Nanomaterials-based biomimetic catalysts with multiple functions are necessary to address challenges in artificial enzymes mimicking physiological processes. Here we report a metal-free nanozyme of modified graphitic carbon nitride and demonstrate its bifunctional enzyme-mimicking roles. With oxidase mimicking, hydrogen peroxide is generated from the coupled photocatalysis of glucose oxidation and dioxygen reduction under visible-light irradiation with a near 100% apparent quantum efficiency. Then, the in situ generated hydrogen peroxide serves for the subsequent peroxidase-mimicking reaction that oxidises a chromogenic substrate on the same catalysts in dark to complete the bifunctional oxidase-peroxidase for biomimetic detection of glucose. The bifunctional cascade catalysis is successfully demonstrated in microfluidics for the real-time colorimetric detection of glucose with a low detection limit of 0.8 μM within 30 s. The artificial nanozymes with physiological functions provide the feasible strategies for mimicking the natural enzymes and realizing the biomedical diagnostics with a smart and miniature device.
Natural enzymes with high substrate specificity and catalytic efficiency prevail to mediate the biological processes in living organisms under mild reaction conditions. However, because protein enzymes suffer from high cost of production and intrinsic instability, nanomaterials-derived artificial enzymes, nanozymes, have been extensively investigated to imitate the protein enzymes in biomimetic chemistry. For example, glucose oxidase (GOx) and horseradish peroxidase (HRP) as the prototype enzyme pair have been often employed in enzyme cascade catalysis particularly for blood glucose monitoring, and various nanozymes have been developed for their applications to enzymatic reactions. Since the first peroxidase-like nanozymes of magnetite was reported, a series of oxide- and carbon-based nanomaterials with good stability and specificity have been employed to mimic HRP for the peroxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)-di ammonium salt (ABTS) in the presence of hydrogen peroxide (H₂O₂) or in cascade glucose detection. In such nanozyme systems, H₂O₂ is generated from glucose oxidation in the presence of GOx and the resulting H₂O₂ is subsequently utilized by nanozymes to oxidize chromogenic substrates through their peroxidase mimicking for colorimetric detection of the glucose level (see Supplementary Table 1). In the development of peroxidase-like nanozymes for cascade glucose detection, the production of intermediate H₂O₂ is the main rate-determining step in the enzymatic reaction, and the glucose-GOx system suffers from the poor atom efficiency. Therefore, the efficient H₂O₂ production using an alternative GOx-like nanozyme that plays the bifunctional roles (both GOx-like and peroxidase-like) is urgently required. Although there have been some reported examples of bifunctional oxidase-peroxidase mimicking nanozymes, all of them are based on expensive noble metal catalysts (see Supplementary Table 2). From this point of view, the metal-free bifunctional nanozymes consisted of earth-abundant elements only are highly desired.

Compared with the common methods of H₂O₂ production such as anthraquinone method and noble metal-based catalysis, the photocatalytic generation of H₂O₂ through the proton-coupled electron transfer to dioxygen (eq. 1) is highly desirable since it does not need H₂ gas reagent and the process operating in ambient condition is eco-friendly. The major challenges in the photocatalytic production of H₂O₂ are to enhance the selectivity of two electron transfer to dioxygen and to minimize the decomposition of in situ produced H₂O₂. The graphitic carbon nitride (g-C₃N₄) is an ideal material that can hinder the in situ decomposition of H₂O₂ since it has lower adsorption for H₂O₂ in addition, chemical functional groups and electronic properties of the GCN can be easily varied through simple modification, which makes it a promising photocatalyst for H₂O₂ production. Although GCN with good biocompatibility has been also employed as a peroxidase-mimicking nanozyme for glucose detection, its GOx-mimicking behaviour has never been explored in enzymatic cascade reactions (domino reactions) for colorimetric detection of glucose.

\[
\text{O}_2 + 2\text{H}^+ + 2e^- \rightarrow \text{H}_2\text{O}_2; E^0 = 0.695 \text{V}_{\text{NHE}}.
\]

Herein, we propose the example of a bifunctional metal-free nanozyme of modified GCN, which performs the dual roles for oxidase-mimicking in glucose oxidation and peroxidase-mimicking in chromogenic substrate oxidation under irradiation and dark condition, respectively. The selectivity for dioxygen reduction and efficient charge separation promote the in situ photogeneration of H₂O₂ from glucose oxidation under visible light. The in situ produced H₂O₂ is then utilized for subsequent peroxidation of a chromogenic substrate on the same modified GCN to complete the bifunctional oxidase-peroxidase mimicking in glucose detection. Finally, the GCN-based bifunctional enzyme-mimicking cascade catalysis is successfully demonstrated in a continuous flow microfluidic reactor for rapid and sensitive real-time monitor of glucose.

### Results

#### Design and characterization of the bifunctional nanozyme

When coupled with the glucose-GOx, in the in situ production of H₂O₂ from glucose oxidation serves for the subsequent HRP-mediated peroxidation of TMB for colorimetric detection of glucose (Fig. 1a). In this work, we employed the modified GCN as an artificial enzyme (nanozyme) that mimics the dual roles of GOx (oxidizing glucose with in situ production of H₂O₂) and HRP (TMB oxidation using in situ generated H₂O₂) in natural enzyme system (Fig. 1b).

The GCN photocatalyst is ideally suited for the visible light-induced synthesis of H₂O₂ since the generated H₂O₂ has little adsorption ability onto GCN surface and its in situ production and intrinsic instability, nanomaterials-derived artificial enzymes, nanozymes, have been extensively investigated to imitate the protein enzymes in biomimetic chemistry. For example, glucose oxidase (GOx) and horseradish peroxidase (HRP) as the prototype enzyme pair have been often employed in enzyme cascade catalysis particularly for blood glucose monitoring, and various nanozymes have been developed for their applications to enzymatic reactions. Since the first peroxidase-like nanozymes of magnetite was reported, a series of oxide- and carbon-based nanomaterials with good stability and specificity have been employed to mimic HRP for the peroxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)-di ammonium salt (ABTS) in the presence of hydrogen peroxide (H₂O₂) or in cascade glucose detection. In such nanozyme systems, H₂O₂ is generated from glucose oxidation in the presence of GOx and the resulting H₂O₂ is subsequently utilized by nanozymes to oxidize chromogenic substrates through their peroxidase mimicking for colorimetric detection of the glucose level (see Supplementary Table 1). In the development of peroxidase-like nanozymes for cascade glucose detection, the production of intermediate H₂O₂ is the main rate-determining step in the enzymatic reaction, and the glucose-GOx system suffers from the poor atom efficiency. Therefore, the efficient H₂O₂ production using an alternative GOx-like nanozyme that plays the bifunctional roles (both GOx-like and peroxidase-like) is urgently required. Although there have been some reported examples of bifunctional oxidase-peroxidase mimicking nanozymes, all of them are based on expensive noble metal catalysts (see Supplementary Table 2). From this point of view, the metal-free bifunctional nanozymes consisted of earth-abundant elements only are highly desired.

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\[
\text{H}_2\text{O}_2 + \text{H}^+ + \text{e}^- \rightarrow \text{OH}^+ + \text{H}_2\text{O}; E^0 = 1.14 \text{V}
\]

\[
\text{H}_2\text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow 2\text{H}_2\text{O}; E^0 = 1.763 \text{V}
\]
photodecomposition can be minimized. The pure GCN, however, has a low photoactivity for H₂O₂ production and therefore the modification of GCN with multiple elements doping has been tried to enhance the photoefficiency significantly. A recent study that synthesized KPF₆-modified GCN achieved an apparent quantum yield of 24% at 420 nm for the production of H₂O₂ in ethanol solution. To further increase the photoefficiency of H₂O₂ production to mimic the high efficiency of GOx enzyme, we developed a modified GCN. The synthesis of pristine GCN via the one-pot thermal polycondensation of melamine was modified by introducing KOH or/and KCl. The modified GCN samples incorporated with KOH, KCl, and both are referred as ACN, KCN, and AKCN, respectively. The resulting AKCN exhibited the main phase of GCN in the XRD spectrum. The fact that the (002) peak shifted along with the disappearance of (100) peak indicates the presence of K interaction among the interlayer and in-plane, which contributed to the formation of tightly stacked layers on modified GCN compared with pristine GCN, judging from the FESEM images. In FTIR spectra, the surface hydroxyl group grafting (N–H) over AKCN was evidenced from the appearance of extra bands at 1000, 1158, and 2152 cm⁻¹ (Fig. 2b), which indicates the replacement of terminal –NH₂ groups by hydroxyl groups after KCl and KOH introduction. In addition, the noticeable appearance of the new band around 2180 cm⁻¹ in ACN, KCN, and AKCN samples can be ascribed to the cyano groups (C≡N) transformed from the terminal –C–NH₂ at the melon structural unit.

The compositions and chemical states in pristine and modified GCN samples were further analysed through XPS survey analysis. In the high resolution C 1s spectra of pristine GCN (Fig. 2c), the typical components around 288.2, 286.4, and 284.8 eV can be indexed as N–C≡N, C–NHₓ, and adventitious carbon, respectively. The markedly enhanced peak around 286.4 eV in ACN, KCN, and AKCN samples can be ascribed to the cyano groups (C≡N) transformed from the terminal –C–NH₂ at the melon structural unit.
Photocatalytic H$_2$O$_2$ production. The ideal atom efficiency of sunlight-driven H$_2$O$_2$ generation (overall reaction as eq. 4) can be up to 100% by coupling the efficient water oxidation (eq. 5) and the selective two-electron reduction of O$_2$ (eq. 1). However, the photocatalytic production of H$_2$O$_2$ has been commonly investigated by utilizing alcohols as an electron donor since the water oxidation (eq. 5) is inefficient$^{21,22}$. This study aims to utilize glucose as an electron donor instead of alcohols and employed GCN as a photoenzyme that mimics the role of GOx under visible light. Since the activity of pure GCN for the production of H$_2$O$_2$ is low, the pure GCN was further modified by incorporating KOH, KCl, and both (KOH/KCl). ACN and KCN were optimized for the content of KOH and KCl and characterized by XRD and FTIR (Supplementary Figure 3 and 4). The photocatalytic production of H$_2$O$_2$ from the resultant samples was tested in the presence of ethanol (see Fig. 3a), which should serve as a proxy test for H$_2$O$_2$ production coupled with glucose oxidation$^{25}$. The highly selective H$_2$O$_2$ formation under visible light was enabled by promoting two-electron reduction of O$_2$ via the rapid formation of 1,4-endoperoxide species in the polymeric GCN structure$^{23}$. The in situ generated H$_2$O$_2$ can be also utilized as a Fenton reagent to accelerate the photocatalytic oxidation process$^{34,38}$. Among various GCN samples modified with different reagents containing alkali metal ions and halide ions, the GCN incorporated with KCl and KOH exhibited markedly higher activities (Supplementary Figure 3d and 4d). To investigate whether the optimal molar ratio can be different when both KOH and KCl are copresent, we carried out an additional optimization of KOH content in the presence of a couple of alkali metal ions and halide ions, the GCN as a photoenzyme that mimics the role of GOx under visible light was enabled by promoting two-electron reduction of O$_2$ via the rapid for-

$$2H_2O + O_2 \rightarrow 2H_2O_2$$

(4)

$$2H_2O + 4H^+ \rightarrow O_2 + 4H^+$$

(5)

It is noted that all the modified GCN (ACN, KCN, and AKCN) exhibited more negative zeta potentials in comparison with pure GCN (Supplementary Figure 6). The higher negative surface charge on the modified GCN should be favourable for H$_2$O$_2$ production (eq. 1) since the supply of protons onto the negatively charged catalyst surface should be facilitated by the electrostatic attraction$^{24,39}$. AKCN produced the highest amount of H$_2$O$_2$ consistently in a wide pH range (pH 3–11) than GCN, ACN, and KCN. When comparing different photocatalysts, the activities were measured at pH 3 because the activity differences among different photocatalysts were most clearly observed at this pH (see Fig. 3d). However, it should be noted that the optimal catalyst composition determined at pH 3 was the same as that determined at phosphate buffer condition (see Supplementary Figure 3c and 4c). In this case, AKCN exhibited the highest enhancement at neutral phosphate buffer solution as a result of the possible specific phosphate promotion (Fig. 3d and Supplementary Figure 5)$^{30,41}$, which concurrently matches the optimal condition of GOx action. The production of H$_2$O$_2$ in the phosphate buffer solution increased in the order of (AKCN > KCN > ACN > GCN), which is the same trend as observed at pH 3, and gradually reached the photostationary state in 16 h irradiation (Supplementary Figure 7). This indicates that the formation and decomposition rate of H$_2$O$_2$ is balanced after a prolonged irradiation$^{42,43}$. In addition, all catalysts (GCN, ACN, KCN, AKCN) exhibited significantly hindered activity for the decomposition of H$_2$O$_2$ under visible light (see Supplementary Figure 7, left axis). Unlike metal oxide photocatalysts with high adsorption for in situ generated H$_2$O$_2$, GCN catalysts with little adsorption ability for H$_2$O$_2$ have insignificant activity for H$_2$O$_2$ decomposition and are ideally suitable for the production of H$_2$O$_2$$^{39,44}$. From the viewpoint of stability, AKCN exhibited a good stability without loss of activity during repeated cycles (Supplementary Figure 8). On the other hand, the characteristics of the electron transfer to O$_2$ on AKCN was investigated by the rotating disk electrode (RDE) analysis, that enables the estimation of the number of electrons (n) transferred to O$_2$ from the slope value of Koutecky–Levich plots (Fig. 3e). The estimated “n” values were close to 2 for pure GCN, ACN, KCN, and AKCN, which indicates that dioxygen molecules is selectively reduced by two-electron transfer only$^{23,25}$.

### Improvement of charge separation

As for the light absorption, the absorption edge of GCN, ACN, and AKCN is progressively redshifted with respect to pristine GCN (Supplementary Figure 9a), which corresponds to the bandgap change from 2.79 eV to 2.70 eV (Table 1) according to the Tauc plot analysis (Supplementary Figure 9a inset). The valence band position could be determined from the valence band XPS (Supplementary Table 1 Structural characteristics of various carbon nitride samples prepared at 550°C

| Samples | Reagent molar ratio | $S_{\text{BET}}$ (m$^2$ g$^{-1}$) | $V_p$ (cm$^3$ g$^{-1}$) | $E_g$ (eV) |
|---------|---------------------|-------------------------------|-------------------|---------|
| GCN     | Melamine            | 7.6                           | 0.05              | 2.79    |
| ACN     | Melamine + KOH/     | 4.8                           | 0.03              | 2.77    |
|         | (1:0.002)           |                               |                   |         |
| KCN     | Melamine + KCl/     | 3.0                           | 0.02              | 2.74    |
|         | (1:0.08)            |                               |                   |         |
| AKCN    | Melamine + KOH + KCl| 2.2                           | 0.01              | 2.70    |

295.2 eV) and Cl 2p (197.3 eV and 198.9 eV) peaks are also significantly shifted from those of KCl (K 2p$_{3/2}$: 293.6 eV; Cl 2p: 199.2 eV), which supports that K$^+$ and Cl$^-$ ions interact with the surrounding C and N atoms$^{37}$. The distribution of K and Cl elements incorporated within AKCN structure is clearly seen from EDS mapping along with the backbone elements of C and N (Fig. 2d). A small O 1s peak was observed in the survey scan (Supplementary Figure 2a) due to the adventitious oxygen-containing species (–C–OH) grafted on the surface, which is consistent with the FTIR result. In comparison with other counterparts, AKCN with higher XPS elemental concentrations of K (8.91 at%) and Cl (0.63 at%) that electronically interact with the chemical structure of GCN should influence the migration and separation of electrons and holes. All the above analysis revealed that AKCN successfully incorporated K and Cl atoms in the GCN framework to bridge the interlayers for efficient charge separation.
Figure 9b). This combined with the above bandgap determination shows that conduction band (CB) and valence band (VB) levels slightly shifted to the positive potential after the modifications of KCl and KOH (Supplementary Figure 9c). However, such a small potential shift cannot provide enough overpotentials for raising the photocatalytic activity of AKCN to the level that is significantly higher than that of GCN, ACN, and KCN. This implies that the markedly high photoactivity of AKCN might be related not only to the thermodynamic factors, but also to the kinetic factors, which should facilitate the charge separation and transfer in the modified structure of GCN. How the photocatalytic activity is strongly enhanced for H$_2$O$_2$ production in AKCN is discussed in the modifiers, which should facilitate the charge separation and transfer in

Glucose oxidase-like activity. AKCN exhibited the superior photoactivity for H$_2$O$_2$ generation in neutral phosphate buffer (0.1 M, pH 7), which happened to be coincident with the typical working condition of glucose oxidase (GOx). When alcohol is replaced by glucose as the electron and proton donor, the GOx-like activity of AKCN can be induced under visible light (OCVD) measurements, AKCN exhibited higher open-circuit voltage and slower photovoltage decay than pure GCN (Fig. 4d), which indicates that the charge recombination in AKCN is hindered for prolonging the lifetimes of charge carriers (Fig. 4d inset)
the photocatalytic oxidation of glucose was negligibly small (2 µmol), compared with that of H₂O₂ (0.8 mM, in the O₂-saturated glucose buffer solution (1 M) after 1 h irradiation). This indicates that the photocatalytic mineralization of glucose is prohibited in the present condition and glucose is selectively phototransformed to gluconic acid on AKCN as illustrated in Fig. 5a. The concentration of H₂O₂ was determined by the colorimetric N,N-diethyl-1,4-phenylene-diamine sulfate (DPD) method (DPD oxidized by H₂O₂ and POD), which exhibited a good linearity up to 1.5 mM H₂O₂ (Supplementary Figure 10).

Fig. 4 Photoelectrochemical behaviours of pure and modified GCN. a Transient photocurrent responses (inset: images of different electrodes). b Electrochemical impedance spectra (EIS). In the simulated electrical equivalent-circuit model (inset), Rs, R1, and CPE represent as solution resistance, charge transfer resistance, and double layer capacitance, respectively. c Time profiles of Fe³⁺/²⁺-redox shuttle-mediated photocurrent collected on a Pt electrode in the catalyst suspension. d Open-circuit voltage decay (OCVD) measurement (inset: average lifetimes of the photogenerated carriers as a function of the Voc). The black open-triangle and pink open-square represent the GCN and AKCN, respectively. Source data are provided as a Source Data file.

Fig. 5 Photocatalytic aerobic oxidation of glucose with the concurrent production of H₂O₂. a Scheme of GOx-like reaction of AKCN (photoenzyme). b H₂O₂ production as a function of glucose concentration (inset: enlarged plot in the linear region) in the phosphate buffer (0.1 M, pH 7) suspension of photoenzyme (0.5 g L⁻¹) under visible light illumination (λ ≥ 420 nm), T = 25 °C. c H₂O₂ generated by AKCN photoenzyme in the presence of different kinds of saturated gas (0.1 M glucose) and carbohydrate substrate (0.1 M). The error bar represents the standard deviation from the repeated experiment after three times. Source data are provided as a Source Data file.
The photocatalytic production of H$_2$O$_2$ on AKCN was negligibly small in Ar-saturated condition (O$_2$-free) and progressively higher in air-saturated and O$_2$-saturated condition (Fig. 5c), which supports that H$_2$O$_2$ production is derived mainly from the selective two-electron reduction of O$_2$ when coupled with the photooxidation of glucose. In accord with its higher AQY in EtOH, AKCN also exhibited highest AQY (close to 100%) for H$_2$O$_2$ production in glucose buffer solution under the visible light (Supplementary Figure 11). Similar to the behaviour of GOx with good specificity for glucose, this photoenzyme of AKCN also exhibited the higher selectivity for glucose oxidation when compared to other glucose analogues, such as fructose, lactose, and maltose (Fig. 5c). Overall, AKCN seems to mimic the behaviour of GOx (producing H$_2$O$_2$ along with the simultaneous oxidation of glucose) under visible light irradiation. The resulting H$_2$O$_2$ can be furthered to combine with peroxidase (HRP) to oxidize chromogenic substrates for colorimetric detection of glucose.

**Peroxidase-like activity.** On the other hand, the intrinsic peroxidase-like activity of AKCN was also tested in the AKCN-TMB-H$_2$O$_2$ system, where the chromogenic substrate of TMB was oxidized under the dark condition (Fig. 6a). The resulting ox-TMB generated deep blue colour (Supplementary Figure 12). This demonstrated the bifunctional biomimetic roles of AKCN: (1) AKCN plays the role of photoenzyme to mimic GOx that oxidizes glucose with the concurrent production of H$_2$O$_2$ under visible light; (2) AKCN mimics HRP that oxidizes the chromogenic TMB to induce blue coloration in the dark. The peroxidase-mimicking activities of AKCN were compared with those of HRP with varying pH and temperature (Supplementary Figure 13). The operating pH and temperature ranges are quite similar between AKCN and HRP, but AKCN with graphitic structure exhibited consistently higher activities than HRP above 30 °C.

The peroxidase mimicking activities of AKCN were systematically investigated by varying one substrate concentration while keeping the other one constant. It was found that the steady-state kinetics well followed the typical Michaelis–Menten model in the tested concentration range of H$_2$O$_2$ (Fig. 6b) and TMB (Fig. 6c). From the Lineweaver–Burk double reciprocal plots (inset), the corresponding kinetic parameters of maximum initial velocity ($V_{max}$) and Michaelis–Menten constant ($K_m$) were obtained from the slopes and intercepts of the fitted lines, which are summarized in Supplementary Table 3. Compared to ferric oxide (154) and HRP (3.7), the lower $K_m$ value of AKCN (0.79) for substrate H$_2$O$_2$ represents its higher binding affinity for H$_2$O$_2$, indicating the lower concentration of H$_2$O$_2$ required to reach the maximal activity of $V_{max}$. In contrast, the $K_m$ value of AKCN with respect to TMB was significantly higher than that of HRP, consistent with the higher TMB concentration required to achieve the maximal activity through the mediation of charge transfer between TMB oxidation and H$_2$O$_2$ reduction.

Same as the case of electrons transfer from graphene to H$_2$O$_2$, the AKCN with graphene-like structure can also facilitate the electron transfer from TMB to H$_2$O$_2$. Inspired by the higher affinity of AKCN to H$_2$O$_2$ in peroxidase-like activity, a colorimetric H$_2$O$_2$ detection with a low detection limit of 0.015 mM was performed (Supplementary Figure 15). As demonstrated above, AKCN possesses both GOx-mimicking and HRP-mimicking behaviors. This motivated us to perform the colorimetric detection of glucose in cascade reactions (sequential
combination of both behaviours) through the in situ H\textsubscript{2}O\textsubscript{2} production from AKCN-catalysed glucose oxidation.

**Enzyme-like cascade reactions in batch and microfluidic modes.** Based on the above GOx- and peroxidase-like activities, the artificial enzymatic cascade reaction carried out by the bifunctional AKCN, instead of GOx-HRP bi-enzymatic reaction, was comparatively tested for glucose detection in a batch and a microfluidic reactor as illustrated in Fig. 7a, c. In the batch reactor with continuous O\textsubscript{2} purging, H\textsubscript{2}O\textsubscript{2} was generated through the photocatalysis of AKCN/glucose for 20 min under visible light irradiation (λ ≥ 420 nm). The in situ generated H\textsubscript{2}O\textsubscript{2} in the photo-stage was then consumed in the following dark-stage where TMB is oxidized by H\textsubscript{2}O\textsubscript{2} on AKCN. The correlation between [glucose] and [H\textsubscript{2}O\textsubscript{2}] generation after the photo-stage in}
the reactor was successfully confirmed (Supplementary Figure 16). After the TMB injection, the in situ generated H₂O₂ was subsequently depleted by the peroxidase-mimicking action of AKCN with simultaneous appearance of blue colour (images inset, indicating the production of ox-TMB)⁵². Similar to the GOx-peroxidase-coupled enzymatic system, the cascade enzymatic mimicking was successfully achieved in the combined system of (1) AKCN/glucose/hv with in situ production of H₂O₂ and (2) AKCN/H₂O₂/TMB (dark). As a result, the production of ox-TMB (monitored by absorption at 652 nm) gradually increased with the glucose concentration ranged from 0.01 M to 1 M (Fig. 7b), and a good linearity was established in the range of 0.01 M < [glucose] < 0.3 M (Fig. 7b inset), which was sensitive enough (with a limit detection of 0.07 mM) to distinguish between the healthy (3–8 mM) and diabetic body (9–40 mM) by monitoring the blood glucose level⁵⁰,⁵¹.

The above enzyme-mimicking cascade reaction can be more facilitated with enhanced mass transfer in a continuous flow microfluidic reactor, which enables to miniaturize the system as a portable real-time monitoring platform⁴⁴. For this purpose, a feasible microfluidic device for glucose detection in a small volume was fabricated by stacking a PDMS substrate with AKCN-coated flow channels (5 mm width, 0.044 mm height, 30 mm length) onto a slide glass (Fig. 7c and inset, see experimental method and Supplementary Figure 17). The cascade reaction was conducted along two flow channels that are serially connected with 30-mm-long channel; the former has the AKCN-coated channel under visible light irradiation (GOx-mimicking part) and the latter mixes the in situ generated H₂O₂ (from the former) with TMB injection (HRP-mimicking part). In other words, the mixed glucose-H₂O₂ from the flow vessel was transferred into the flow channel by a syringe-pump, where the AKCN (0.25 mg per chamber) immobilized on the channel wall catalyses the H₂O₂ generation under visible light irradiation. The outlet flow from the first channel was continuously fed into the second flow channel (shielded from the irradiation) for the subsequent peroxidase-mimicking reaction on AKCN. TMB was introduced through a separate inlet in the head of the second flow channel. The solution in the second flow channel was immediately turned blue (Supplementary Figure 17e) due to the formation of ox-TMB and the resulting outflow out of the channel can be analysed for its absorbance at 652 nm in real time. Since H₂O₂ was equilibrated proportion to the oxidized glucose (one molar molecule of glucose converted to one molar H₂O₂) in GOx mimicking, the further continuously catalysis of quantitative [TMB] with accurate [H₂O₂] (detected from DPD method) might offer a promising indicator in glucose assay originated from the calibration curve of [glucose] against the TMB and H₂O₂ (Supplementary Figure 18).

The initial reaction rate ($v_i$) can be calculated from the plot of the ox-TMB absorbance as a function of time (Fig. 7d). The $v_i$ was linearly correlated with [glucose] (Fig. 7e), which enables the quantification of the glucose concentration from the calculated $k_{\text{apo}}$ (1.7 Abs. s⁻¹ mM⁻¹). It is worth noting that this analytical method based on the photonic microfluidic cascade reaction can be used to detect the unknown glucose concentration from the calibration curve within 30 s by measuring $v_i$ (Abs = $ecl$, $e$ = 39000 L·mol⁻¹·cm⁻¹ for TMB). It exhibited the limit of detection around 0.8 μM in microfluidic device, which is sensitive enough for practical application since the clinical glucose concentration is greater than 1 mM. The microfluidic biomimetic cascade exhibited the superior turnover frequency (9.1 h⁻¹) of H₂O₂ generation as 5.3 times high as that of the batch process (1.7 h⁻¹), which can be ascribed to the intrinsic characteristics of the microfluidic reactors such as efficient mass transfer and high surface-to-volume ratio (see detailed discussion in Supplementary Note). While all the previously reported bifunctional nanozymes required hours for glucose detection (Supplementary Table 2), the present AKCN-based nanoenzyme employed in a microfluidic device exhibited a much faster cascade catalysis (~30 s) and a lower detection limit (0.8 μM). In addition, the detection limit of the microfluidic device is orders of magnitude lower than that of the batch reactor. The merits of the present nanozyme–microfluidic sensor are highly desirable for point-of-care diagnosis. Overall, this study successfully demonstrated the performance of the microfluidic reactor for biomimetic cascade catalysis reaction as a miniaturized tool for rapid and facile real-time monitor of glucose.

**Discussion**

The successful performance of AKCN that achieved the ideal efficiency of about 100% AQY implies that the photogenerated charge carriers in AKCN are efficiently separated with directional charge migration and selective reduction of O₂ to H₂O₂. To understand how the modification of GCN with KCl and KOH modified the structure and charge distribution, density functional theory (DFT) calculations were performed. The optimized location of Cl, K, and OH in the carbon nitride structure and the relative energy of each modified GCN structure are shown in Supplementary Figs. 19–22. It is evident that the electron-rich nitride pots in the three-fold N-bridge linking triazine units are liable to capture and confine the alkali cations of K⁺ in the adjacent layers through the ion-dipole interaction⁵⁵. The K-doped GCN structure with weak interlayer bridging made a relatively large number of electrons accumulated on the first layer (~2.34 e of layer charge) than on the second one (~0.99 e of layer charge) (Fig. 8b), whereas the Cl-doping in GCN did not induce such localization of electrons between the layers (Fig. 8a). As a result, the K-GCN exhibited a high value of the charge difference between the adjacent layers (|Δq| = 1.35 e) while that on Cl-GCN (|Δq| = 0.06 e) is insignificant. The presence of doped K atoms induces the anisotropic electron density distribution, which is preferably accumulated on the first layer but the additional doping of Cl atoms makes the electron distribution more balanced between the layers (Fig. 8c). In other words, when both K and Cl are copresent in the carbon nitride structure, the K-induced electron density polarization can be counterbalanced by Cl to lower |Δq| (0.16 e) significantly. This implies that the charge transfers between the layers in KCl-doped carbon nitride are more facilitated than those in pristine GCN, which may provide an explanation for its higher photocatalytic activity. When OH groups are additionally introduced in AKCN (noted as KCl-OH-GCN in Fig. 8d), the DFT calculation predicts that the hydroxyl group is preferably bonded to the surface carbon (Supplementary Figure 22) with inducing an outstanding electron depletion region around the OH group (blue colour) on the first layer (see Fig. 8d). This further decreases |Δq| from 0.16 e (Figs. 8c) to 0.06 e (Fig. 8d), which should help the facile charge transfer between adjacent layers. It should be noted that the formation of charge delivery channels in AKCN is critically important to extend the π-conjugated system for facilitating the directional carrier migration between the adjacent layers and assisting the interlayer charge separation⁵⁷,⁶⁶.

The DFT calculation also shows how the presence of OH group changes the charge distribution over different atoms. The enlarged top view of KCl-OH-GCN (Fig. 8e) shows that the surface carbon atom attached to the OH group carries more positive charge (1.43 e) than the corresponding carbon atom in the pristine GCN (0.99 e in Fig. 8f), furthered to be arising for the neighbour N atoms carry more negative charges (~1.09 e and ~1.08 e in Fig. 8e) compared with those on the pristine GCN (~0.95 e and ~1.01 e in Fig. 8f)⁵⁶. Based on this calculated result,
the proposed reaction scheme is illustrated in Fig. 8g. Upon photoexcitation (Fig. 8g–I), electrons and holes surviving from the fast recombination migrate onto the surface of AKCN (Fig. 8g–II). The holes are preferentially trapped at the N sites (electron rich) adjacent to the C–OH group in the melem unit and subsequently abstract two H atoms to form >NH+ sites (blue H atoms in Fig. 8g–III) through the glucose oxidation to gluconic acid57. On the other hand, a dioxygen molecule reacts with two electrons trapped in the melem unit, which concurrently abstracts two protons from the >NH+ sites (protonated through glucose oxidation), and consequently generates H2O2 as a product through the intermediate of 1,4-endoperoxide. The sequential reactions of holes and electrons regenerate the melem unit. Therefore, the synergistic improvement of spatial charge separation and local polarization between the interlayers and in-plane is critical for efficient H2O2 production on AKCN.

On the other hand, in the peroxidase mimicking part, the electrophilic carbon atom in the cyano group abstracts an O atom from H2O2 and subsequently an H atom from TMB (hydroxyl group formation, bottom part of Fig. 8g–v) with generating ox-TMB58. The resulting hydroxyl group further abstract an H atom from another TMB to yield a water molecule and another ox-TMB (hydroxyl group depletion, upper part of Fig. 8g–v). The above two ox-TMB can be protonated in acid buffer to form bluish ox-TMBH+. Overall, two TMB molecules are oxidized by one molecule of H2O2 that is in situ photogenerated on bifunctional AKCN, consistently with their higher affinity of H2O2 than TMB in the peroxidase-mimicking activity. The combined
processes (photogeneration of H₂O₂ coupled TMB oxidation in the dark) occurring on AKCN successfully complete the enzymatic cascade reaction.

In summary, a metal-free nanoyzme based on modified GCN demonstrated bifunctional enzyme-mimicking behaviours, which combined the roles of oxidase (GOx) and peroxidase (HRP) in a sequential light-dark process. With the intrinsic GOX-like mimicking, the in situ generated H₂O₂ from photocatalytic oxidation of glucose served for the subsequent peroxidase-mimicking part. The bifunctional AKCN exhibited a near 100% quantum efficiency of H₂O₂ generation and enabled the coupled cascade reactions for colorimetric glucose detection. In addition, such biomimicking catalytic process could be highly accelerated in a microfluidic device, which enabled the real-time monitor of H₂O₂ and glucose with a detection limit of 0.8 μM in 30 s. Our results not only certify the successful modification but also clarify the importance of charge separation for cascade reaction with combination of theoretical and experimental fundamental insight. This study provides a design strategy for bifunctional nanoyzme capable of generating and subsequently utilizing in situ H₂O₂ under ambient condition, which can be potentially applied to a variety of eco-friendly and biomimicking processes involving H₂O₂.

**Methods**

**Materials.** All chemicals were purchased from Sigma–Aldrich or Alfa–Aesar with the highest purity and used without further treatment. Melamine, alkalis chloride and potassium halide, 3.3.S.5 tetramethylbenzidine dihydrochloride (TMB) were bought from Sigma–Aldrich and used as received. Hydrogen peroxide (35 wt %) was bought from Junsee. Milli-Q water was used for all the experiments.

**Photocatalysts preparation.** GCN was simply synthesized as following process36–38. 1.5 g of melamine was put into porcelain cup with a cap and calcined at 550 °C for 4 h with a ramping rate of 2.2 °C min⁻¹. After heating, the resulting product was gently ground and treated under ultrasonication for 3 h as an aqueous solution (1 g L⁻¹). Then the powder was filtered, washed, and dried at 80 °C for further tests. Alkalinized GCN (ACN) was synthesized by similar procedure to GCN, but the proper amount (0.3, 1.0, 2.0, 3.0, 4.0 mmol) of potassium hydroxide was mixed with melamine and grounded together before calcination process. For further comparison of the effect from alkaline metals, 0.8 mol alkali chlorides (ammonium chloride, sodium chloride, barium chloride, or 0.08 mol potassium halide (potassium fluoride, potassium bromide, potassium iodide, potassium sulfate) were, respectively, mixed with melamine instead of potassium hydroxide. K-incorporated GCN (KCN) was synthesized by similar procedure to GCN, but the proper amount of potassium hydroxide (2.0 mmol) and potassium chloride (0.08 mol) was mixed with melamine and grounded together before calcination process.

**Photocatalysts characterisation.** The phase structures of resultant catalysts were characterised by a PANalytical X’Pert diffractometer with an X’Celerator detector using Cu Kα line (1.2536 eV) and ATR-FTIR spectroscopy (Thermo Scientific Nicolet 6700 FTIR). A Zeosorb E (80/100 mesh) diatomaceous earth reflectance UV absorption spectra (DR-UVS) were obtained by Shimadzu UV-2600 with an integrating sphere attachment. The reference material of BaSO₄ was used before the measurement. Zeta potentials of the aqueous suspension (10 mM of NaNO₃) with reference electrodes, respectively. Photocatalysts (1 g L⁻¹) were suspended in aqueous solution consisting 1 mM NaClO₄ (electrolyte) and 1 mM Fe(III) chloride at pH 1.7 with a bias of +0.7 V (vs. Ag/AgCl). Electrochemical oxygen reduction reaction (ORR) was investigated through linear sweep voltammetry (LSV) (using a Gamry Reference 600 potentiostat) in KOH electrolyte (0.1 M) under continuous O₂ purging. To prepare the catalyst-coated electrode, the catalyst slurry with Nafion (0.5 wt%) was loaded on the surface of glassy carbon disk (615 μg cm⁻²). The resultant working electrode was scanned at a rate of 10 mV s⁻¹ to cathodic direction in LSV. The reference and counter electrodes were Ag/AgCl (in saturated KCl) electrode and platinum wire, respectively. The rotating disk analysis was performed at the speed of 400–1600 rpm during the ORR. The electron transfer number in ORR can be calculated from the detection of Koutecky-Levich plot, which was constructed according to the Koutecky-Levich equation (eq. 7).

**Photocatalytic H₂O₂ generation.** Photocatalytic H₂O₂ generation was conducted under visible light irradiation (λ ≥ 420 nm) with continuous O₂-purging. The colorimetric method employing N,N-diethyl-1,4-phenylene-diamine sulfate (DPD, 97%, Aldrich) was used to determine the concentration of H₂O₂. The sample aliquots were collected intermittingly during the reaction and then mixed with phosphate buffer, DPD solution, and peroxidase (POD, horseradish, Aldrich) under vigorous stirring. The production of H₂O₂ was monitored by measuring the absorbance at 531 nm using a UV/visible spectrophotometer (LAMBDA 950, PerkinElmer). The detailed method is described elsewhere32,33. The apparent quantum yield (AQY) was calculated through the equation (eq. 6).

\[
\phi_{AQY} = \frac{\text{Number of produced H}_2\text{O}_2 \text{molecules} \times 2}{\text{Number of incident photons}} \times 100
\]

Where the incident wavelength was adjusted by a monochromator (Newport, Oriel 77250) and the light intensities was measured using a low-power detector (Newport, 818-UV).

**Electrochemical analysis.** The transient photocurrent and electrochemical impedance spectroscopy were done by the catalysts coated on ITO glass via spin coating. The measurements were conducted on a potentiostat (Gamry, Reference 600) by three electrode system, where Pt wire as counter electrode, Ag/AgCl as reference electrode, and catalyst coated on ITO as working electrode. 0.2 M NaSO₄ was used as an electrolyte and pH were adjusted to 3 under continuous Ar purging. The slurry photocurrent measurements were carried out in three electrode system, consist of Pt wire, graphite rod, and Ag/AgCl as working, counter and reference electrodes, respectively. Photocatalysts (1 g L⁻¹) were suspended in aqueous solution consisting 1 mM NaClO₄ (electrolyte) and 1 mM Fe(III) chloride at pH 1.7 with a bias of +0.7 V (vs. Ag/AgCl).

**Oxidation of glucose.** The glucose oxidation was performed in a quartz cuvette (5 mL) containing a solution of glucose with different concentrations (0.01, 0.025, 0.05, 0.075, 0.1, 0.25, 0.4, 0.5, 0.75, and 2 M) and 2 mg photocatalyst in 4 mL phosphate buffer solution (0.1 M, pH 7). The photoreaction tests were done under continuous O₂-purging with 30 min irradiation. After filtration, the colorimetric sensing of photogenerated H₂O₂ was analysed for the detection of glucose oxidation. For further comparison of the selectivity, the same photoreaction was carried out as a glucose except replacing the glucose by 0.1 M fructose, lactose, and maltose, respectively. The effluent of CO₂ in the closed reactor with O₂-saturated glucose buffer solution (1 M) was tested from the calibrated gas chromatography with flame ionization detector (GC-FID, Agilent).

** Peroxidase-like activity mimic.** In a typical peroxidation reaction, 200 μL of substrate (TMB, 4 mM) was added to 3.6 mL acetate buffer solution (0.1 M, pH 4) containing 3 mM H₂O₂ (800 μL) and 1 mg mL⁻¹ 4-molybdenum peroxides. During the 10 min incubation at 25 °C, the kinetics of peroxidase-like activity was measured by monitoring the absorbance at 652 nm after filtration, which represents the
concentration of the oxidized product of TMB. The optimized condition was modulated from the variable pH and temperatures. To measure the steady-state kinetics, the various concentrations of substrates (H_2O_2 (3 mM) and H_2O_2 (TMB, 4 mM)) were used, separately. For colorimetric detection of H_2O_2, 200 μL of substrate of TMB (4 mM) was added to 3.8 mL of acetate buffer solution (0.1 M, pH 4) containing 0.5 mg mL^{-1} photoenzyme and H_2O_2 with various concentrations. After incubation for 10 min in dark, the concentrations dependent blue colour at 652 nm was recorded via UV–Vis spectroscopy. The kinetic parameters were calculated using the Michaelis–Menten equation (eq. 9):

\[ V = \frac{V_{\text{max}}[S]}{K_m+[S]} \]  

(9)

where [S] is the concentrations of substrates.

**Enzymatic cascade reaction.** In a quartz cuvette, 2 mg photocatalyst was added into 2 mL phosphate buffer solution (0.1 M, pH 7) containing different concentrations of glucose. After 20 min irradiation with continuous O_2-purging, 200 μL acetate buffer solution (0.1 M, pH 4.0) were added into above reaction solutions, which were then incubated for another 10 min in dark. The final reaction solution was recorded by UV–Vis spectroscopy after filtration.

**Enzymatic cascade reaction in a microfluidic photoreactor.** The AKCN were immobilized on the poly(dimethylsiloxane) (PDMS) wall with 3- glycidoxypropyltrimethoxysilane (GLYMO) brush, originated from the shadow-mask of lithograph defined shape and location in spatial distribution, and finally sealed with the packed slide glass. The microfluidic device was systematically set up with light source and syringe-pump connection through the silica fibers (Supplementary Figure 17a). For the redox-coupled enzymatic mimicking in separated chambers, the chip was positioned on a home-built holder to control the light irradiation on required area. The reaction volume was consisted of a length of 30 mm, a depth of 0.044 mm from a cross section, and a wide of 5 mm (Supplementary Figure 17c and 17d). The enzymatic cascade reaction in microfluidic photoreactor were carried out as the same conditions as aforementioned batch process. A phosphate buffer solution (0.1 M, pH 7) containing different concentrations of glucose was pumped (12 μL min^{-1}) into the microfluidic channels from the solution inlet. Meanwhile, the continuous O_2 was pumped (12 μL min^{-1}) into the flow channels from the adjacent gas inlet leading to mix with the glucose solution at cross point. As the O_2-solution flowed into the paralleled reactive chamber, the GOx mimicking occurred on AKCN (0.25 mg cm^{-1}) under the back irradiation (see the image of setup in Supplementary Figure 17e). For the real-time detection of glucose in the sequential processes, the TMB solution (4 mM) was pumped (12 μL min^{-1}) from the downstream inlet for the continuous peroxidase-like activity in the individual chamber (0.25 mg cm^{-1}, AKCN) without irradiation. The reactive solution was splitted from the outlet and collected for the ox-TMB detection through the colorimetric method. As the same procedure without TMB injection, the concentration of H_2O_2 in the splitted solution was determined from DPD method. The turnover frequency (TOF) in the fluidic photoreactor was systematically set up using a Monkhost-Pack scheme and the relaxed structural parameters of bulk GCN are determined from DPD method. The turnover frequency (TOF) in the individual chamber and batch system was calculated based on the H_2O_2 generation in each reaction volume.

**Computational details.** Density functional theory (DFT) calculations were conducted using the Vienna Ab Initio Simulation Package (VASP). The Perdew-Burke-Ernzerhof (PBE) functional, which is based on the generalized gradient approximation (GGA), was used to treat the exchange-correlation energy. The semi-empirical DFT-D3 method was employed to consider dispersion force. The kinetic cutoff energy of 400 eV was employed for the expansion of the plane wave. A conjugate gradient algorithm was applied to relax the geometries until the forces on all the unrestricted atoms were less than 0.03 eV Å^{-1}. The width of Gaussian smearing for the occupation of electronic levels is 0.2 eV. The convergence criteria for electronic structure iteration was set to be 10^{-4} eV. The Brillouin zone is sampled with a 5 × 5 × 3 for (1 × 1 × 1) supercell of bulk GCN and 3 × 3 × 1 for (2 × 2) slab structure using a Monkhost–Pack scheme. The relaxed structural parameters of bulk GCN are a = b = 7.124, c = 12.328. We used the three-layer Pack scheme. The relaxed structural parameters of bulk GCN are a = b = 7.124, c = 12.328. We used the three-layer Pack scheme. The relaxed structural parameters of bulk GCN are a = b = 7.124, c = 12.328. We used the three-layer Pack scheme.

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

**Data availability**

The data sets within the article and Supplementary Information of the current study are available from the authors upon request.

Received: 15 June 2018 Accepted: 21 January 2019

Published online: 26 February 2019

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