Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Electrochemically controlled cleavage of imine bonds on a graphene platform. Towards new electro-responsive hybrids for drug release

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1. Materials and chemicals

Synthetic spherical graphite (SG17, ≥ 99.95 % C, Bonding Chemical, USA) with tap density ≥ 1 g/ml and a specific surface area ≤ 6.5 m^2/g was used after annealing under vacuum (300 °C).
Commercial reagents and anhydrous solvents were purchased from ABCR GmbH, Aldrich Chemical Co., or Strem Chemicals Inc., and were used without further purification. Kaiser test kit and Sephadex LH-20 were purchased from Sigma-Aldrich. All anhydrous solvents for graphite intercalation compound (GIC) were used and stored in glovebox.
The adherent mouse astrocyte cell line C8-D1A was purchased from ATCC-LGV and cultured in Dulbecco's Modified Eagle's Medium (DMEM) completed with 1 mM sodium pyruvate, 2nM L-glutamine, 100 $\mu g \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin and 10 % heat-inactivated fetal bovine serum from Gibco. The PBS buffer was purchased in Sigma Aldrich in tablets and prepared following manufacture procedures, corresponding to 10 mM phosphate buffer containing 137 mM NaCl and 2.7 Mm KCl at ph 7.4. The 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) -based assay cell Proliferation kit, CellTiter 96® AQueous One Solution Cell Proliferation Assay was purchased from Promega.

2. Equipment and Characterization

Glove Box: Sample synthesis and preparation was carried out in an argon filled glovebox, equipped with a gas purifier and solvent vapor removal unit: oxygen content < 0.1 ppm, water content < 0.6 ppm.

Raman Spectroscopy: The Raman spectroscopic characterization was performed using an Invia Renishaw microspectrometer (50) with an excitation wavelength of 532 nm. The spectra were processed with WiRE 4.4 RENISHAW software.

X-ray photoelectron spectroscopy (XPS): XPS experiments were made in a SPECS HR100 spectrometer in high vacuum atmosphere with a pressure around 10^{-6} Pa (or 10^{-8} mbar) using a non-monochromatic Mg source with energy of 1253.6 eV. An electron flood gun was used in order to neutralize for charging.

Nuclear magnetic resonance spectroscopy (NMR) ^1H spectrum was recorded at 500 MHz instrument.

Ultra-performance liquid chromatography-mass spectrometry (UPLC-

MS): Instrument: UPLC Acquity (Waters): Column: Acquity C18 100x2.1mm 1.7µm, Mobile phase: 100mM Ammonium formate in H_2O (A) / ACN (B), Injection volume = 10 µL / Column temperature =30 °C

Time (min)	Flow (µL/min)	%A	%B
0	300	95	5
0,5	300	95	5
1	300	70	30
15	300	20	80
16	300	1	99
28	300	1	99
28,5	300	95	5
30	300	95	5

Mass spectrometry: Instrument: LCT Premier XE (Waters), Source: Electrospray positive mode / W mode m/z range 100-2000, Capillar volt = 3000 / Cone volt = 50, Desolvation temp = 300 °C / Source temp = 120 °C, Cone gas flow 50 $L \cdot h^{-1}$ / Desolvation gas flow = 600 $L \cdot h^{-1}$

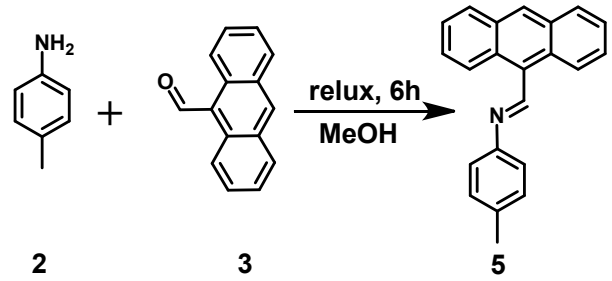
Electrochemical workstation*:* All electrochemical experiments were performed with three-electrode electrochemical cell driven by an Autolab MSTAT204 Potentiostat/Galvanostat. Ag/AgCl was used as the reference electrode. Working and counter electrodes were used according to the experiments as shown in experimental detail for each time.

Microplate spectrophotometer*:* Instrument: GENios Pro (Tecan). Soluble formazan product was measured by absorbance at 492nm.

3. Experimental Details

3.1 Synthesis of (E)-N-(anthracen-9-ylmethylene)-4-methylaniline (5): **5** was prepared according the literature.[1] Anthracene-9-carboxaldehyde (**3**, 1 mmol) was added to a toluidine solution (**2**, 1 mmol) in anhydrous MeOH (20 mL). Then the resulting mixture was refluxed for 6 h. Subsequently, the

reaction solution was slowly evaporated on a rotary evaporator to remove solvent and crystals were obtained. Yield: 262 mg (89%). The resulting product was characterized by ^{1}H NMR and MALDI-MS.^{1}H NMR (500 MHz, $CDCl_3$), δ: 2.45 (s, 3H, CH_3), 7.31 (d, *J* = 8.13 Hz, 2H), 7.36 (d, *J* = 8.25 Hz, 2H), 7.50–7.59 (m, 4H), 8.06 (d, *J* = 8.28 Hz, 2H), 8.56 (s, 1H), 8.73 (d, *J* = 8.89 Hz, 2H), 9.70 (s, 1H, CH=N) ppm. MALDI-MS [M+H$^+$] calculation 296.143, found 296.544.



Scheme S1. Synthesis route of **5**.

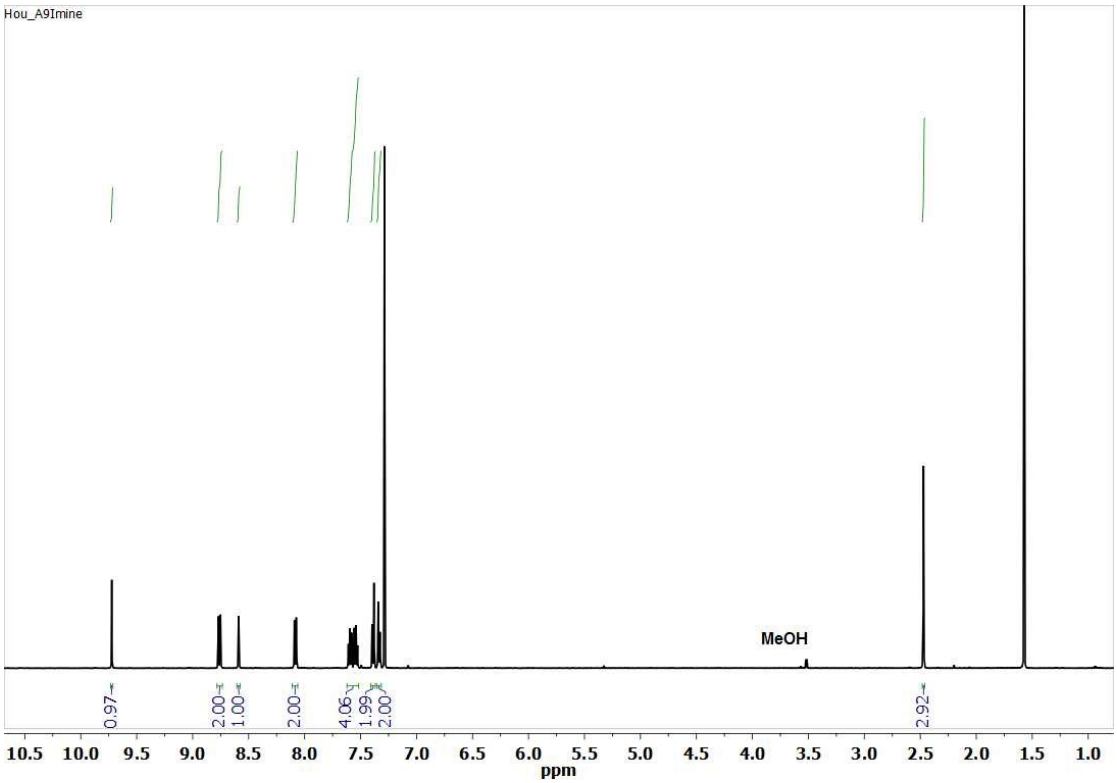


Fig. S1. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of **5**.

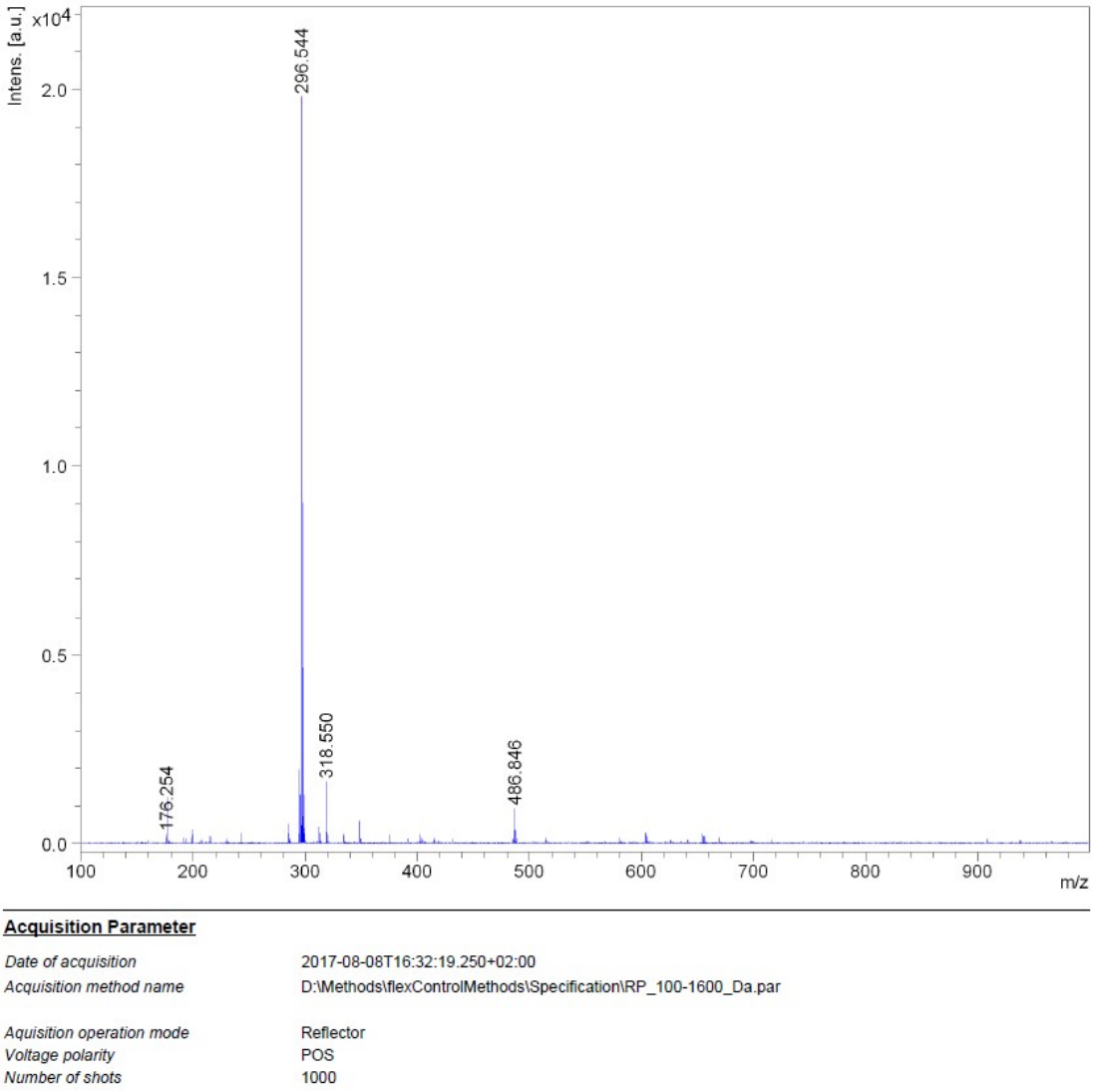
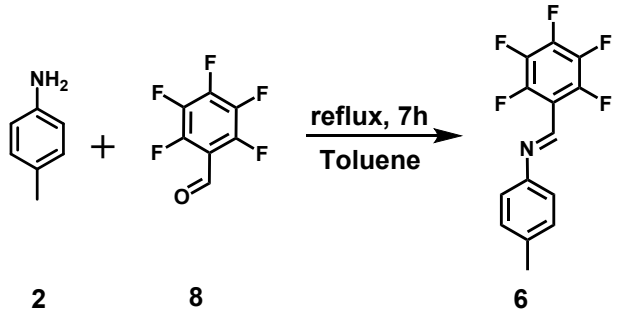


Fig. S2. MALDI-MS of **5**.

3.2 Stability of 5 in serum: 10 mg of **5** was put into 5 mL serum and incubated for 3 days at 37 ºC, 5% CO_2. Then the resulting 4 was obtained by firteration and washed with distilled water. After drying under vacuum, it was mesured ^{1}H NMR.

3.3 Synthesis of (E)-4-methyl-N-(perfluorobenzylidene)aniline (6): 6 was prepared according the literature.[2] The mixture of the 2,3,4,5,6-Pentafluorobenzaldehyde (**8**, 1 mmol) and toluidine (**2**, 1 mmol) in anhydrous toluene (50 mL) was stirred and heated under reflux in a Dean-Stark apparatus for 7 h. Subsequently, the reaction solution was evaporated on a rotary evaporator to remove solvent. The residual solid was recrystallized from HPLC grade ethanol, giving crystals in 227 mg (79%). The resulting product was characterized by ^{1}H NMR and MALDI-MS.^{1}H NMR (500 MHz, $CDCl_3$), δ:

2.39 (s, 3H, CH_3), 7.16 (d, *J* = 8.29 Hz, 2H), 7.23 (d, *J* = 8.28 Hz, 2H), 8.58 (s, 1H, CH=N) ppm. MALDI-MS [M+H$^+$] calculation 286.065, found 286.025.



2 **8** **6**

Scheme S2. Synthesis route of **6**.

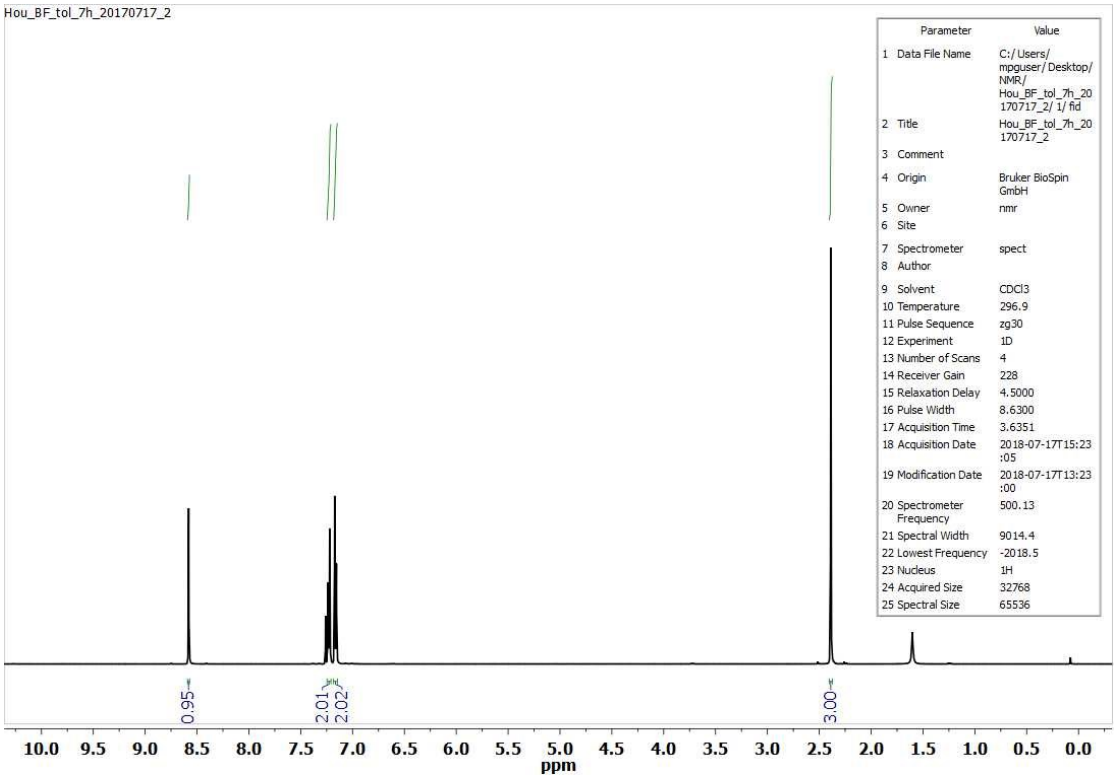


Fig. S3. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of **6**.

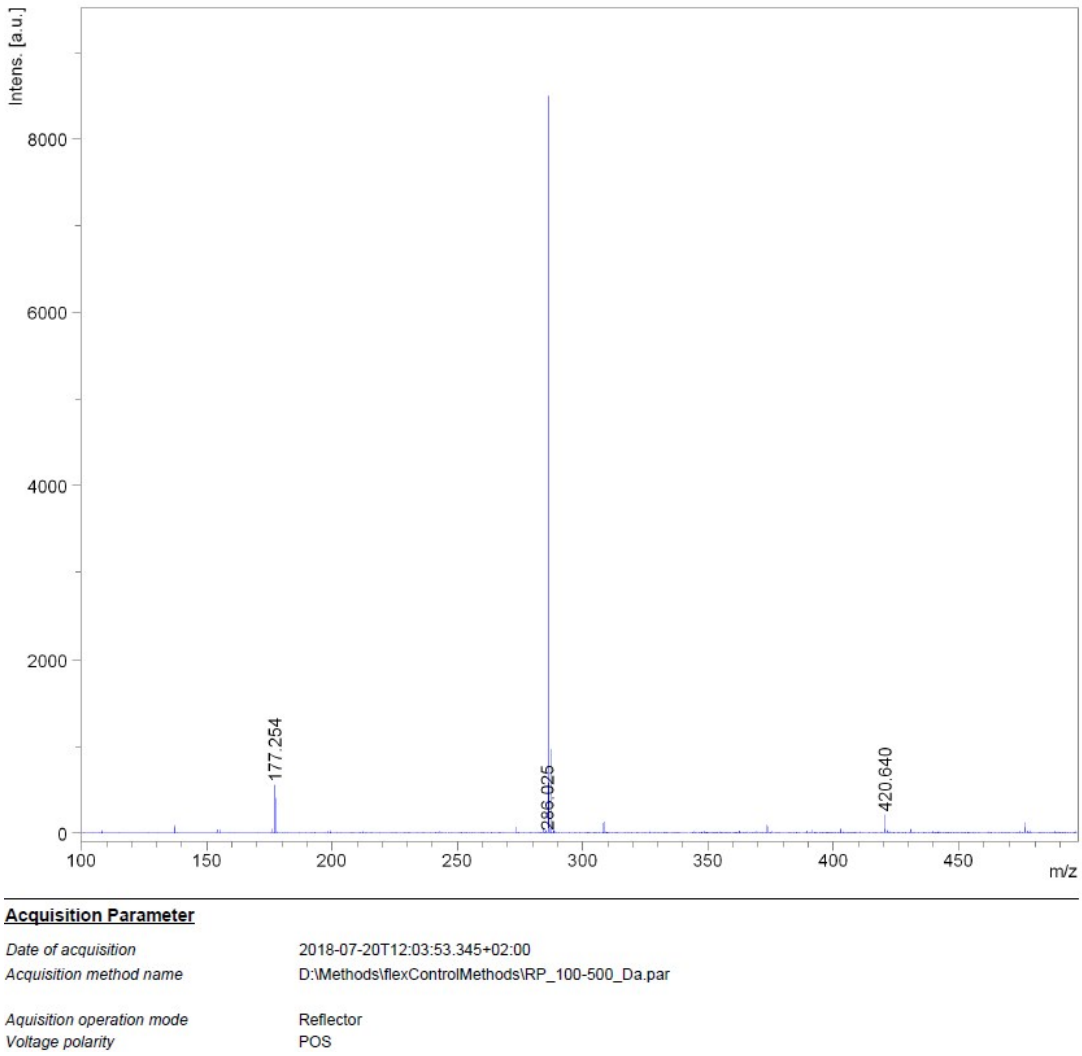
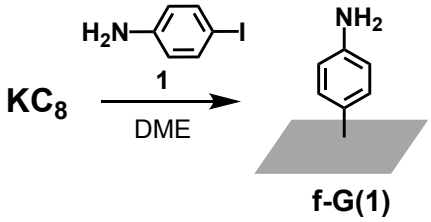


Fig. S4. MALDI-MS of **6**.

3.4 Synthesis of potassium intercalation graphite (KC_8): KC_8 was prepared according to previous report.[3] Pristine spherical graphite (SG17) was heated at 300 °C under vacuum for 72 hours and transferred into the glovebox with Ar atmosphere. Then 96 mg (8 mmol C) graphite and 39.5 mg (1 mmol K) potassium were mixed and heated at 180 °C overnight in order to obtain the brown golden KC_8 graphite potassium intercalation compound.

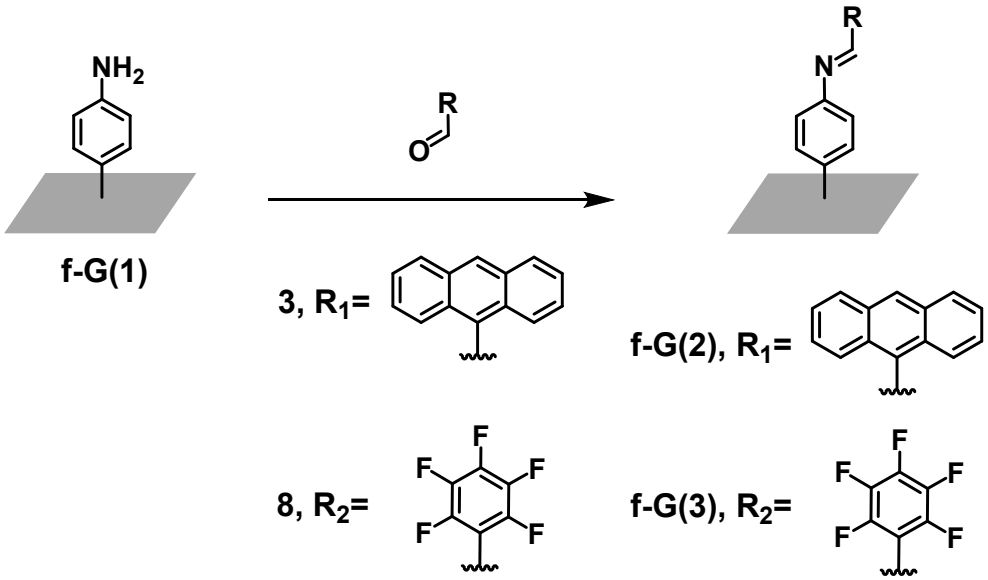
3.5 Synthesis of f-G(1): f-G(**1**) was prepared according to previous report.[3] Firstly, KC_8 (68 mg, 0.5 mmol, 1 eq.) was dissolved in 60 mL of anhydrous DME at room temperature and sonicated for 5 min with tip sonicator in glovebox. Subsequently, 4-iodoaniline (**1**, 2 mmol, 4 eq., **1**) was added. After 4 hours stirring, the reaction was quenched by the addition of 10 mL PhCN. 100 mL cyclohexane was added to the solution, which was then washed five

times with 100 mL water. The organic phase was filtered over a reinforced cellulose membrane filter (0.45 µm pore). The grey solid was washed with acetone, THF, toluene, and DMF.



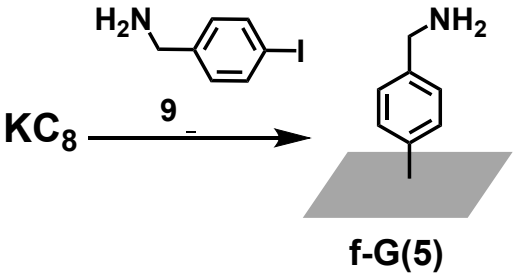
Scheme S3. Synthesis route of f-G(**1**)

3.6 General procedure for the imination of f-G(1): The corresponding aldehyde (1 mmol) was added to a f-G(**1**) (20 mg) suspension in anhydrous MeOH (20 mL) and the resulting mixture was refluxed for 6 h. The suspension was filtered over a reinforced cellulose membrane filter (0.45 µm pore). The grey solid was washed with acetone, THF, toluene, and DMF.



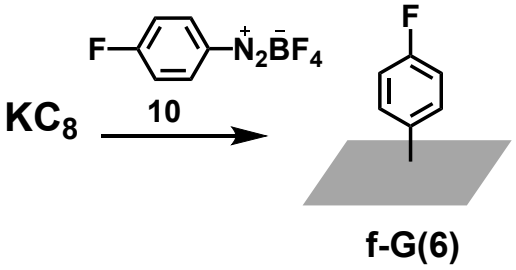
Scheme S4. Imination reaction of-G(**1**).

3.7 Synthesis of f-G(5): The procedure was similar to those for preparation of f-G(**1**), but using 4-Iodobenzylamine (**9**) instead of 4-iodoaniline (**1**). The amount of amine of f-G(**5**) is 24 µmol/g according to Kaiser Test (Fig. S13).



Scheme S5. Synthesis route of f-G(**5**).

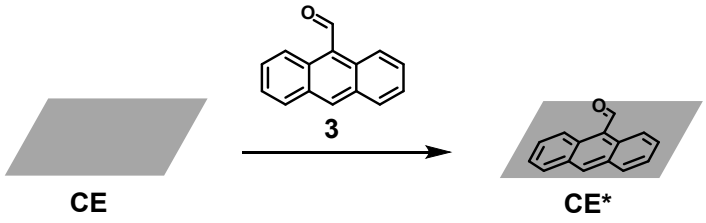
3.8 Synthesis of f-G(6): KC_8 (68 mg, 0.5 mmol, 1 eq.) was dissolved in 60 mL of anhydrous DME at room temperature and sonicated for 5 min with tip sonicator in glovebox. Subsequently, 4-fluorobenzediazonium tetrafluoroborate (**10**, 2 mmol, 4 eq., **1**) was added. After 4 hours stirring, the reaction was quenched by the addition of 10 mL PhCN. 100 mL cyclohexane was added to the solution, which was then washed five times with 100 mL water. The organic phase was filtered over a reinforced cellulose membrane filter (0.45 µm pore). The grey solid was washed with acetone, THF, toluene, and DMF.



Scheme S6. Synthesis route of f-G(**6**).

3.9 Control experiment (CE): The procedure was similar to those for preparation of f-G(**1**), but without addition of 4-iodoaniline.

3.10 Synthesis of CE*: CE (20 mg) was treated with a solution of **3** (1 mmol) in anhydrous MeOH (20 mL) under reflux for 6 h. The reaction mixture was filtered over a reinforced cellulose membrane filter (0.45 µm pore). The grey solid was washed with acetone, THF, toluene, and DMF.



Scheme S7. Synthesis route of CE*.

3.11 Cyclic voltammetries (CVs) of 5: A three-electrode cell was used for CV measurements and a glassy carbon, a platinum wire were used as working electrode and counter electrode, respectively. Ag/AgCl was used as reference electrode. DMF with 0.1 M $LiClO_4$ was used as electrolyte.

3.12 Controlled potential electrolysis of 5: The chemical electrolysis of **5** was performed in a three-electrode cell. Flexible Indium Tin Oxide (ITO) coated PET slices (Sigma-Aldrich) or Pt plate were used as both working and counter electrodes. Ag/AgCl was used as reference electrode. DMF with 0.1 M $LiClO_4$ was used as electrolyte.
5 (25 mg) was dispersed in 10 mL DMF 0.1 M $LiClO_4$ and electrolyzed at 1.35 V vs Ag/AgCl until the current decreased to 10^{-4} A or reached to the theoretical number of electrons required for oxidation (one electron each molecule) to avoid the over oxidation. At the end of the process, the solution was diluted in HPLC grade acetonitrile for UPLC-MS analysis (Fig. S10 and S11). In addition, distilled water was added to the solution and the precipitated product (expected to be mainly **3**) was filtered and dried. The resulting solid was characterized by ^1H NMR in CD_3Cl (Fig. S14 and Fig 1a).

3.13 Controlled potential electrolysis of f-G(2): The procedure was similar to those for electrolysis of **5**, but using f-G(**2**) (36 mg) instead of **5**.

3.14 Fluorescence measurements of eluent of f-G(2) before electrolysis: f-G(**2**) (36 mg) was put in 10 mL DMF with 0.1 M $LiClO_4$ and stirred for 2 hour and the solution was filtered over a reinforced cellulose membrane filter (0.45 μm pore) and the eluent was measured the fluorescence.

3.15 Fluorescence measurements of eluent of f-G(2) after electrolysis: After the electrolysis of f-G(**2**) (20mg), the solution was filtered over a reinforced cellulose membrane filter (0.45 μm pore) and the eluent was measured the fluorescence.

3.16 Controlled potential electrolysis of f-G(3): The procedure was similar to those for electrolysis of **4**, but using ITO with conductive copper tape for loading f-G(**3**) (4 mg) as working electrode instead of pristine ITO, and electrolyzed for 10 s, 30 s, 70 s, and 120 s, respectively. Each one of electrolysis was repeated 3 times. After the electrolysis, the working electrodes were carefully washed by ethanol and acetone. The resulting

products were dried under air at room temperature and measured XPS.

3.17 Controlled potential electrolysis of CE*: The procedure was like those for electrolysis of **5** but using **CE*** (20 mg) instead of **5**.

3.18 Fluorescence measurements of eluent of CE* after electrolysis: After the electrolysis of CE* (20 mg), the solution was filtered over a reinforced cellulose membrane filter (0.45 μm pore) and the eluent was measured the fluorescence.

3.19 Viability of C8-D1A cells on functionalized graphene

Cell culture and counting

C8-D1A cells were cultured in complete DMEM at 37 °C in humidified atmosphere of 5 % CO2 in tissue culture-treated 75 cm2-flasks (Nunc). For cell passage, adherent cells were lifted from the flasks by incubation at 37 °C with trypsin-EDTA solution 1x (2.5 g porcine trypsin and 0.2 g EDTA • 4 Na per liter of Hanks' Balanced Salt from Sigma), span at 1000 g for 5 min and the pellet re-suspended in 1 ml media. For cell counting, the cell suspension was serially diluted 1:10 in PBS and 1:2 in the exclusion dye Trypan Blue solution (0.4 % in 0.8 % sodium chloride and 0.1 % potassium phosphate, dibasic from Sigma Aldrich), 10.0 μL of the diluted cell suspension was counted in a haemocytometer chamber under transmitted light in an inverted microscope (DMIL, Leica).

Cell viability assay

Cell viability of C8-D1A cells was measured with the colorimetric MTS (Owen´s reagent) assay. 10^4 cells were seeded in 100 μL per well of complete media at 37 ºC and 5% CO_2 in a 96-microwell plate and cultured for 4 days. At days 2 and 3, media was replaced with 100 μL complete media alone (untreated control) or containing CE and fG(**1**), at several concentrations (100, 20 and 4 μg/mL) and incubated at 37 ºC and 5 % CO_2 for 72 h and 24 h, respectively. Then, the graphene suspension was replaced by complete media containing MTS to a final dilution of 1:20. Following incubation at 37 ºC and 5 % CO_2 for 30 min, the absorbance at 492 nm of the soluble formazan product generated by the metabolically active cells were measured in a micro plate spectrophotometer (Genios Pro, TECAN). This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes.Data are expressed as a percentage of absorbance of treated cells

related to the untreated control cells (Blank) and represented as means of triplicates ± S.D.

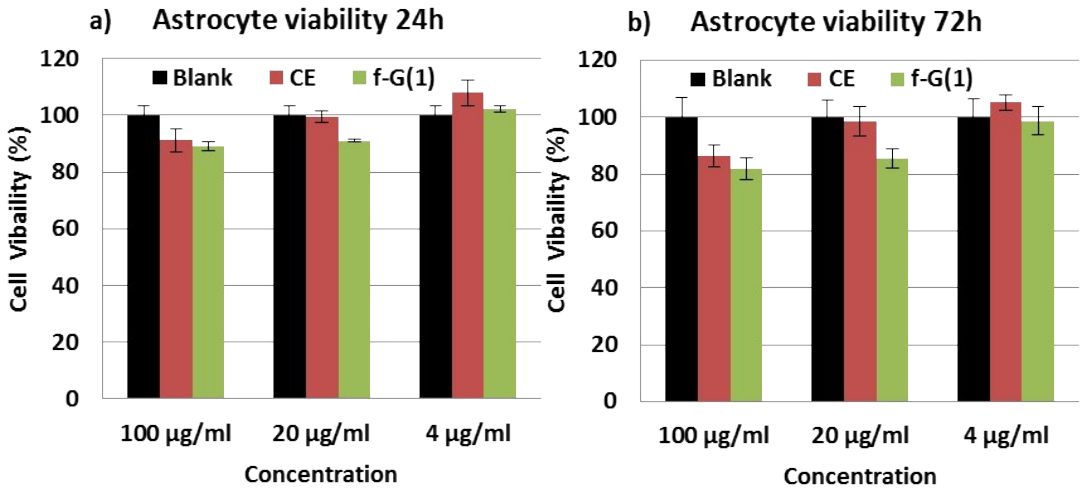


Fig. S5. MTS-based cytotoxicity assay. Cellular viability of astrocytes after incubation with different concentrations of graphene-based materials for (a) 24 h and (b) 72 h, respectively. The absorbance of treated cells is related to the untreated control cells as a percentage.

4. Supporting Figures and Tables

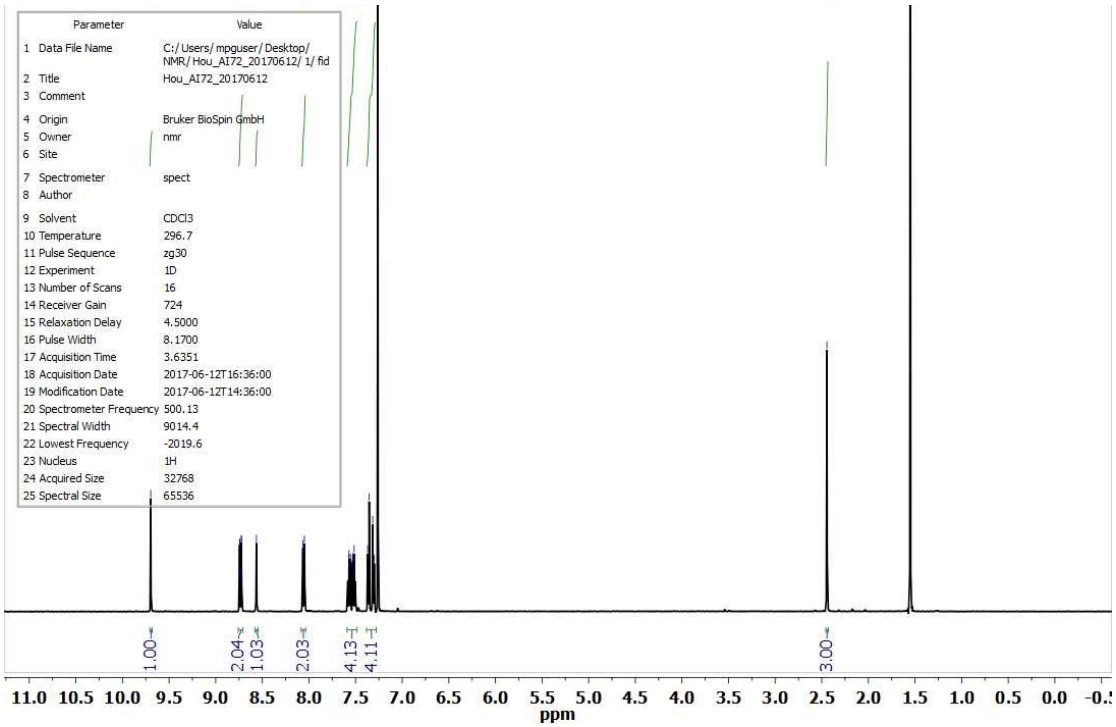


Fig. S6. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of **5** after incubation in serum for 3 days at 37 ºC, 5% CO_2.

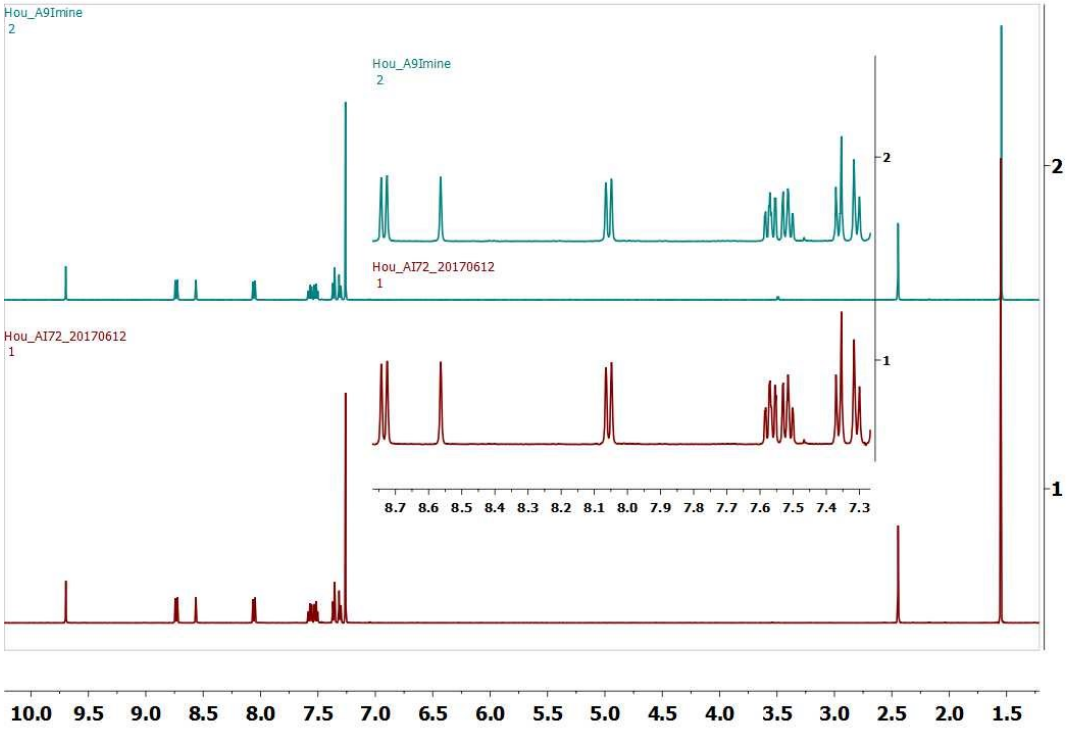


Fig. S7. Comparison of ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of **5** before (top) and after (bottom) incubation in serum for 3 days at 37 ℃, 5% CO_2.

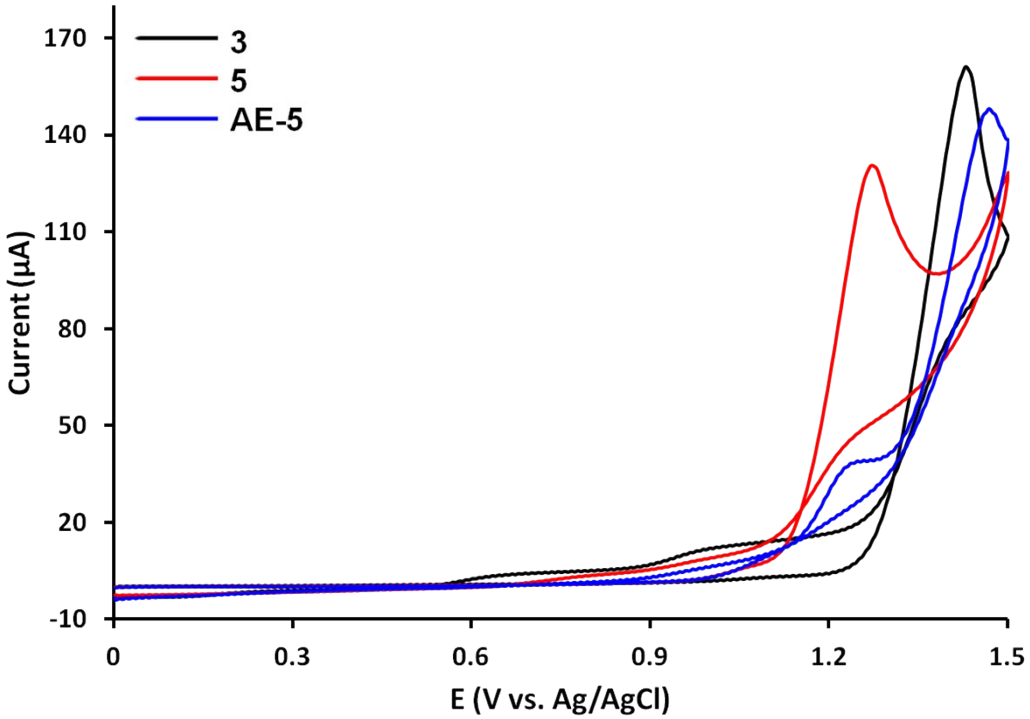


Fig. S8. CVs in DMF with 0.1 M $LiClO_4$, vs. Ag/AgCl.

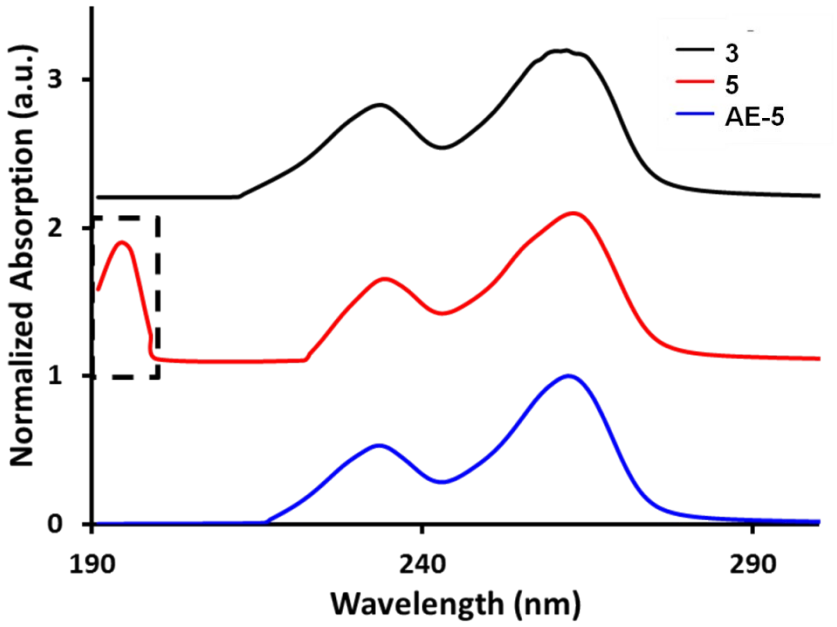


Fig. S9. UV-vis of **3**, **5** and AE-**5** in CH_3CN.

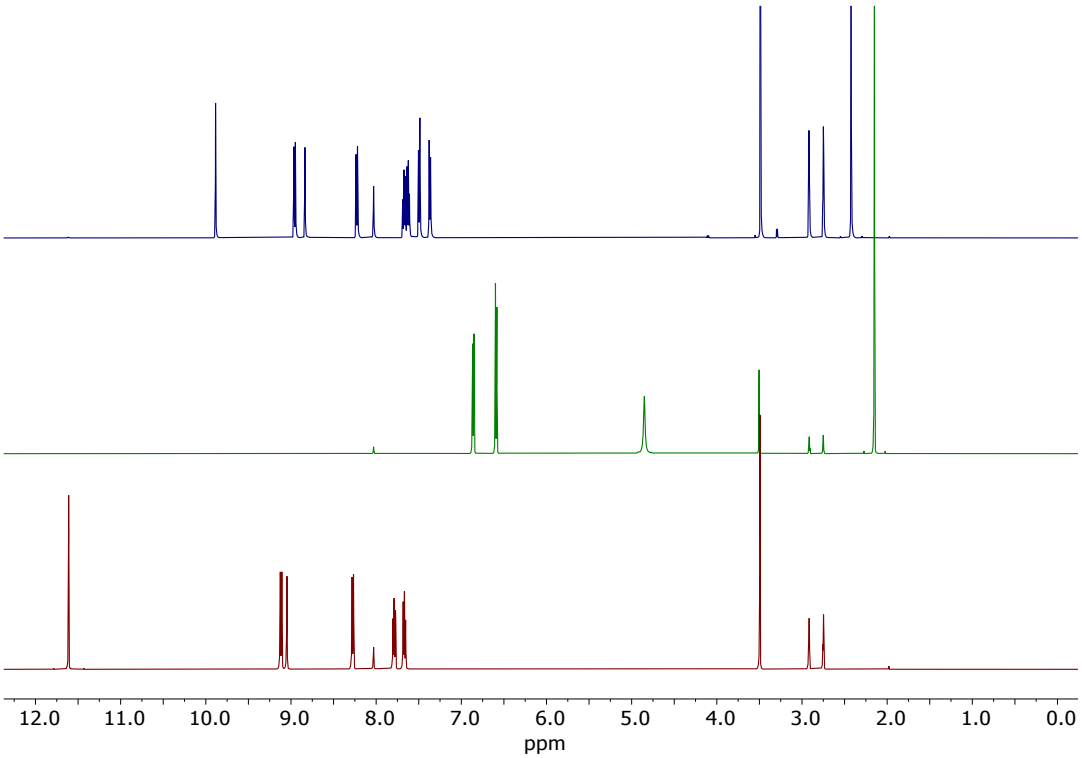


Fig. S10. From the bottom: (red) 1H NMR of 9-anthraldehyde (**3**); (green) 1H NMR of *p*-toluidine (**2**); (blue) 1H NMR of the imine **5** in DMF-d_7.

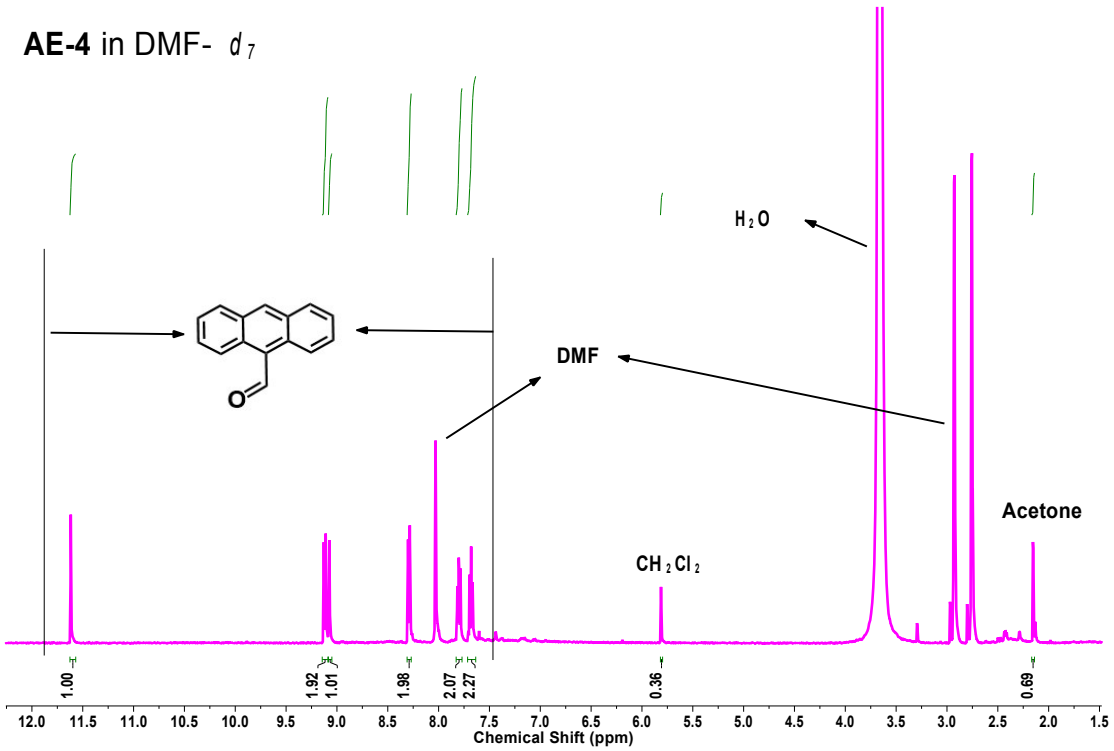


Fig. S11. ^{1}H NMR of AE-**5** in DMF-d_7 without any workup.

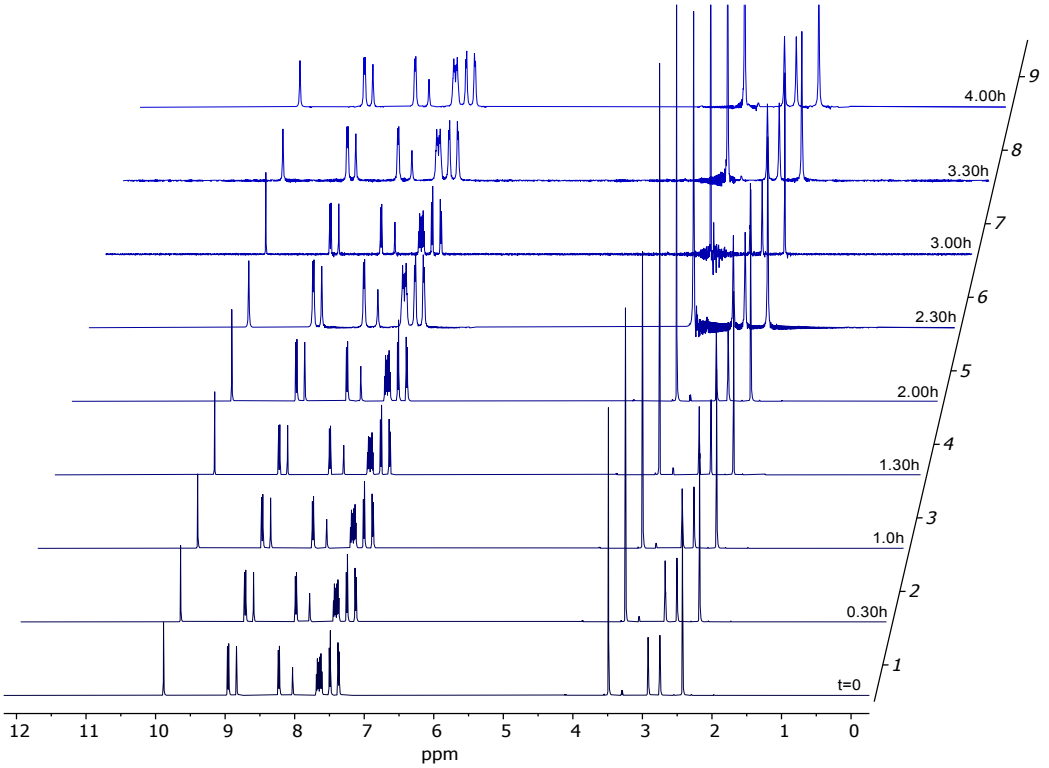


Fig. S12. Evolution in time of compound **5** in DMF-d_7 in a 4-hour time window with ^{1}H NMR spectra recording each 30min.

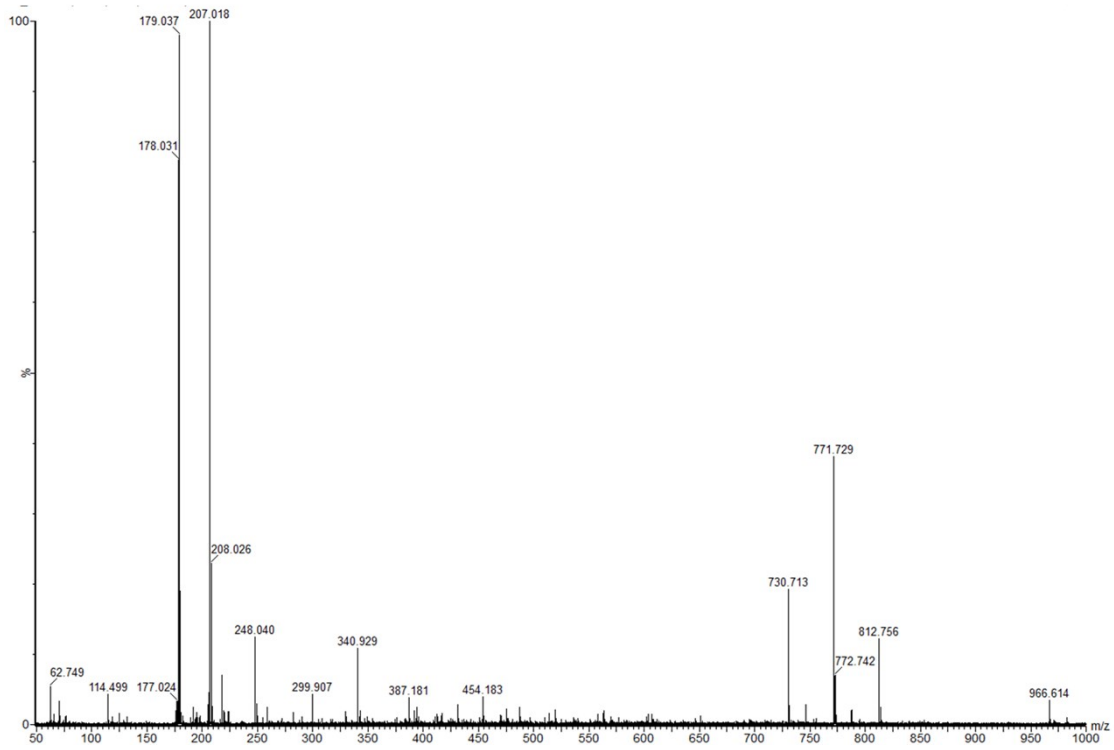


Fig. S13. TOF-MS of peak at 9.6 min of **3**.

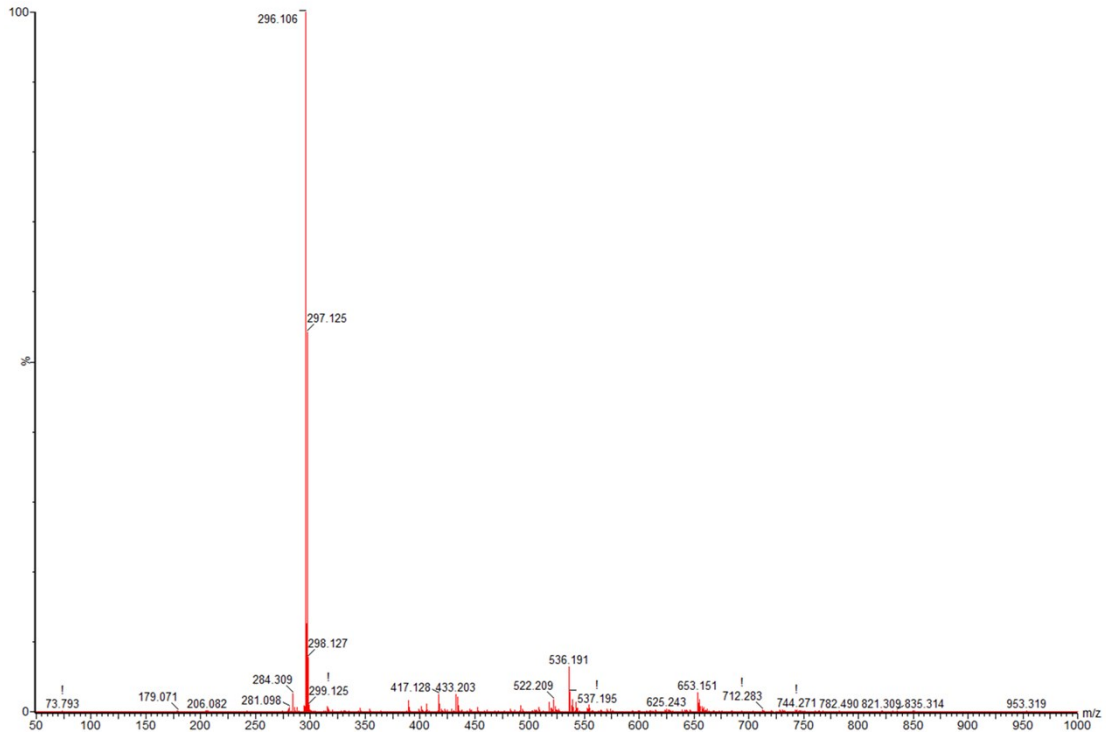


Fig. S14. TOF-MS of peak at 9.5 min of AE-**5**.

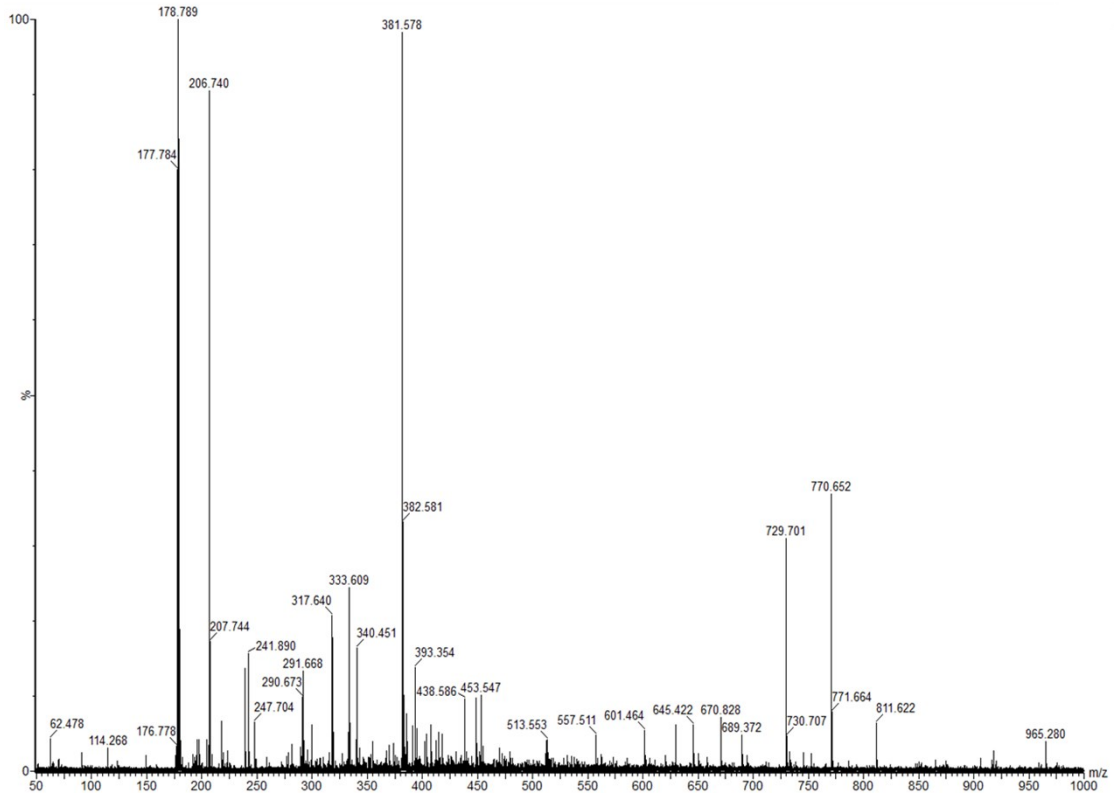


Fig. S15. TOF-MS of peak at 9.5 min of AE-**5**.

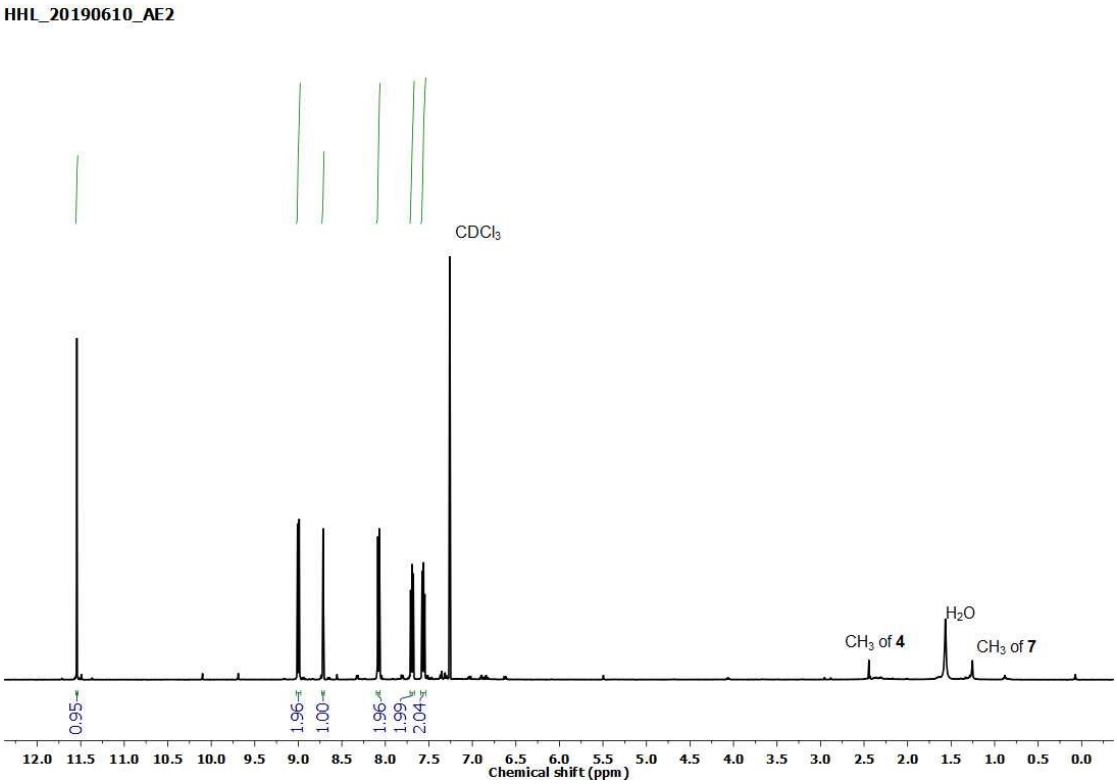


Fig. S16. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of product of after electrolysis of **5** (precipitated from water).

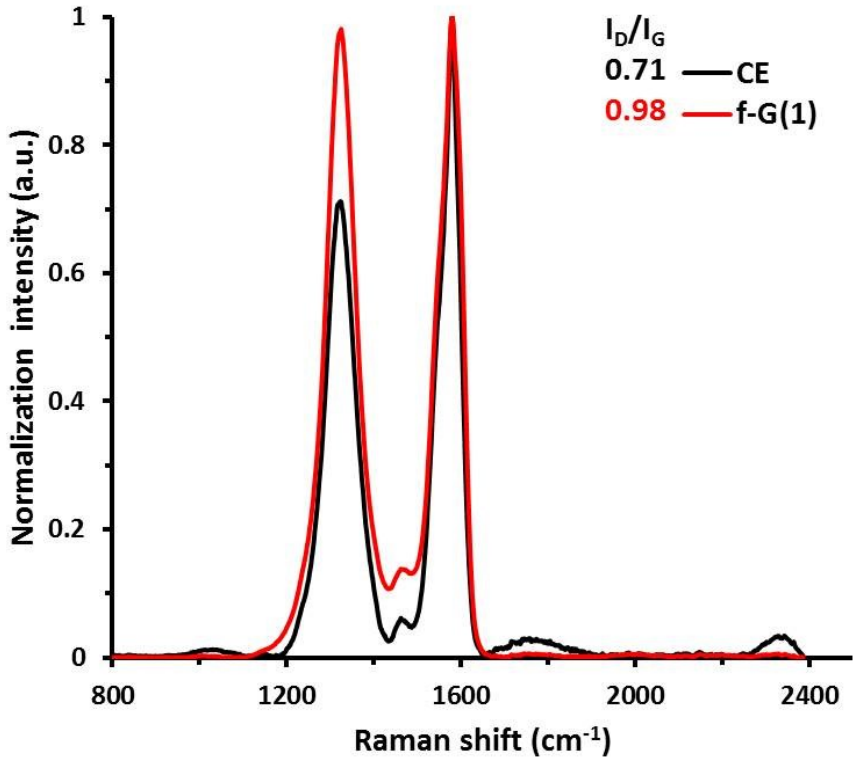


Fig. S17. Averaged Raman spectra of CE and f-G(**1**) (more than 1000 single-point spectra, λ_{exc} = 532 nm).

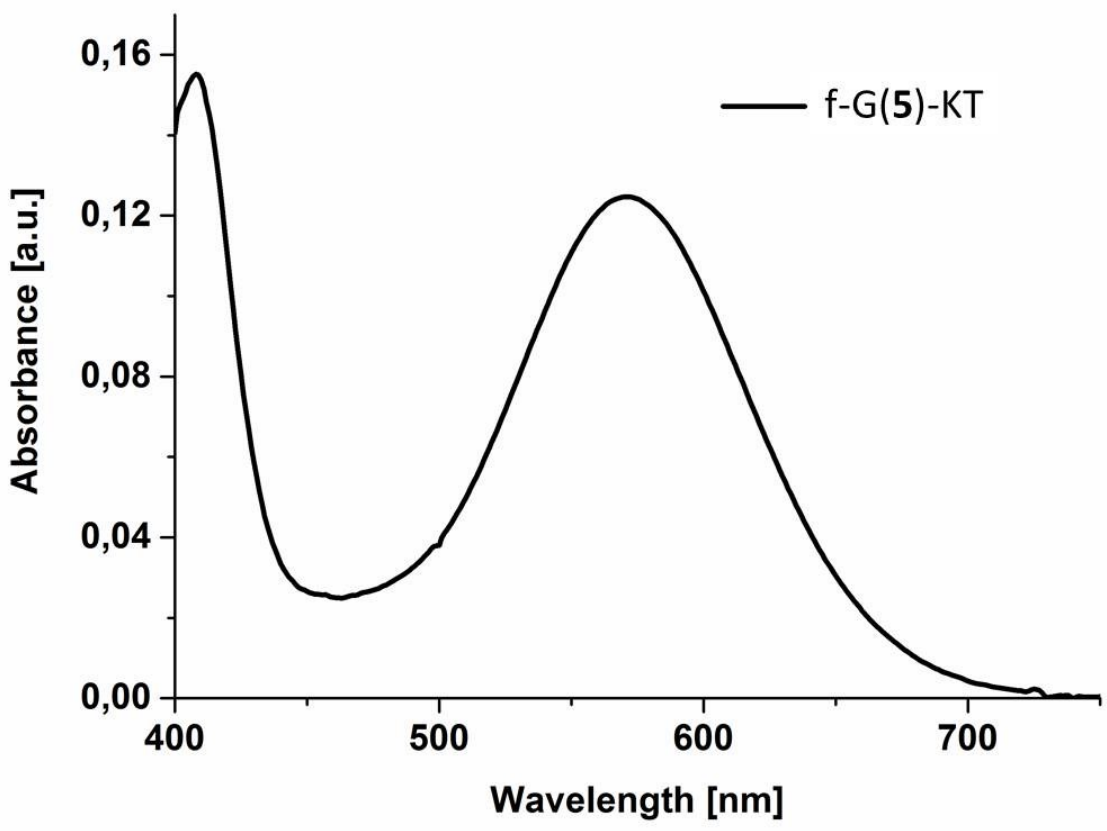


Fig. S18. UV-vis of f-G(**5**) for Kaiser Test.

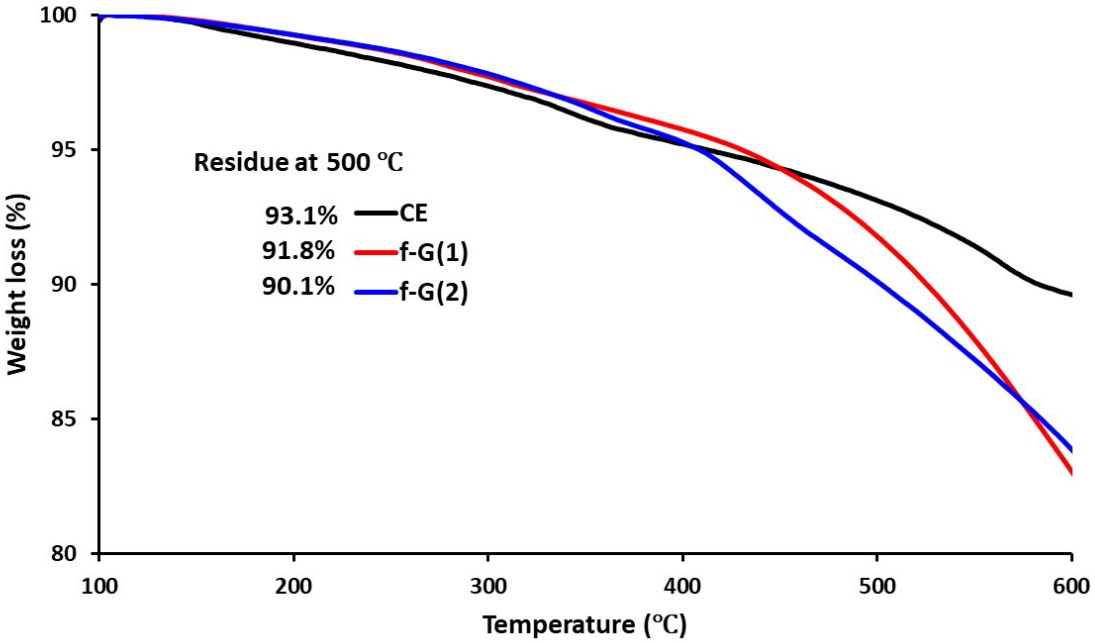


Figure S19. TGA profiles under N_2 atmosphere for graphene derivatives.

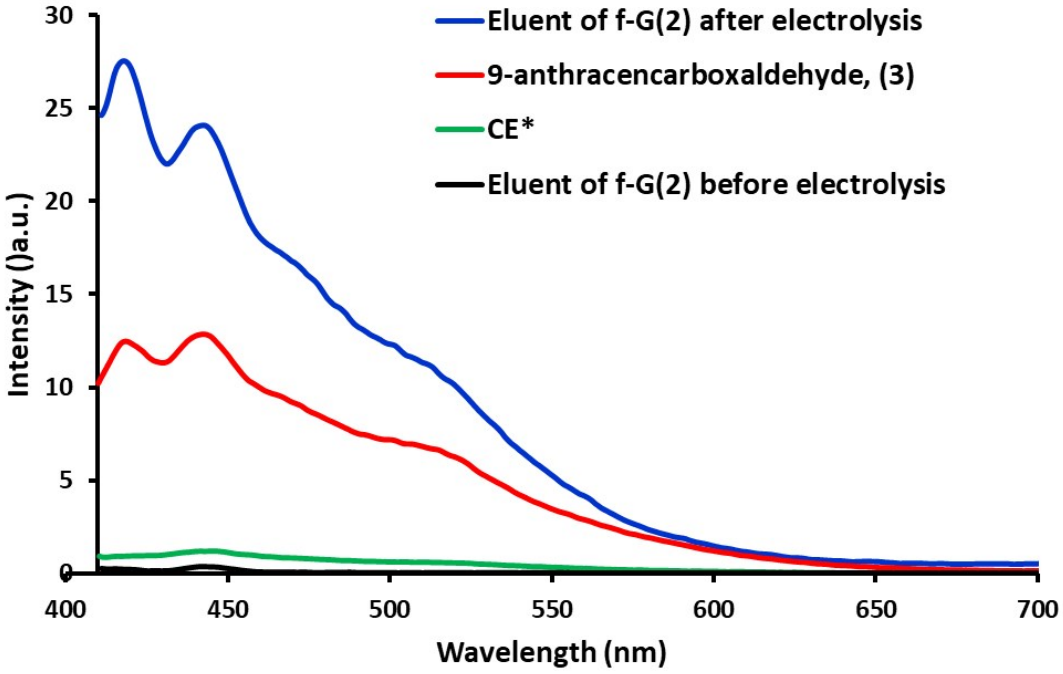


Fig. S20. Fluorescence of 9-antheracnecarboxaldehyde (**2**, red), eluent before (blue) and after electrolysis of f-G(**2**) (red) in DMF. Fluorescence of and after

electrolysis of of CE* (blue) in DMF.

Fig. S21. Home-made working electrode by pasting f-G(**3**) (black surface) on ITO electrode (grey surface) with conductive copper tape (orange surface).

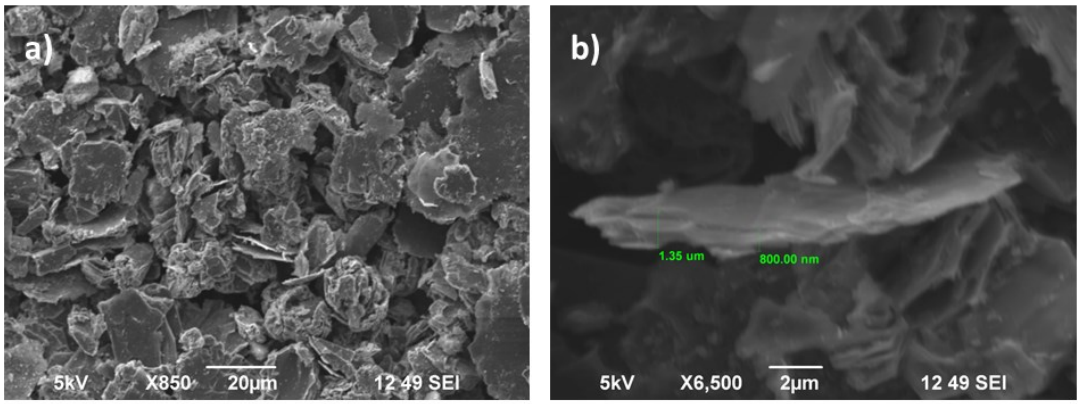


Figure S22. SEM images of the electrode composed of f-G(**3**) deposited on ITO and copper tape at different magnifications. It can be clearly observed that the thickness of a deposited f-G(**3**) (in green).

Table S1. Atomic concentration percentage of f-G(1) obtained by XPS.

Elements	**C**	**O**	**N**	**F**
At. Conc. (%)	**95.28**	**3.75**	**0.98**	**--**

Table S2. Atomic concentration percentage of f-G(**6**) before and after electrolysis at 1.35 V vs. Ag/AgCl obtained by XPS.

Elements	**C**	**O**	**F**

At. Conc. (%) before	**88.9**	**8.3**	**2.8**
At. Conc. (%) after	**89.3**	**7.8**	**2.9**

Table S3. Atomic concentration percentage of f-G(**3**) obtained by XPS.

Elements	**C**	**O**	**N**	**F**
At. Conc. (%)	**94.98**	**3.48**	**0.35**	**0.72**

Table S4. Atomic concentration percentage of f-G(**4**) obtained by XPS.

Elements	**C**	**O**	**N**	**F**
At. Conc. (%)	**69.74**	**28.21**	**1.24**	**0.81**

Table S5. Atomic concentration percentage of f-G(**5**) obtained by XPS.

Elements	**C**	**O**	**N**
At. Conc. (%)	**96.36**	**2.94**	**0.70**

In order to draw quantitative information from thermogravimetric plots, we performed the following calculation to obtain functionalization degree (FD); where *L* corresponds to the weight loss observed at 500ºC (in %), after having subtracted the analogous loss from the pristine material. The molecular weight (*Mw*) is set for the expected desorbed moiety. The conversion factor (10^4) provides data in the desired unities (μmol/g).

$$\frac{L(\%)\cdot 10^4}{Mw(g/mol)} = FD\ (\mu mol/g) \qquad \textit{Equation (S1)}$$

Table S6. Functionalisation degree (FD) from TGA data (*Equation S1*). [a] Weight loss determined at 500°C toward CE. [b] All the groups are considered as imine group.

Samples	***L* (%)[a]**	**FD (μmol FG / g G)**
f-G(1)	1.3	140
f-G(2)	3.0	107[b]

5. References

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