

Graphene

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Graphene Oxide Restricts Growth and Recrystallization of Ice Crystals

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Abstract: We show graphene oxide (GO) greatly suppresses the growth and recrystallization of ice crystals, and ice crystals display a hexagonal shape in the GO dispersion. Preferred adsorption of GO on the ice crystal surface in liquid water leads to curved ice crystal surface. Therefore, the growth of ice crystal is suppressed owing to the Gibbs-Thompson effect, that is, the curved surface lowers the freezing temperature. Molecular dynamics simulation analysis reveals that oxidized groups on the basal plane of GO form more hydrogen bonds with ice in comparison with liquid water because of the honeycomb hexagonal scaffold of graphene, giving a molecular-level mechanism for controlling ice formation. Application of GO for cryopreservation shows that addition of only 0.01 wt % of GO to a culture medium greatly increases the motility (from 24.3% to 71.3%) of horse sperms. This work reports the control of growth of ice with GO, and opens a new avenue for the application of 2D materials.

Understanding and controlling ice formation is important in both fundamental research and practical applications.^[1] Nature has unique ways of regulating ice formation, for example, antifreeze proteins (AFPs) protect organisms from freeze damage by regulating ice formation^[2] by controlling the arrangement of hydroxy groups.^[3] Recently, both experiments^[4] and theoretical calculations^[5] have shown the coexistence of large oxidized and unoxidized graphene regions on the surface of graphene oxide (GO; Figure 1 a). The hydroxy (-OH) and epoxy (-O-) groups are located at oxidized regions of the basal plane of GO, whereas the carboxyl groups mainly localize at the periphery of GO.^[6] The plane of GO

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Figure 1. Arrangement of oxidized groups on the basal plane of GO matches with the ice crystal and effects of GO on the growth and shape of ice crystal. a) Illustration of the structure of GO shows the location of hydroxy and epoxy groups on the basal plane of GO with the repeated honeycomb hexagonal carbon ring scaffold. The atoms are colored as follows: C, blue; O, red; H, white. b) The side view illustrates the possible hydrogen bonds (colored by green) between the ice crystal and hydroxy groups on the basal plane of GO. c, d) Optical images exhibit a completely different growth behavior and shape of ice crystals with and without the addition of GO in pure liquid water. The concentration of GO is 1.0 mg mL⁻¹. *c* axis and *a* axis of the ice crystal are indicated by the arrows. Scale bars = 40 μ m. The growth and shape of ice crystals in a NaCl solution of 8.0 mg mL⁻¹ with and without GO are shown in Figures S8–S13, which consolidate the profound effect of GO on ice formation.

consists of repeated honeycomb hexagonal carbon rings (7.35 Å×4.26 Å). This honeycomb scaffold structure arranges the hydroxy groups on GO to match with the ice crystal lattice^[7] (Figures 1 a,b), which is reminiscent of the organization of hydroxy groups on the ice-binding surface of AFPs.^[8] This implies that GO may mimic AFPs in controlling ice formation.

The effectiveness of GO in controlling the shape and growth of ice crystals is shown in Figures 1 c,d and S1–S7. A typical hexagonally shaped ice crystal was observed in the GO dispersion after a disc-shaped single ice crystal was kept at -0.40 °C for 240 s, afterwards the ice crystal maintained its shape without any observable growth (7200 s was demonstrated; Movie S1). This is in stark contrast with the ice growth without the addition of GO; a typical flat disc-shaped ice crystal grows rapidly at -0.40 °C^[10] (Figure 1 d, Movie S2) and the whole observation window is full of ice within 30 s (Movies S3 and S4).

GO of various concentrations in liquid water shape ice crystals in exactly the same way, but the growth rate (r) of ice



Figure 2. Mechanism of GO in controlling the growth and shape of ice crystal. a) The variation of the growth rate (r) with the change of the GO concentration (c). All of the data were obtained at a supercooling temperature of 0.04° C (the temperature below the equilibrium melting temperature, Δ T). Each data point represents an average of at least 9 measurements. b) The effect of GO with different concentrations on the growth rate (r) of ice crystals along *a* axis at various Δ T. c) Ice crystal growth rate (r) investigated on GO of the same size (500 nm) but with various C/O ratios. d) The size effect of GO on the growth rate (r) of ice crystals, and the effect of carboxyl-functionalized graphene (GCOOH) on the ice growth rate is also shown for comparison. GCOOH is used as a negative control owing to its lack of observable effect on ice formation. e) Ice affinity experiment for confirming the adsorption of GO on ice crystal surface. Ice crystals were obtained by placing liquid N₂ above the dispersion. In each cycle, an aliquot amount of ice melt was extracted to calculate the concentrations of samples in ice. The above procedure was repeated for more than 20 times. After each cycle, the freezing rate was decreased (details of the experiments are shown in the Figure S23). f) Proposed mechanism of ice growth inhibition, the curve formed between adsorbed GO sheets depresses the freezing of ice.

crystals first decreases and then saturates with the increase of concentration of GO ($C_{\rm GO}$). It decreases from 3.07 $\mu m s^{-1}$ $(C_{\rm GO} = 0.01 \text{ mg mL}^{-1})$ to 0.94 μ m s⁻¹ $(C_{\rm GO} = 8.0 \text{ mg mL}^{-1})$ at a supercooling temperature of 0.04°C (Figure 2a), which is slower than the growth rate in pure water $(3.68 \ \mu m s^{-1})$ at the same supercooling temperature. One important feature is that a discontinuity (highlighted by arrows in Figures 2b-d and S11-S13) of the growth rate can be observed as the supercooling and concentration of GO dispersion increases. Further investigations on the effect of the carbon to oxygen ratio (GO of various C/O ratios with the size being the same) and the size of GO show that the effectiveness of GO in controlling ice growth decreases with increases in the C/O ratio and size of GO (Figures 2b,c, S14-S16). For comparison, carboxyl-functionalized graphene (GCOOH; Figure S2a) was also investigated, and displayed no observable effects on the growth and shape of ice crystals (Figures S8, S17-S22). This indicates that GO possesses a thermal hysteresis (TH, the difference between the equilibrium melting and freezing temperatures of an ice crystal), which is a typical feature shared by all AFPs.^[11]

The experimental data points in Figure 2 a can be well fitted by $r = 1.14 + 2.36e^{(-C_{GO}/0.13)}$; and the fitting can be explained by making two reasonable assumptions: i) GO adsorption onto the ice crystal is described by a Langmuirtype kinetic model,^[12] and ii) the rate of ice crystal growth is

proportional to the uncovered ice crystal surface area. Therefore, we propose that preferred adsorption of GO on the ice crystal surface in great excess of liquid water leads to curvatures on the ice crystal surface, which suppress the further growth of ice crystals owing to the Gibbs–Thomson effect,^[2] that is, the surface curvature depresses the freezing point (Figure 2 f). The adsorption of GO on the ice crystal surface was verified by a modified ice affinity experiment on *Microdera punctipennis dzungarica* antifreeze protein (*Mpd*AFP), GO 10 nm, and GCOOH (Figure 2 e; for specific procedures, see Figure S23). An adsorption equilibrium was observed on *Mpd*AFP and GO, while no adsorption equilibrium was observed on GCOOH.

Molecular dynamics (MD) simulation^[13] revealed the molecular-level mechanism for GO to preferably adsorb onto the ice crystal surface. Figure 3a shows that no ice growth can be observed on the ice crystal surface with GO throughout the whole 400 ns simulation time, whereas continuous growth is observed on the ice crystal surface without GO. For comparison, the same simulation was also carried out on GCOOH. The GCOOH nanosheet model consisting of 100% –COOH groups without any –OH (hydroxy) or –O– (epoxide) groups on the basal plane was generated by replacing all of the oxidized groups (–OH and –O–) on the GO nanosheet using –COOH groups, which is consistent with our experiment results. The GCOOH and GO were placed atop the fastest



Figure 3. MD analysis for the molecular level mechanism of the preferred adsorption of GO on the ice crystal surface. a,b) Representative simulated trajectories of the effect of GO and GCOOH on the growth of ice crystals, the snapshot time is shown on the top of each frame. The ice crystal is covered by light blue, and liquid water is shown in red lines. The atoms are colored as follows: C, cyan; O, red; H, white. The rotation is along the z-axis (the axis shown in Figure S33). c) The average number of hydrogen bonds between the oxidized groups on the basal plane of GO (or GCOOH) and water or ice, which are calculated by dividing the number of hydrogen bonds formed between the corresponding oxidized group on GO (or GCOOH) and ice or water by the total number of oxidized groups on GO (or GCOOH). d) The snapshot of the molecular dynamics (MD) trajectories shows that the formation of ice-like water atop of GO due to the 2D-controlled arrangement of hydroxy groups on GO matching with the ice crystal lattice, and oxygen of water molecules highlighted by green ball, form hydrogen bonds with hydroxy groups of GO. Inset is the side view.

growth face of ice crystal $(1 \ 1 \ \overline{2} \ 0)$,^[14] with a distance of 6 Å as the initial position and can freely move in water. The simulation showed that GCOOH is pushed away as ice grows (Figure 3b). Evaluation of the center of mass of GO/ GCOOH atop the ice crystals with the simulation time further verified the distinct performance of GO and GCOOH on ice crystal growth (Figure S24). Figure 3 a clearly shows a curve forms on the ice surface between adsorbed GO sheets (Figure S25), that is, the Gibbs–Thomson effect for the inhibition of ice growth.

We adopted a commonly used geometry definition of hydrogen bonding to determine if the hydrogen bonds are formed. The hydrogen bond between liquid water or ice water molecules and oxidized groups on the GO/GCOOH basal plane were defined as the O···O distance less than 3.5 Å and simultaneously the angle H–O···O less than 30°.^[15] Detailed analysis showed that the average number of hydrogen bonds that each –OH and –O– group on the basal plane of GO forms with the ice crystal (HB_{GO-Ice}) are 0.89 and 0.65 (1.54 in

all), which exceeds those with liquid water (HB_{GO-Water}, 0.85 and 0.55 respectively, 1.40 overall; Figure 3c). By contrast, the average number of hydrogen bonds that each COOH group on the basal plane of GCOOH forms with liquid water (1.03) is higher than that with ice (0.96). It is worth mentioning that the average lifetime of HB_{GO-Ice} is clearly longer than that of HB_{GO-Water} (Figure S26). A snapshot of the MD simulation shows the formation of an ice-like liquid water layer atop of GO (Figure 3 d, connected by dotted blue lines, Figure S25) owing to the 2D arrangement of hydroxy groups on the basal plane of GO. In all, it is favorable for GO to be adsorbed on the ice crystal surface in the liquid water environment.

Hydrophobic interactions and hydrogen bonding between AFPs and ice crystals have been regarded as the main mechanisms for AFPs to bind preferentially to the ice crystal surface, although which one dominates is still elusive.[11] Our experimental investigation on the C/O ratio displays that the increase of the C/O ratio leads to a decreased effectiveness of GO in controlling ice formation. The increase of the C/O ratio of GO can be translated to the increase of the hydrophobic graphite region and the reduced

density of hydroxy groups on the basal plane of graphene. Therefore, our investigation leads to the conclusion that hydrogen bonding is more important than the hydrophobic interactions for GO to adsorb onto the ice crystal surface consistent with our MD simulation analysis. This enhances the molecular-level understanding of the preferred adsorption of AFPs to the ice crystal surface and consequently the ice formation.

Previous reports have indicated that the adsorption of AFPs onto the ice crystal surface could endow the AFPs with the activity of ice recrystallization inhibition (IRI).^[11] Therefore, quantitative evaluation of the IRI activity of GO was investigated (Figures 4 a,b, S27, and S28), and showed that the addition of 5.0 mgmL⁻¹ of GO to a NaCl solution (8.0 mgmL⁻¹) can reduce the average grain size up to one order of magnitude, indicating a high IRI activity of GO when compared with some reported IRI agents (Figure S29, Table S2).^[16] The behavior of GO in controlling ice formation resembles that of *longsnout poacher* (*LpAFP*),^[17] that is, both

Communications



Figure 4. Ice recrystallization inhibition activity of GO and its application for cryopreservation. a, b) Microscopic images of ice crystals grown in the NaCl solution of 8.0 mg mL⁻¹ with and without GO (5 mg mL⁻¹, 500 nm in size) after annealing at -6° C for 30 mins, and the insets are the grain size distributions. c) Quantitative assessment of the grain size of ice crystals obtained from a NaCl solution (8.0 mg mL⁻¹) and the culture medium for horse sperm with the addition of GO, and grain sizes of ice crystals obtained from two aqueous solutions with the addition of glycerol, PVA, GCOOH, and *Mpd*AFP are shown for comparison. d) The motility of cryopreserved horse sperm with different cryoprotectants. Error bars represent the standard deviation.

possess a high IRI activity and a low TH activity, which is desired for cryopreservation. $^{\left[18\right] }$

The suitability of GO as a cryoprotectant was demonstrated by the cryopreservation of horse sperm. We first investigated the IRI activity of GO in the culture medium in comparison with glycerol (a conventional cryopretectant for horse sperm), polyvinyl alcohol (PVA, molecular weight $31\,000$ to $50\,000$ g mol⁻¹ with an average degree of hydrolysis of 100 %),^[19] GCOOH, and MpdAFP (Figure 4 c). The results showed that GO unambiguously exhibits a high IRI activity both in culture medium and NaCl solution. The motility of cryopreserved horse sperm thawed at 37°C with GO as the cryoprotectant is shown in Figure 4d, as compared with the other cryoprotectants: glycerol, GCOOH, and MpdAFP. Consistent with previous reports,^[16] employing MpdAFP as the cryoprotectant reduces the motility of horse sperm owing to the high TH activity, because the addition of MpdAFP triggers no sperm death before the programmed cooling and thawing procedure (Figure S30). In sharp contrast, the motility of horse sperm with GO as the cryoprotectant was greatly increased from 24.3% to 71.3%, and the membrane integrity was maintained (Figure S31, Movies S5-S9) when GO of 0.01 wt% concentration was used as the cryoprotectant, which is much higher than that with glycerol as the

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Conflict of interest

The authors declare no conflict of interest.

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cryoprotectant (3.5 wt%, the optimized concentration; Figure S32). The cryopreservation studies clearly demonstrate that GO can be utilized as an effective cryoprotectant.

In summary, we report the effectiveness of GO in restricting ice growth and recrystallization, and it is speculated that GO may mimic AFPs in regulating ice formation. MD simulation demonstrated the preferred binding of GO to the ice crystal surface in great excess of liquid water, consistent with our experiments observation, which endows GO the capability to control ice formation. Molecularly speaking, the 2D-controlled arrangement of hydroxy groups on the basal plane of GO leads to the formation of more hydrogen bonds with ice crystals in comparison with liquid water, as revealed by MD simulation analysis. Moreover, GO was demonstrated as an effective cryoprotectant for horse sperm, which opens a new variety of avenues for the application of 2D materials. In the future we will further utilize GO for the cryopreservation of cells and organs.



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