**Super-Resolution Imaging**

### Nanographenes: Ultrastable, Switchable, and Bright Probes for Super-Resolution Microscopy

**Xiaomin Liu**, Shih-Ya Chen, Qiang Chen, Xueling Yao, Márton Gelléri, Sandra Ritz, Sachin Kumar, Christoph Cremer, Katharina Landfester, Klaus Müllen, Sapun H. Parekh, Akimitsu Narita, and Mischa Bonn*

**Abstract:** Super-resolution fluorescence microscopy has enabled important breakthroughs in biology and materials science. Implementations such as single-molecule localization microscopy (SMLM) and minimal emission fluxes (MINFLUX) microscopy in the localization mode exploit fluorophores that blink, i.e., switch on and off, stochastically. Here, we introduce nanographenes, namely large polycyclic aromatic hydrocarbons that can also be regarded as atomically precise graphene quantum dots, as a new class of fluorophores for super-resolution fluorescence microscopy. Nanographenes exhibit outstanding photophysical properties: intrinsic blinking even in air, excellent fluorescence recovery, and stability over several months. As a proof of concept for super-resolution applications, we use nanographenes in SMLM to generate 3D super-resolution images of silica nanocracks. Our findings open the door for the widespread application of nanographenes in super-resolution fluorescence microscopy.

**Introduction**

Super-resolution fluorescence microscopy has enabled visualizing structures with a resolution substantially better than that prescribed by the physics of diffraction-limited conventional microscopy. Several important approaches to super-resolution fluorescence microscopy rely on fluorophores switching between bright, emissive (on) and dark (off) states stochastically when they are continuously excited by light. This phenomenon underlies single-molecule localization microscopy (SMLM), for example, stochastic optical reconstruction microscopy (STORM), photoactivated localization microscopy (PALM), and minimal emission fluxes (MINFLUX) microscopy in the localization mode. Presently, four classes of photoactivatable fluorophores are used in such applications: small-molecule organic dyes, inorganic quantum dots (QDs), and carbon dots (CDs). Each class of fluorophores has its strengths and weaknesses from either a photophysical or application perspective. Briefly, organic dyes, such as Alexa 647, the gold standard in SMLM, are small molecules with excellent blinking properties like high photon numbers and low on-off duty cycles. However, their photophysical properties are strongly environment-dependent (buffer) dependent, restricting their use for super-resolution imaging to specific environments, and the imaging buffer typically has a limited chemical reaction lifetime (less than several hours) after preparation. Photoactivatable/switchable fluorescent proteins have exquisite target molecule specificity but have the issue of low photon numbers resulting in a lower localization precision compared to organic dyes. QDs are very photostable and bright, but their large size (∼10 nm) and high on-off duty cycle limit their function as reliable minimally invasive probes. Also, the toxicity of QDs contain-

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**[+] Dr. X. Liu, Q. Chen, X. Yao, Dr. S. Kumar, Prof. Dr. K. Landfester, Prof. Dr. K. Müllen, Dr. S. H. Parekh, Prof. Dr. A. Narita, Prof. Dr. M. Bonn Max Planck Institute for Polymer Research Ackermannweg 10, 55128 Mainz (Germany) E-mail: liuxiaomin@mpip-mainz.mpg.de bonn@mpip-mainz.mpg.de S. Chen, Dr. M. Gelléri, Dr. S. Ritz, Prof. Dr. C. Cremer Institute of Molecular Biology gGmbH (IMB) Mainz (Germany) Prof. Dr. C. Cremer Department of Physics, University of Mainz (JGU) Mainz (Germany) and Institute for Pharmacy and Molecular Biotechnology (IPMB) and Kirchhoff Institute for Physics (KIP) University of Heidelberg Heidelberg (Germany) Prof. Dr. K. Müllen Institute of Physical Chemistry Johannes Gutenberg-University Mainz Mainz (Germany) Dr. S. Kumar, Prof. Dr. S. H. Parekh Department of Biomedical Engineering University of Texas at Austin Austin, TX (USA) Prof. Dr. A. Narita Organic and Carbon Nanomaterials Unit Okinawa Institute of Science and Technology Graduate University Okinawa (Japan)**

**[+] These authors contributed equally to this work.**

**[++] co-first authors**

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ing heavy metal atoms may restrict their applicability in biomedicine.\textsuperscript{[29]} CDs have environment-independent blinking properties and are smaller than QDs; however, the undefined/mixed chemical structures not only induce broader fluorescence absorption and emission spectra but also make target-specific binding difficult.

Graphene quantum dots (GQDs) are nanoscale graphene fragments with well-defined, quantized energy levels and have recently been proposed as an environmentally friendly alternative to CDs and QDs.\textsuperscript{[20]} GQDs are, in principle, superior for microscopy imaging due to their small size (typically < 10 nm) and low toxicity. The synthetic approaches to GQDs can be classified in general as top-down or bottom-up approaches. The top-down synthesis method uses physical or chemical techniques to “cleave” graphene\textsuperscript{[24]} or other carbon allotropes such as $C_{60}$ fullerene\textsuperscript{[21]} and double-walled carbon nanotubes.\textsuperscript{[22]} Such methods produce GQDs varying in size and chemical structure, similar to CDs, which results in heterogeneous CD-like photophysical properties. The nanographenes employed in this work are molecularly defined GQDs with a monodisperse character, that is, a precise chemical structure and elemental composition, which are more specifically called (nano)graphene molecules.\textsuperscript{[23,24]} They are obtained by a bottom-up synthesis method which is stepwise derivatization from small organic molecules as starting materials. This method, based on synthetic organic chemistry, not only permits creating nanographenes with well-defined absorption and emission spectra defined by their precise chemical structures, but also enables the introduction of various functional groups\textsuperscript{[20,25]} similarly to organic dyes, e.g., for binding to specific ligands or reactions with specific target molecules. The spectral (absorption and emission) properties of nanographenes can be tailored by engineering the shapes and edge structures with the extension of carbons.\textsuperscript{[26]} These advantages pave the way to develop nanographenes as a new series of fluorophores for optical imaging. While stable fluorescence, i.e., nonblinking emission from nanographenes has been demonstrated\textsuperscript{[27–30]} this stable, nonblinking, fluorescence has precluded the application of nanographenes from SMLM applications.

**Results and Discussion**

Here, we demonstrate that, in contrast to previous reports,\textsuperscript{[22–30]} nanographenes show outstanding blinking behavior, combining many of the superior photophysical properties of existing chromophores; they are very small and display blinking independent of the (buffer or air) environment, making them ideal candidates for SMLM. This discovery can be traced to improved nanographene synthesis and better control over the nanographene aggregation behavior. We show that a broad portfolio of nanographenes, for which the chemical structures, and hence absorption and emission spectra, can be precisely tuned, exhibit near-ideal properties as a new class of probes for super-resolution microscopy allowing multiplexing and 3D SMLM.

In this work, we characterize the properties of four types of graphene molecules (GM): DBOV-Mes, GM-C60, GM-C78, and GM-C96 as blinking probes, benchmarked against Alexa 647, a widely used organic fluorophore in SMLM. These properties include 1) size, 2) absorption and emission spectra, 3) duty cycle (fraction of time a molecule resides in its fluorescent state), and 4) photon number (detected photons per switching event). Figure 1a,b shows the chemical structures and spectra of DBOV-Mes, dibenzo[hi.j]alvalene (DBOV) with two mesityl (Mes) groups,\textsuperscript{[31,32]} and GM-C60\textsuperscript{[33]} with alkyl side chains. DBOV-Mes (GM-C38) and GM-C60 consist of 38 and 60 sp²-hybridized carbons in their aromatic cores, both with an approximate size of 1 to 2 nm. The chemical structures and spectra of GM-C78, GM-C96, and Alexa 647 are shown in the Supporting Information, Figures S1 and S2. Table 1 shows general fluorescent characteristics of nanographenes in an organic solvent and Alexa 647 in an aqueous solvent. All of the measured nanographenes exhibit well-defined absorption and emission spectra with different emission wavelengths. In particular, DBOV-Mes has very narrow absorption and emission spectra comparable to those of commonly used organic dyes, e.g., Alexa 647, making this nanographene ideal for multicolor imaging.

As mentioned above, Alexa 647 and other organic dyes maintain proper blinking properties in a special blinking buffer. Here we used a standard buffer containing an enzymatic oxygen scavenger system with glucose oxidase and a primary thiol (cysteamine, MEA).\textsuperscript{[4]} Most often due to oxidation reactions or side reactions, these imaging buffers degrade over time, and blinking conditions are maintained only for a few hours.\textsuperscript{[43]} We measured the blinking properties of nanographenes in different environments (i.e., without any blinking buffer) and compared it to Alexa 647 under the optimized conditions (Table 2 and Figures S3–10). As a straightforward measurement to demonstrate the blinking performance of nanographenes in different environments, based on the observation of blinking events, we quantified the duty cycles and the average photon numbers per blinking event for nanographenes in air, in Dulbecco’s phosphate-buffered saline (DPBS) buffer, or embedded in a polystyrene (PS) film. As shown by the representative dataset shown in Figure 1c, nanographene GM-C60 exhibits intrinsic blinking when exposed to air. Figure 1d,e shows the duty cycle and the mean photon number per switching event of GM-C60 as compared to the properties of Alexa 647. Figure 1f and Table 2 show that DBOV-Mes and GM-C60 in each environment have average photon numbers per blinking event about 1.3-fold higher than those of Alexa 647 in the optimized blinking buffer while maintaining comparable duty cycles and blinking times. This result demonstrates the versatility of nanographene fluorophores compared to small dye molecules; they retain the required properties for SMLM: good blinking in ambient conditions and no special buffer chemistry is needed. Regarding the blinking mechanism of nanographenes, their blinking behavior in different environments indicates a mechanism different from that of traditional organic dyes,\textsuperscript{[34]} semiconductor quantum dots,\textsuperscript{[35,36]} and carbon nanodots.\textsuperscript{[37,38]} Elucidating the blinking mechanism of nanographenes is the topic of ongoing research.
In addition to the excellent blinking properties, we further investigated the photo- and chemical stability of nanographenes decreased with time over 16 min (20 000 frames) from the beginning of the experiment. In the following 15 min, an

Table 1: Fluorescence properties of different nanographenes and Alexa 647 in solution.

| Dye         | Excitation max. [nm] | Emission max. [nm] | FWHM of excitation spectrum [nm] | FWHM of emission spectrum [nm] | Extinction [m$^{-1}$ cm$^{-1}$] | Quantum yield | Brightness [m$^{-1}$ cm$^{-2}$] |
|-------------|----------------------|--------------------|----------------------------------|-------------------------------|---------------------------------|---------------|-----------------------------|
| Alexa 647   | 650$^{[a]}$          | 665$^{[a]}$        | 38$^{[h]}$                      | 35$^{[h]}$                    | 239 000$^{[h]}$                 | 0.33$^{[h]}$  | 78 870                      |
| DBOV-Mes (GM-C38) | 610                | 614                | 15$^{[j]}$                      | 22$^{[j]}$                    | 70 000                          | 0.79          | 55 300                     |
| GM-C60      | 412$^{[j]}$          | 701                | 84$^{[j]}$                      | 220                           | 22 000                          | 0.1           | 2200                       |
| GM-C78      | 513$^{[j]}$          | 80$^{[j]}$         | 80$^{[j]}$                      | 54 000$^{[j]}$                | 0.02                            | 1080          |
| GM-C96      | 491$^{[j]}$          | 650                | 83$^{[j]}$                      | 61 000$^{[j]}$                | 0.01                            | 610           |

[a] Spectral maxima, extinction coefficient, and quantum yield of Alexa 647 from Ref. [9]. [b] Full width at half maximum (FWHM) of the excitation and emission spectra of Alexa 647 from the dye manufacturer. [c] Peak wavelength and FWHM of GM-C78 excitation spectrum not available due to the wavelength limitation of the spectrometer. The fluorescence properties of nanographenes were measured in toluene solution.
additional recovery irradiation (405 nm laser) was administered for 1–2 seconds and was applied on every ≈1000 frames. The number of blinking events clearly shows that a substantial number of nanographenes were switched back into the on state following each 405 nm irradiation. Although both Alexa 647 and DBOV-Mes can be transferred into a reversible off state and stochastically activated, the reversible nature of Alexa 647 remains only when it is freshly prepared blinking buffer. On the contrary, the DBOV-Mes sample, in air, maintains its blinking behavior even 125 days after preparation, as demonstrated in Figure 2d. From a practical point of view, such reversible activation of the photorecovery property of DBOV-Mes, even in air, strongly broadens the potential applications of nanographenes in super-resolution microscopy.

As a proof-of-concept for super-resolution microscopy using nanographenes, we prepared nanoscale crevices in glass and imaged them with 3D-SMLM. Afterward, the resulting 3D SMLM image was compared with atomic force microscopy (AFM). Figure 3a shows a schematic of the procedure of depositing nanographenes onto the surface of coverslips and into crevices used for 3D SMLM and AFM imaging. The coverslip was initially examined by conventional brightfield microscopy (Figure S12a) and fluorescence microscopy (Figure S12b). For SMLM, a 2D time series stack of images was acquired, and the raw images were processed to localize nanographenes in 3D within the sample. For 3D localization we first calibrated the microscopy system (Figures S13 and S14), and the DBOV-Mes molecules were localized over an axial (z) interval of ±300 nm with an average localization precision of 17.2 nm. A detailed description of the 3D localization and a direct image comparison between conventional microscopy and the 3D SMLM method can be found in the Supporting Information, Section 2. The molecular localizations were sub-

### Table 2: Summary of blinking properties of different nanographenes and Alexa 647.

| Dye          | Alexa 647 | DBOV-Mes (GM-C38) | GM-C60 | GM-C78 | GM-C96 |
|--------------|-----------|-------------------|--------|--------|--------|
| Environment  | blinking buffer[a] | DPBS buffer | air     | poly-styrene | DPBS buffer | air     | poly-styrene | poly-styrene |
| Detected photons per switching event | 3438 | 4918 | 5570 | 4902 | 3673 | 4960 | 4690 | 5740 | 5020 |
| Duty cycle [×10⁻¹⁰] | 2.1 | 1.3 | 4.7 | 8 | 5.4 | 1.2 | 3.2 | 2.7 | 1.7 |
| Blinking time [ms] | 65 | 87 | 108 | 54 | 75 | 79 | 96 | 83 | 94 |

[a] Blinking properties of Alexa 647 measured in the presence of an enzymatic oxygen-scavenging system (glucose oxidase with catalase [GLOX]) and a primary thiol (MEA, 10 mM). Two representatives of nanographenes, DBOV-Mes and GM-C60, were measured in three different environments: air, DPBS, and embedded in PS, while the other two nanographenes, GM-C78 and GM-C96, were measured only in a PS film.
Figure 3. 3D super-resolution microscopy images of nanometer-sized crevices in a glass substrate. 

(a) Imaging procedure for the “etched” coverslips showing crevice features and subsets of molecules localized on (shaded green) or right below (shaded red) the macroscopic (planar) glass surface. After deposition of nanographenes (DBOV-Mes) on the coverslips (left), the structure was imaged using 3D SMLM (middle) and AFM (right). The resulting blinking localization of 3D SMLM was classified into two subsets, which indicate the distribution of nanographenes close to (green) and right below (red) the glass–air interface in the cracks. 

(b) For the intensity projection along the z-axis between 300 and −10 nm (subset 1) above the “etched” structure, a relatively random spatial distribution of nanographenes was observed. 

(c) For the region between −10 and −300 nm (subset 2), the spatial structure of crevices is apparent. 

(d) AFM reveals the surface structure in the region of interest in (b) and (c). 

(e) Overlay of the SMLM image in (c) and AFM image in (d) shows strong overlapping features after image registration. 

(f, g) Line profiles from AFM (d) and SMLM (c) images show an FWHM about 70 nm and 80 nm, respectively.

Conclusion

In summary, nanographenes with atomically defined chemical structures exhibit ideal properties for various super-resolution microscopy methods due to their environmentally independent blinking behavior, large photon numbers, good stability, and well-defined excitation and emission spectra. In our case, the highly pure synthetic production and sample preparation allowed us to discover these properties, which stand in contrast to the general knowledge that nanographenes are constant fluorescent emitters. Atomically precise nanographenes blink in various environments and have high photon numbers per blinking event and low duty cycles in comparison to Alexa 647, the current gold standard small-molecule dye. Moreover, the nanographene dyes exhibit extremely high sample stability of more than 1000-fold longer than Alexa 647. The nanoscale structural imaging of etched cracks in glass demonstrates the potential of nanographenes in super-resolution fluorescence microscopy for applications in materials science. Also, the exquisite chemical control and blinking environment-independent properties of nanographenes offer a great opportunity to investigate blinking mechanisms, which is challenging for organic dyes due to complex buffer dependence and the relatively minimal shelf-life of buffer solutions. Moreover, specific functional side groups can be incorporated at peripheral positions of the nanographenes, for example, hydrophilic groups to enhance the water solubility and binding functionality for protein targeting making nanographenes very promising dyes for bioimaging applications.
Experimental Section

Microscopy

3D SMLM was performed with a SR GSD 3D (Leica) equipped with four lasers: 488 nm (300 mW), 532 nm (500 mW), and 642 nm (500 mW) for excitation, and 405 nm (30 mW) for fluorescence reactivation. An HCX PL APO 160 x 1.43 NA OIL CORR-TIRF (1.43 NA Oil CORR-TIRF) mode was used for single-molecule blinking measurements and for super-resolution imaging. For all measurements of nanographenes, the 532 nm laser and the emission bandpass filter (550–650 nm/449–451 nm) were used. For the measurements using Alexa 647, the 642 nm laser and the emission bandpass filter (660–760 nm) was used. The microscope was equipped with a back-illuminated EMCCD camera (iXonDU-897, Andor). The resulting images have a pixel size of 100 nm. The detailed description of the setup can be found in the Supporting Information, Section 4.1.

Atomic force microscopy (AFM, Dimension FastScan, Bruker) was done in the tapping mode in air. A standard V-shaped silicon nitride cantilever which has a spring constant of 26 Nm−1 with a pyramidal tip from Bruker AFM probes (OTESPA-R3) was mounted. The tip has on average a height of 15 nm and a radius of curvature of around 10 nm with a vertex angle of 35° and 18°. Further information about the AFM can be found in the Supporting Information, Section 4.2. AFM images were acquired with a 0.5 Hz line rate, and images with 512 pixels × 512 pixels were acquired.

Experiments for Blinking Properties of Single Nanographenes and Alexa 647

For evaluation of the blinking properties including photon per switching event and on-off duty cycle, nanographene samples were prepared in different embedding media including air, DPBS, and polystyrene. For comparison, additional samples with Alexa 647 were prepared in an oxygen-scavenging system. Detailed information can be found in the Supporting Information, Section 5.

Multiple series of 20000 frames were recorded for every nanographene variant. Nanographenes were excited continuously during imaging with the appropriate wavelength. Images were acquired at an exposure time of 50 ms and an EM gain of 100.

To extract single-molecule time traces, first a maximum intensity projection of the recorded time series was generated. Molecules were localized in the maximum-intensity projection image, returning the positions of all molecules visible throughout the recorded time series. Single-molecule time traces were then extracted as background-corrected intensities at these positions over all recorded frames. Photon counts and blinking times were determined by localization of single-molecule positions in all frames and merging into blinking events. Resulting histograms of photons per blinking event and blinking duration were fit with a single-exponential function. The reported mean values and errors were extracted from the respective fits. For the detailed description, see the Supporting Information, Section 6.

To investigate the photorecovery characteristics of nanographenes, 40000 frames were acquired with an exposure time of 50 ms and an EM gain of 100. At the first 20000 frames, images were acquired by continuous laser irradiation at 532 nm with an irradiance of 4 kW cm−2. While acquiring images from frame 20000 to frame 40000, additionally the 405 nm laser with an intensity of 0.30 kW cm−2 was administered for 1–1.65 seconds and this was repeated around every 1000 frames. The images were processed and localizations were determined for analysis.

Experiments for Imaging 3D Crevices Structures

A mild chemical treatment was used to etch nanoscale "cracks" on the surface of a borosilicate coverslip (iBidi, Gridded glass coverslips Grid-50). For fluorescence imaging, 3 μL (1 nM) nanographene in toluene was applied on the coverslip. The detailed sample preparation is described in the Supporting Information, Section 7. For super-resolution imaging of the 3D crevices on the coverslips, the TIRF mode was used to excite the samples with an intensity of 10 kW cm−2 at a laser wavelength of 532 nm. 40000 frames with an exposure time of 50 ms and an EM gain of 100 were acquired and processed to determine the 3D localization. AFM images of the same region were acquired, and the resulting image is shown in Figure S15a. The complete overlay image of AFM and SMLM after image registration is shown in Figure S15b for comparison.

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Conflict of interest

X. Liu, A. Narita, S. Parakh, Q. Chen, C. Cremer, K. Landfester, K. Müller, and M. Bonn are listed as inventors on a patent application (application no. 18199451.8 – EPO) and A. Narita, Q. Chen, K. Müller, X. Liu, S. Parakh, and M. Bonn are listed as inventors on a patent application (application no. 18199447.6 – EPO) related to the work presented in this manuscript. All other authors have nothing to disclose.

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