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Reporter-encapsulated liposomes on graphene field effect transistors for signal enhanced detection of physiological enzymes†

Hu Chen,[abc] Seng Koon Lim,[ad] Peng Chen,[ad] Jingfeng Huang,[ab] Yi Wang,[ad] Alagappan Palaniappan,[ad] Mark Platt,[c] Bo Liedberg*[ad] and Alfred Iing Yoong Tok*[ab]

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A novel approach for enzymatic assay using reporter-encapsulated liposomes on graphene field effect transistors (FET) is proposed. This approach involves real time monitoring of drain current (I_d) of reduced graphene oxide (rGO) upon rupture of reporter-encapsulated 1-palmitoyl-2-oleoyl-*sn*-*glycero*-3-phosphatidylcholine (POPC) liposomes triggered by enzymes. For validation of the proposed approach, 2,4,6-trinitrophenol (TNP) is used as the reporter for specific detection of phospholipase A_2 (PLA_2), a key enzyme in various membrane related physiological processes. Experimental results revealed that I_d increased with PLA_2 concentration, which is attributed to the interaction between released TNP and rGO. The limit of detection (LOD) achieved by the proposed approach was 80 pM, which is superior to most assays reported previously and much lower than the cut-off level of circulating secretory PLA_2 (2.07 nM). Besides the high accuracy of the electronic detection methodology, the signal enhancement effect realized by the excess concentration of TNP (approximately 1 mM) in liposomes is believed to be the main reason for the significantly enhanced sensitivity of the proposed assay, indicating great potential for further improvement in the sensitivity by increasing the concentration of TNP. In addition, the proposed approach is rapid (incubation time $\leq$ 10 min) and label-free, thus showing great potential for practical applications in the future.

Introduction

Assays enabling rapid and ultra-sensitive monitoring of physiologically relevant enzymes have attracted increasing attention[1] due to the significant roles of enzymes in various physiological processes. These assays include enzyme-linked immunosorbent assay (ELISA), fluorescence-based assays,[2,3] colorimetric assays[4,5] and electronic detection platforms.[6] Among physiologically relevant enzymes of interest, efforts have been devoted to assay development for phospholipase, an enzyme that play a key role in various physiological processes such as inflammation response, intercellular signalling and membrane remodelling.[7] A typical phospholipase that has been intensively studied is the phospholipase A_2 (PLA_2). PLA_2 is a superfamily of enzymes that degrade phospholipids to produce free fatty acids and lysolipids, achieved by cleavage of the *sn*-2 acyl ester bond in glycerophospholipids.[8] Dysregulation of PLA_2 can be an indicator for various pathological conditions including atherosclerosis,[9] acute sepsis[10] and certain forms of cancer.[11] Owing to its diverse biological roles, PLA_2 has been targeted by many assays. The commercially available sensors based on ELISA (as in Table S1, ESI†) for PLA_2 may not be ideal candidates for practical applications as they are resource intensive (expensive equipment and well-trained personnel) and can only provide limited information on the enzyme activity, in spite of its excellent sensitivity.[12] Therefore, Aili *et al.*[13] reported a colorimetric approach for the detection of phospholipase concentration and activity using Au nanoparticles (AuNP) and polypeptides. In this approach, naked eye detection is achieved (no measuring equipment required), while the lag time is relatively long (around 20 min). Another assay reported[14] involving hybrid nano-constructs as label-free optical probes for surface plasmon resonance (SPR) – based detection of PLA_2. In this approach, the specificity is excellent and the assay time is significantly reduced (less than 2 min), while the sensitivity is relatively low (LOD = 1.82 nM). Fluorescent-based detection of phospholipases using dye-encapsulated liposomes

[a] *School of Materials Science and Engineering, Nanyang Technological University, Singapore 639798. E-mail: MIYTOK@ntu.edu.sg; Tel: 65 6790 4953*
[b] *Institute for Sports Research, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798*
[c] *Department of Chemistry, Centre for Analytical Science, Loughborough University, Loughborough, Leicestershire LE11 3TU, UK*
[d] *Center for Biomimetic Sensor Science, Nanyang Technological University, Singapore 637553. E-mail: BLIEDBERG@ntu.edu.sg; Tel: 65 6316 2957*

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has also been reported previously.[15] The fluorescence from dyes is quenched drastically when encapsulated at high concentrations within liposomes.[16] Nevertheless, these assays are limited by the cross-talk between different dye molecules (in other words interferences from other dye molecules), yielding a relatively poor spectral discrimination of the fluorescence emission.[17] Moreover, sophisticated instrument such as the fluorescence spectrometer is required for these assays, in contrast to the proposed methodology that could be miniaturized in the form of a small electronic circuitry.

To overcome these limitations (including long lag time, cross-talk and insufficient sensitivity, as mentioned above), an approach for ultra-sensitive and label-free enzymatic assay using reporter-encapsulated 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phosphatidylcholine (POPC) liposomes on graphene field effect transistors (FET) is proposed. As a single layer of sp^2 hybridized carbon atoms, graphene has emerged to be an exciting class of nanomaterials for biosensing applications[18,19] in virtue of their unique electrical and physical properties.[20–22] Therefore, reduced graphene oxide (rGO), a p-type semiconducting graphene,[23,24] was utilized for the fabrication of an electronic sensing platform. 2,4,6-Trinitrophenol (TNP) was utilized as the reporter due to its excellent chemical stability[25] and the benzene ring structure, which enables its stacking on rGO *via* π–π interactions.[26,27] PLA_2-triggered rupture of liposomes with TNP molecules leads to the release of these molecules, which subsequently adsorb on rGO, thereby modulating the conductance of rGO. A schematic of the enzymatic assay proposed is shown in Fig. 1.[13] Due to the presence of electron withdrawing NO_2 groups in these molecules, a remarkable increase in the drain current (I_d) positively related to the concentration of PLA_2 was observed. In virtue of the signal enhancement effect realized by the excess concentration of TNP in liposomes, the proposed approach achieved a limit of detection of 80 pM, which is superior to previously reported colorimetric and SPR assays and much lower than the cut-off level of circulating secretory PLA_2 (2.07 nM). The sensitivity may be further improved by increasing the concentration of TNP. Cross-talk, which is a common problem for fluorescent-based detection, has not been observed in the proposed assay. This approach is rapid (incubation time $\leq$ 10 min) as compared to conventional ELISA assays[28] and label-free, thus showing great potential for practical applications in the future. Furthermore, to the best of our knowledge, this is the first report on an electronic platform for enzymatic assays using reporter-encapsulated liposomes.

Results and discussion

The Field Emission Scanning Electron Microscope (FESEM) image of rGO on a SiO_2 substrate is shown in Fig. 2(a). The bright regions are the SiO_2 substrate while the dark regions are deposited rGO flakes. The size distribution and polydispersity of liposomes fabricated were checked by Dynamic Light Scattering (DLS) and the results are shown in Fig. 2(b). As can be seen, both liposomes (with and without TNP) exhibit narrow size distributions (the polydispersity index was measured to be 0.195 and 0.21 for liposomes with and without TNP, respectively), indicating good monodispersity.[29] The average sizes of liposomes with and without TNP were measured to be 188 and 179 nm, respectively; indicating that encapsulation of TNP has no significant effect on the self-assembly process of POPC liposomes. In addition, both liposomes remained stable for at least 5 days at

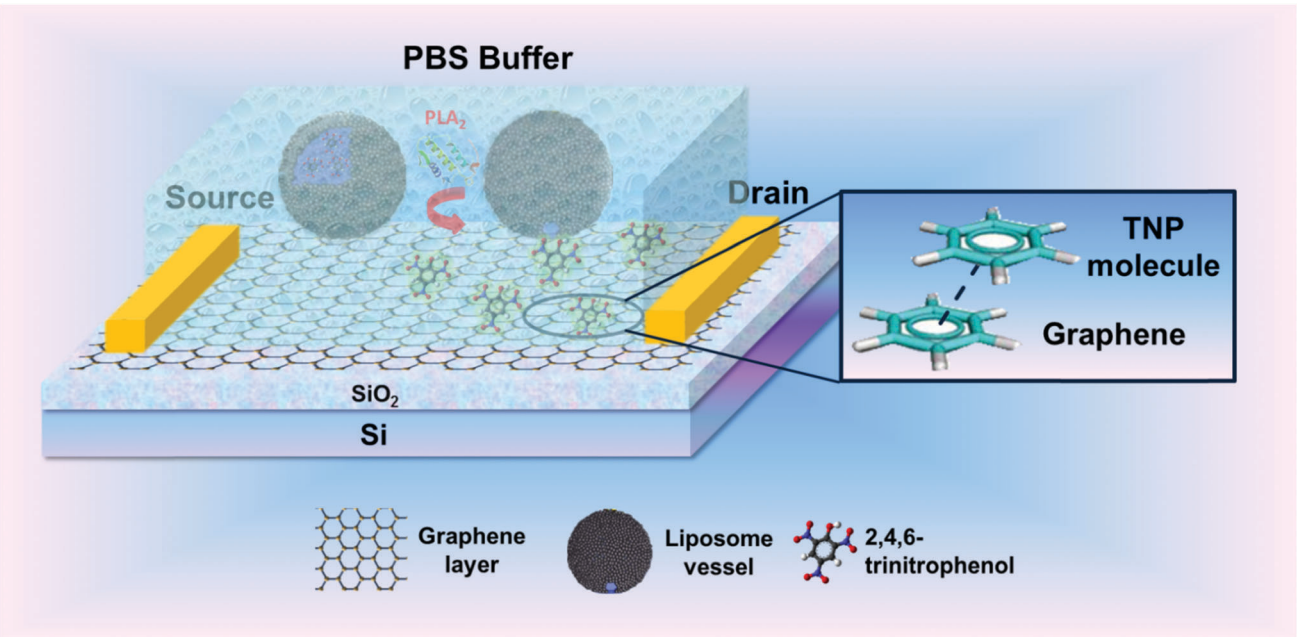

Fig. 1 Schematic illustration of reporter-encapsulated liposomes on rGO field effect transistors for PLA_2 detection.[13] TNP molecules released from the liposomes stack on the graphene surface *via* π–π interaction.

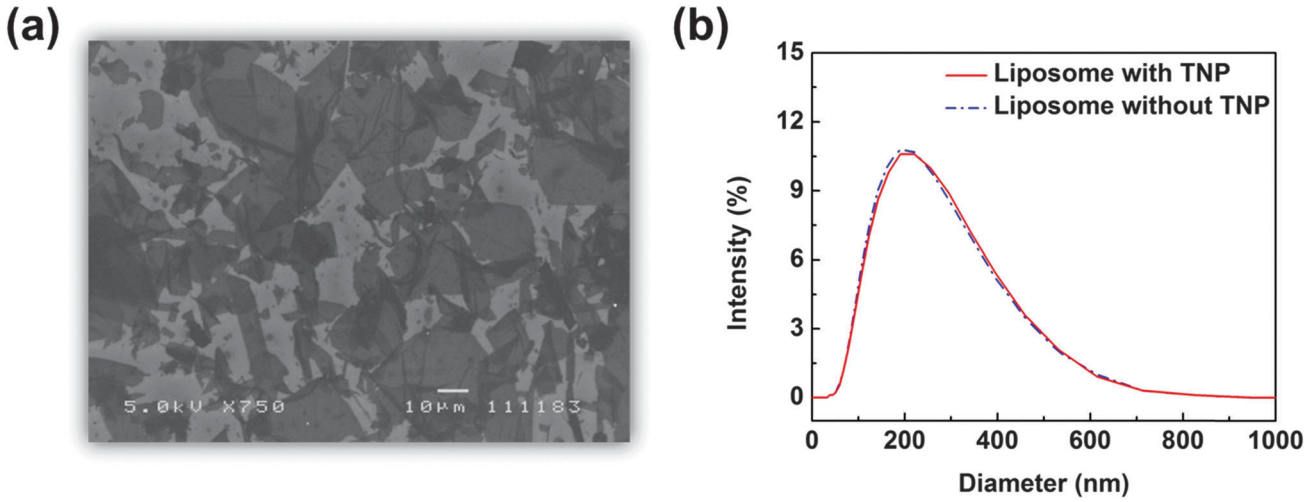

Fig. 2 (a) Field Emission Scanning Electron Microscope (FESEM) image of rGO on a SiO_2 substrate; (b) size distribution of liposomes with and without TNP obtained by Dynamic Light Scattering (DLS).

room temperature as no significant aggregations were observed, which is consistent with previous reports.

The effect of TNP physisorption on the conductance of rGO was investigated in order to validate the role of TNP in this assay.

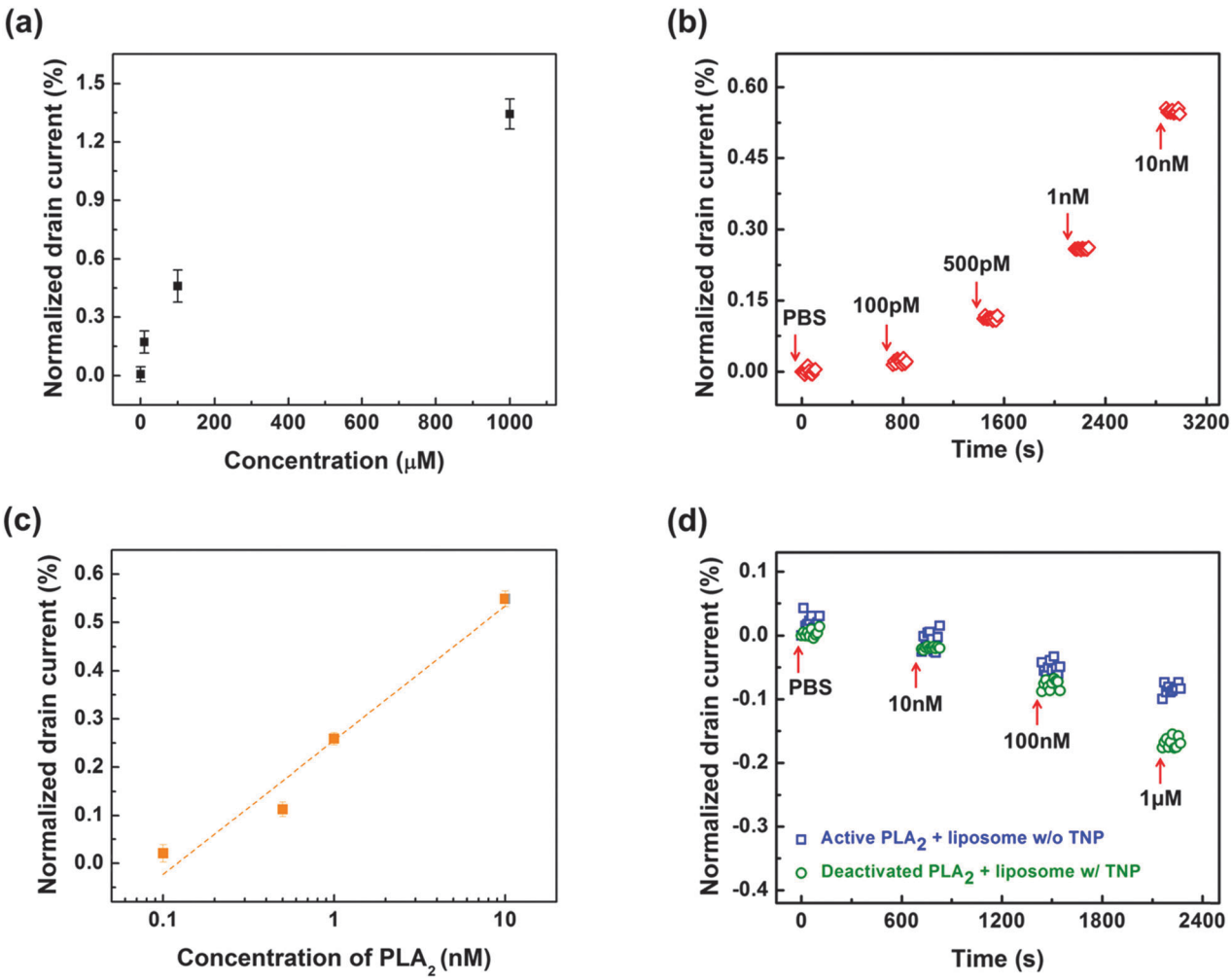

Fig. 3 (a) Effect of TNP physisorption on the conductance of rGO-FET; (b) kinetic measurements of PLA_2 at different concentration levels; (c) I_d response of rGO devices *vs.* PLA_2 concentration in kinetic measurements; (d) real time I_d responses of control groups A and B.

As shown in Fig. 3(a), I_d increases proportionally with the concentration of TNP (up to 100 μM), which is in agreement with previous reports.[26] This suggests that the interaction between TNP at μM levels and rGO is detectable by the developed device.

Since TNP at 1 mM leads to a significant I_d response (1.2% increase in I_d), the concentration of TNP used for the encapsulation process for all the measurements in this study was set to be 1 mM. Kinetic measurements of PLA_2 were conducted and the results are shown in Fig. 3(b). 10 min incubation in mixed solutions of liposomes (concentration = 0.5 mg mL^{-1}) and PLA_2 resulted in a detectable increase in I_d, indicating that PLA_2 is active and yields detectable I_d responses. More importantly, I_d increases with the concentration of PLA_2 in the mixed solution in a logarithmic pattern (up to 10 nM, as shown in Fig. 3(c)), indicating that the concentration of PLA_2 can be measured by the proposed methodology. The LOD can be calculated using the $3\sigma/S$ approach.[30] Herein, σ stands for the standard deviation of device response to PBS, which was 2.3 nA in this case, as obtained from the experimental data; S is defined as the sensitivity, namely the slope of the linear sensor response range. Calculation of S was executed as follows:

$$S = \frac{\text{Id corresponding to 100 pM} - \text{Id corresponding to buffer}}{100\ \text{pM} - 0}$$

where the average I_d corresponding to 100 pM was 39.93823 μA and the average I_d corresponding to PBS was 39.93017 μA. Both values were determined from the experimental data.

The LOD obtained is 80 pM, which is superior to most PLA_2 assays reported previously. Furthermore, the proposed assay can be used for detection of PLA_2 at clinically relevant concentration ranges as the cut-off level of circulating secretory PLA_2 is 2.07 nM.[31] Besides the high accuracy of the electronic detection method,[32] the enhanced sensitivity of the proposed methodology can be attributed to the signal enhancement effect by the excess concentration of TNP (approximately 1 mM, provided no significant leakage occurs) in liposomes. In virtue of the excess concentration of TNP encapsulated in the liposomes, a considerable quantity of the reporter is released upon rupture of a small amount of liposomes, leading to enhanced signals as compared to other assays.

In addition, two control experiments were conducted to investigate the effects of the enzyme and liposome on the rGO-FET responses. Liposomes without TNP were incubated with active enzymes (control group A) and liposomes with TNP were incubated with deactivated enzymes (control group B). The deactivation was achieved by heating the enzymes at 45 °C for 30 min. As shown in Fig. 3(d), negligible I_d responses as compared to the responses in kinetic measurement (0.01% and 0.02% at 10 nM of PLA_2 for control measurements A and B, respectively, while that for the kinetic measurement was 0.55%) were obtained for both control measurements, indicating that neither the PLA_2 nor the POPC liposomes significantly influences the sensor response. Furthermore, liposomes with TNP in control group B led to negligible I_d responses, indicating that these liposomes are stable (no leakage of TNP) in the absence of active enzymes. A comparison between I_d responses in control measurements and that in the kinetic measurement indicates ultra-high signal-to-noise ratios (defined as the ratio of normalized I_d in kinetic measurement to normalized I_d in control measurements at the same PLA_2 level) of the proposed assay. For instance, the signal-to-noise ratio at 10 nM of PLA_2 is 50. Despite the slight decreases in I_d (as observed in control measurements) due to non-specific binding of liposomes and/or enzymes,[33] the control measurements confirm that the signal is indeed generated by the interaction between rGO and TNP. In addition, the control experiment B shows that the activity of PLA_2 as an enzyme is detectable by the proposed methodology.

Future studies will focus on sensitivity enhancement studies *via* substrate passivation and optimization of liposome composition/TNP encapsulation. Further studies will emphasize on surface passivation methodologies that provide anti-fouling properties while retaining the capability of TNP capturing as TNP interaction with rGO (π–π interactions) is much stronger than non-specific binding of enzymes and liposomes.[34] Additionally, algorithms for drift-compensation could be established upon systematically monitoring I_d responses for enzymes and liposomes at different concentrations. Liposome modification and alternative feasible encapsulation molecules will also be evaluated for assay development of other lipid-degrading enzymes and membrane toxins.

Experimental section

Materials

TNP and PLA_2 were purchased from Sigma-Aldrich Inc. and used without further treatment. POPC was purchased from Avanti Polar Lipids and the POPC liposomes were fabricated by a self-assembly method reported elsewhere.[35,36]

Methods

Synthesis of rGO. The rGO was prepared by extended growth of graphene oxide (GO) on SiO_2 substrates, as reported previously.[36,37] Briefly, GO flakes in aqueous solution were synthesized and attached to SiO_2 substrates functionalized with (3-aminopropyl)triethoxysilane. Then, these substrates were placed on a Si holder and inserted into a quartz tube. Before the growth process, the reaction tube was purged with Ar at 300 sccm for 40 min to avoid oxidation of rGO. Subsequently, a mixture gas of Ar (100 sccm) and H_2 (20 sccm) was introduced into an ethanol bubbler, before entering the reaction tube. The bubbler and reaction tube were kept at room temperature and 950 °C, respectively, for 2 h, followed by cooling down to room temperature. Finally, the synthesized rGO on SiO_2 was characterized using the Field Emission Scanning Electron Microscope (FESEM, JSM-7600F).

Preparation of POPC liposomes. The dried POPC film from 5 mg mL^{-1} POPC was rehydrated with 1.0 mL of 1 mM TNP in PBS. This is followed by multiple extrusion cycles (20 times) through the membranes with 200 nm pores. Non-encapsulated

TNP molecules were removed by centrifugation using an ultra-centrifuge (Sorvall Legend Micro 21 R, provided by Thermo Scientific) for 20 min at 10 000 g at 15 °C.[38] After the removal of the supernatant, the residual liposomal pellet was re-suspended in 1 mL of PBS, and the centrifugation was repeated another three times. The final liposomal pellet was re-suspended in 0.5 mL of PBS to obtain a liposome solution with a concentration of 13.2 mM. The size distribution and polydispersity of liposomes fabricated were checked by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS (Malvern, UK). A 20× diluted liposome sample (in PBS) was measured at a fixed scattering angle of 173°; three measurements, each consisting of 3 runs of 30 s duration, were recorded at room temperature.

Development of the rGO-based biosensor. After graphene synthesis, 2 mm wide, 100 nm thick Au source and drain electrodes (spacing = 200 μ) were evaporated on the substrates. A voltage bias of 10 mV is applied between source and drain electrodes and gate potential is applied *via* a reference electrode (3 M KCl) (FLEXREF from World Precision Instruments). A PDMS reservoir was utilized to confine test solutions between the electrodes.

Detection of PLA_2. TNP-encapsulated liposomes in equal quantity were mixed with PLA_2 of different concentrations (100 pM, 500 pM, 1 nM and 10 nM) and kept for 30 min to allow complete reaction. For kinetic measurements, the devices were incubated in the mixed solution for 10 min, followed by removal of the solution and electrical measurements in buffer (liquid-gated measurement). All measurements were conducted under ambient conditions (25 °C and atmosphere pressure).

Conclusions

A novel approach for rapid and label-free enzymatic assay using reporter-encapsulated liposomes on graphene field effect transistors (FET) was described. As a demonstration, detection of PLA_2 in terms of both activity and concentration was achieved using TNP-encapsulated POPC liposomes. In virtue of the signal enhancement effect by the excess concentration of TNP encapsulated in liposomes, the developed sensor responded to pM levels of target molecules. The LOD measured was 80 pM and the incubation time is less than 10 min, indicating significantly improved performance compared with assays reported previously. The proposed approach could be extended for detection of other lipid-degrading enzymes and membrane toxins upon appropriate selection or modification of liposomes and encapsulation molecules.

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