovery kinetics of CD80-GFP, EGFP-GPI, TIR, Lyn-GFP and EGFR-GFP on GCs and control cells assessed by FRAP. For GCs with 4 to 20 wt% hydrogel densities, all examined membrane proteins had similar lateral mobility as on live cells. Error bars represent geometric mean with 95% confidence interval. Membrane protein mobility on 40 wt% GCs relative to their corresponding mean mobility on live cells. Error bars represent geometric mean with 95% confidence interval (n = 7–12). Statistical analysis was performed using a two-tail Student t test, ****p<0.0001.

Gelated dendritic cells for antigen presentation. To highlight the potential utility of GCs, we prepared gelated dendritic cells (G-DCs) and assessed its antigen presenting capability. Effective T-lymphocyte expansion by DCs hinges on the presence of multiple, mobile, membrane-bound lymphocyte activation signals, and replicating these biological features remains a primary engineering objective in the development of artificial APC systems. In our system, we hypothesized that G-DCs could trigger T-cell expansion through MHC class I-TCR and CD80-CD28 interactions (Fig. 5a). To prepare G-DCs, DCs were first activated by subjecting JAWSII murine DCs to SIINFEKL peptide pulsing and lipopolysaccharide stimulation. G-DCs were then prepared using 4 wt% PEG-DA with both activated and nonactivated DCs (Supplementary Fig. 16A, B). DCs fixed with glutaraldehyde were prepared as a control. Flow cytometric analysis of DC surface markers showed the expression of H-2Kb/SIINFEKL and CD80 were largely similar before and after the gelation process (Fig. 5b–c). Among the activated DC samples, expression of H-2Kb/SIINFEKL complexes in live and gelated DCs were 98.2% and 89.5%, respectively (p > 0.05). Likewise, the live and gelated DCs showed comparable CD80 levels at 79.3% and 63.4% respectively (p > 0.05). The nonactivated live and gelated DCs also shared similar basal levels of H-2Kb/SIINFEKL and CD80 expression, demonstrating that the cell-surface protein signature was effectively preserved following the gelation process. The interaction between G-DCs and T-lymphocytes was further...
examined by incubating CD8+ T cells derived from OT-I transgenic mice with activated G-DCs. The G-DCs effectively expanded the target T lymphocytes in a time- and cell ratio-dependent fashion (Fig. 5f and Supplementary Fig. 16C, D), validating preservation of functional cell membrane interface by intracellular hydrogelation.

Gelated APCs dynamically interact with T cells. T-cell interaction with activated G-DCs was visually examined through confocal microscopy. Upon incubation, multiple T cells were observed to interact with G-DCs actively, forming cell–cell conjugates (Fig. 6a and Supplementary Movie 8). Time-lapse imaging revealed continuous morphological changes and movements.
among the T cells in apposition to the G-DCs, and each engagement event lasted approximately 2–3 h (Supplementary Movie 8). The interaction dynamic between the G-DCs and the T cells was similar to the many reports on live APCs and T cells,26,27, and this long-lasting yet nonstatic engagement has been deemed an essential process for immunological synapse formation and T-cell activation. Between glutaraldehyde-fixed DCs and T cells, no interaction was observed. We also examined CD80 distribution on G-DCs upon T-cell engagement by incubating CD80-GFP-transfected G-DCs with antigen-specific T cells. Following 3 h of incubation, fluorescent CD80 clusters were observed at G-DC/T-cell junctions (Fig. 6b and Supplementary Fig. 16E). Presence of these CD80 clusters attests to the fluid membrane interface on GCs and indicates that the T cells were able to sample and recruit these co-stimulatory signals on the G-DC surfaces. With CD80 clusters being a hallmark of immunological synapse, their presence helps justify the prominent T-cell expansion triggered by the G-DCs.

Gelated APCs for ex vivo and in vivo T-cell expansion. We then compared the antigen presentation capability of G-DCs to that of live DCs. An ex vivo T-cell proliferation assay using CFSE-labeled CD8+ OT-I T cells was first performed with G-DCs, live DCs, and glutaraldehyde-fixed DCs derived from the same cell source. Compared to live DCs that expanded the T cells by 78.7%, the G-DCs induced 56.2% of T-cell proliferation, which retained their spherical morphology (Supplementary Fig. 16B), ably triggered antigen-specific T-cell expansion in vivo (Fig. 7e, f). In contrast, nongelated DCs showed prominent disintegration and had significantly reduced capacity in expanding T cells. In addition, G-DCs mechanically disrupted by ultrasonication resulted in reduced T-cell expansion, further corroborating hydrogel’s function in maintaining plasma membrane functionality.

Discussion
In summary, intracellular assembly of hydrogel polymers was made possible through photoactivated cross-linking, presenting a unique cellular fixation strategy that seamlessly bridges the robustness of synthetic materials with the biochemical complexity of natural cells. In contrast to common fixation techniques based on chemical fixatives, intracellular hydrogelation avoids cross-linking of membrane-bound components, preserving fluid and functional plasma membrane interfaces for biological interactions. Several studies have previously examined de novo generation of globular and filamentous hydrogels in cells to mimic RNA granules,29, stimulate the phase transition of RNA/protein bodies,30, and induce cellular apoptosis.31 However, cellular fixation and cell membrane preservation were not observed in these works. The present work differs from the aforementioned approaches in that the rapid, photoactivated assembly of covalently bonded hydrogel networks permits fast cytosolic immobilization, thereby enabling cellular fixation while obviating cellular reorganization and other cellular responses. Future development of the gelated cellular systems could further benefit from the growing arsenal of membrane manipulation strategies for intracellular hydrogel delivery,32 which may be less disruptive as compared to the freeze–thaw approach adopted in the present work. Toward biomimetic materials engineering, intracellular hydrogelation permits facile preparation of stable, cell-like constructs, offering a robust platform for device development. As cell membranes are a widely present interface with broad biological implications, the present system sees broad potential applications in biomembrane research and biomaterials engineering.
Intracellular gelation of suspension and adherent cells. Gelation buffers were first prepared by mixing protease inhibitors (Pierce™ Protease Inhibitor Mini Tablets; ThermoFisher), 1 wt% of 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure D-2959; Sigma-Aldrich), and poly(ethylene glycol) diacrylate (PEG-DA; Mn = 700 Da; Sigma-Aldrich) ranging from 4 to 40 wt% in 10 mM phosphate buffer. For fluorescent labeling of the hydrogel network, the gelation buffers were supplemented with 0.05 wt% of 2-hydroxy-5-fluorescein diacetate (Sigma-Aldrich). For cross-linking cells in suspension, adherent cells (i.e., HeLa and JAWSII cells) were detached using an enzyme-free cell dissociation buffer (ThermoFisher). For cross-linking adherent cells, cells grown on a tissue culture plate were washed twice in PBS for further experiments.

Quantification of intracellular PEG-DA concentrations. Quantification of intracellular PEG-DA concentrations was performed using an iodine-based quantification method. Briefly, following PEG-DA infusion, 1 x 10^6 HeLa cells were washed and suspended in PBS to 1 mL. The collected cells were then sonicated in a bath sonicator for 1 min to release the entrapped PEG-DA, and the cellular debris was spun down via centrifugation at 3000 g for 5 min. The supernatants were collected and mixed with BaCl2 and iodine solutions in an 8:2:1 ratio. Following color development for 15 min, PEG-DA concentrations in the supernatants were collected and mixed with BaCl2 and iodine solutions in an 8:2:1 ratio. Following color development for 15 min, PEG-DA concentrations in the supernatants were quantified using absorbance at 600 nm. The resulting GCs were washed twice in PBS to 1 mL. The collected cells were then sonicated in a bath sonicator for 1 min to release the entrapped PEG-DA, and the cellular debris was spun down via centrifugation at 3000 g for 5 min. The supernatants were collected and mixed with BaCl2 and iodine solutions in an 8:2:1 ratio. Following color development for 15 min, PEG-DA concentrations in the supernatants were quantified using absorbance at 600 nm. The resulting GCs were washed twice in PBS for further experiments.

Transmission electron microscopy. Cellular samples were fixed using 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 overnight at 4 °C. After postfixation in 1% osmium tetroxide and pre-embedding staining with 1% uranyl acetate, tissue samples were dehydrated and embedded in Agar 100. Sections measuring 80 nm were then examined using an FEI Tecnai G2 TWIN microscope equipped with a field emission gun.
**Cryogenic scanning electron microscopy.** For cryogenic scanning electron microscopy (cryo-SEM) imaging, an FEI Quanta 200/Quorum PQ2000TR FEI, 2007 high-resolution SEM was used. Briefly, HeLa cells were seeded on Aclar embedding films for 24 h prior to PBS or gelation treatments. Before imaging, the samples were washed with PBS and suspended in RO water for freezing by liquid nitrogen. The samples were then etched under vacuum and imaged at an acceleration voltage of 3 kV by cryo-SEM.

**Examination of membrane order in GCs.** Examination of membrane lipid order in GCs was carried out according to a previously described protocol. Briefly, GCs and control cells were stained in media containing 100 μg mL⁻¹ of Laurdan dye and 0.5% of DMSO to 200 μL of serum-free media (G-DC) or 200 μL of 1× PBS (normal DCs) for 24 h prior to PBS or gelation treatments. Before imaging, the cells were washed with PBS or gelation and placed into RPMI1640 complete medium with 10% FBS. In order to harvest single splenocytes, the spleens were removed and stained with the tip of a 5 µl syringe against a sterilizer (BD Biosciences, #352340). Splenocytes were incubated with BD Pharm Lyse lysing buffer (BD Biosciences, #555899) for 3 min to remove RBCs. OT-I cells were subsequently isolated from the splenocytes using a Mouse CD8α⁺ T Cell Isolation Kit (BD Biosciences, #198553). OT-I cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) by incubating the cells with 5 µM CFSE (Sigma-Aldrich, #21888) at 37 °C for 5 min. The cells were washed three times with complete medium. CFSE-labeled cells were harvested for further experimental studies.

**Examination of G-DC/T-cell interaction.** For observation of G-DC/T-cell interactions, adherent JAWSII DCs were gelated using 4% PEG-DA and subsequently stained with CellTracker™ Deep Red dye (Molecular Probes) at 37 °C for 30 min. Stained G-DCs were washed twice using PBS and resuspended in RPMI1640 complete media supplemented with 10% FBS. CFSE-labeled OT-I cells were subsequently added to the G-DCs. The interaction between G-DCs and OT-I CD8 T cells was subsequently imaged using a confocal microscope (Zeiss LSM780 confocal microscope system, Zeiss) and analyzed using LSM Image Browser software (Zeiss). To examine CD80-GFP expression, G-DCs were transfected with CD80-GFP plasmids using the TransIT-TKO transfection reagent (Mirus, #2154) following a previously described protocol. Briefly, plasmids were prepared using a Qiagen Plasmid Midi kit (QIAGEN, #21243). Transfection mixtures consisting of 5 µL of serum-free DMEM, 20 µg of plasmids, and 40 µL of transfection reagent were prepared and transfected into JAWSII DCs. Following 48 h of incubation, a total of 10% of complete medium was added to the cells. 48 h after transfection, CD80-GFP-expressing JAWSII cells were gelated with 4 wt% PEG-DA and used for examining CD80 clustering upon incubation with antigen-specific T cells.

**Proliferation assay ex vivo.** CFSE-labeled OT-I cells were co-cultured with live DCs, G-DCs or glutaraldehyde-fixed DCs at different ratios. Co-cultured cells in 96-well v-bottomed plates were cultured at 37 °C for indicated time periods. After harvesting, cells were stained with allophycocyanin-conjugated rat anti-mouse CD8α antibodies (eBioscience, #100712, Clone 53-6.7, 1:100) and analyzed by flow cytometry. Proliferation analysis platform in FlowJo was used to analyze cell division. For experiments involving stored G-DCs and DCs, G-DCs, and DCs were stored in PBS for 4 days at 2°C for 21 days. Homogenized G-DCs were prepared by sonication 21-day-old G-DCs using a Fisher Scientific 150E Sonic Dismembrator at 80% power pulse (3 s on/1 s off) for 1 min. The G-DC, DC, and glutaraldehyde-fixed DC samples were derived from the same cell source for each separate experiment.

**T-cell proliferation assay in vivo.** CFSE-labeled splenocytes were adoptively transferred into 6–8 week-old C57BL/6 mice via tail vein injections in a volume of 100–200 µL per animal. In a single experiment, 3 x 10⁷ T cells were challenged with live DCs, G-DCs or Glut-fixed DCs at a cell concentration of 10⁸ cells/100 µL, 24 h after aerosol delivery of 10⁶ CFSE-labeled T cells. In order to determine the duration of T-cell engagement, spleens were harvested from the mice and stained with allophycocyanin-conjugated rat anti-mouse CD8α and CD4 antibodies followed by flow cytometry and FlowJo analysis. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Academia Sinica. The G-DCs and D-DCs were derived from the same source of activated or nonactivated DCs.

**Reporting summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** All relevant data are available in the authors and/or are included within the manuscript and Supplementary Information.
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Author contributions
J.C.L., C.Y.C., Y.I.C., H.W.C. and C.M.J.H. conceived the experimental designs. J.C.L., C.Y.C., Y.I.C., J.Y.C., B.Y.Y., N.N.L., Z.S.F. and W.Y.C. performed the optimization and characterization of the intracellular hydrogelation protocol. C.Y.C., Y.I.C., C.L.L. and B.Y.Y. performed the membrane fluidity analysis. J.C.L and Y.H.L. performed the immunological assays. J.C.L., C.Y.C., C.L.L. and C.M.J.H. prepared the paper. All authors have read and approved the paper.

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