A biosupramolecular approach to graphene: complementary nucleotide-nucleobase combinations as enhanced stabilizers towards aqueous-phase exfoliation and functional graphene-nucleotide hydrogels

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Abstract

The ability to use RNA/DNA nucleotides as colloidal stabilizers for graphene would be an important asset, as a close graphene-nucleotide association would facilitate access to hybrid systems where the rich covalent and supramolecular chemistry of these biomolecules could be exploited alongside graphene in a number of applications. Unfortunately, single RNA/DNA nucleotides are inefficient graphene dispersants. Here we propose and demonstrate a supramolecular strategy which overcomes this limitation, affording aqueous dispersions of high quality graphene flakes with much improved colloidal stability. A nucleotide is combined with its complementary nucleobase yielding stable hydrogen-bonded supramolecular entities that adsorb more strongly on the graphene surface than their individual components. Based on this approach, graphene-nucleotide hybrid hydrogels could be readily obtained, where the graphene flakes were intimately and uniformly intermixed with the nucleotide-based gel phase. Such hydrogels exhibited higher uptakes and/or slower release profiles of dyes and drugs (rhodamine B, methylene blue and tetracycline) than their graphene-free counterparts. Cell proliferation tests suggested the graphene materials obtained with nucleotide-nucleobase stabilizers to be biocompatible. The present results constitute a novel strategy in the processing and molecular integration of graphene that could be extended to other (bio)molecules of interest towards the realization of functional materials for different applications.

1. Introduction

Driven by its exceptional physical properties and the promise of a strong impact in many key technological fields (e.g., electronics, photonics, energy conversion/storage,¹ biomedicine or sensing)², graphene has become one of the most extensively investigated materials of the last decade and the focus of many prominent national and international research programs worldwide.³ However, although the high potential of this two-dimensional (2D) form of carbon in a remarkably broad range of practical uses is already well established, its widespread implementation in real-life applications that directly benefit society has been notoriously lacking. While it is true that new or improved technologies based on novel materials may take several decades to develop and reach the market, in the case of graphene there is general agreement that current limitations in our ability to produce and process it by efficient and affordable means are one of the main reasons behind this state of affairs.⁴

At present, realistic prospects towards the scalable production of processable graphene nanosheets for use in many large-scale applications are mostly circumscribed to three types of top-down approaches, each of which having its own advantages and drawbacks.⁵ These approaches are (1) the so-called graphite oxide route,⁶ (2) the electrochemical delamination of graphite,^{7,8} and (3) the direct, ultrasound- or shear-induced liquid-phase exfoliation of graphite.⁹ Graphenes derived from graphite oxide can be readily prepared in very large quantities in the form of single-layer entities (referred to as reduced graphene oxide nanosheets), but they typically possess a highly defective structure that is difficult to fully revert and degrades many of the attractive characteristics associated to their more defect-free counterparts.¹⁰ Electrochemical (anodic or cathodic) delamination of graphite electrodes generally affords few-layer (< 5) graphene nanosheets in high yields, but attaining largely defect-free, pristine materials requires the use of organic solvents and/or sophisticated, expensive electrolytes,¹¹ thus compromising its wider utility. Direct liquid-phase exfoliation, on the other hand, suffers from low production yields (typically <5 wt%), but the resulting few- to several-layer graphene flakes boast a very high structural quality that for the most part is unmatched by any of the other two approaches.^{12,13} Furthermore, its extreme simplicity and relatively high throughput make the direct liquid-phase exfoliation strategy probably the best production option when large volumes of defect-free, solution-processable graphene nanosheets are needed.^{9,14}

Pioneering work by the group of Coleman determined that pristine graphene can be directly exfoliated from graphite and colloidally dispersed via ultrasonic treatment in solvents with surface energies close to that of graphite/graphene ($\sim 70\text{--}80 \text{ mJ m}^{-2}$).^{15,16} These usually include high boiling point and non-innocuous organic solvents, such as *N*-methyl-2-pyrrolidone, *N,N*-dimethylformamide or cyclopentanone. However, with a view to a greener production and processing of graphene, the use of water as the solvent medium is clearly preferable,^{17,18} but its surface energy is much higher than that of graphite/graphene. Thus, even though some recent reports have shown that pristine graphite can be exfoliated and dispersed in water under neutral or basic conditions,¹⁹⁻²¹ the concentration of the resulting graphene dispersions is too low ($< 0.02 \text{ mg mL}^{-1}$) to be of far-reaching practical relevance. This issue can be addressed with the use of amphiphilic additives that act as colloidal stabilizers of the graphene nanosheets. Indeed, extensive work carried out since 2009 has identified a large number of both ionic and non-ionic (polymeric) surfactants, mostly of synthetic origin, for the exfoliation/dispersion of pristine graphene in aqueous medium at significant concentrations.^{9,13} Nevertheless, in the pursuit of greener approaches to graphene production and processing, dispersants of natural origin (i.e., biomolecule-based) have recently emerged as an attractive alternative to synthetic ones.²² In addition to the question of environmental friendliness, some assets of biomolecule-based dispersants include improved biocompatibility and better integration of the graphene materials in bio-related applications, or the possibility of (bio)chemical derivatization of the graphene nanosheets using well-developed protocols for the biomolecules.

So far, mainly large-sized biomolecules such as proteins, RNA/DNA or polysaccharides have been used as natural stabilizers for the production of pristine graphene dispersions in aqueous medium, whereas small amphiphilic biomolecules have received relatively little attention towards this end (with a few notable exceptions, namely, bile salts and flavin mononucleotide).²²⁻²⁶ Many small biomolecules exhibit rich covalent and non-covalent chemistries, and therefore their use as graphene dispersants could expand significantly the functionality and application scope for this 2D material (e.g., by facilitating its incorporation in supramolecular assemblies). This could be particularly the case of RNA and DNA nucleotides, whose covalent and supramolecular chemistry has been extensively investigated and developed.²⁷⁻²⁹ Unfortunately, very recent work has provided indication that single RNA/DNA

nucleotides are quite inefficient dispersants for graphene.³⁰ Such a result implies that these nucleotides adsorb rather weakly on the graphene surface and constitutes an obstacle to fully exploiting the hybrid systems that could result from a close graphene–nucleotide association. However, the very fact that the nucleobases in RNA/DNA nucleotides readily engage in non-covalent, supramolecular interactions (especially hydrogen bonding interactions)^{29,31} could offer a solution to this issue.

Inspired by the relatively strong hydrogen bonding between complementary nucleobases in double-stranded DNA and RNA, i.e. the well-known Watson–Crick base pairs,³¹ as well as the concept of supramolecular approaches to functional graphene,³² we hypothesized that rather than using a single nucleotide as a graphene dispersant, the combination of a nucleotide with its complementary nucleobase would give rise to supramolecular entities (e.g., cyclic dimers³¹) that could adsorb more strongly onto graphene and thus could be more efficient stabilizers of the nanosheets. We demonstrate here that such a hypothesis is correct and that pristine graphene suspensions with much improved colloidal stability in water can be obtained using proper nucleotide–nucleobase combinations. Furthermore, the tighter graphene–nucleotide associations derived from this strategy could be exploited in a number of practical applications. For example, previous reports on graphene-based composites have presented them as good candidates for dye adsorption due to the contribution of graphene to their adsorption capacity.³³ As an illustration of the different possibilities of application, we also report here graphene-containing, nucleotide-based hydrogels that exhibit improved uptakes and/or slower release profiles of drugs and dyes compared with those of their graphene-free counterparts, suggesting a potential use as delivery systems. Finally, preliminary tests carried out with murine fibroblasts indicated that the graphene flakes stabilized by these supramolecular nucleotide-nucleobase entities are biocompatible.

2. Experimental section

2.1. Materials and reagents

The following materials and chemicals were obtained from Sigma-Aldrich and used as received: natural graphite powder (particle size < 150 μm , ref. 496588); the nucleotides adenosine 5'-monophosphate disodium salt (AMP), guanosine 5'-monophosphate disodium salt (GMP), thymidine 5'-monophosphate disodium salt

(TMP) and cytidine 5'-monophosphate disodium salt (CMP); the nucleobases adenine (A), guanine (G), thymine (T) and cytosine (C); the dyes methylene blue (MB) and rhodamine B (RB); the antibiotic tetracycline (TC); phosphate buffer saline (PBS) solution (1 M, pH 7.4); ZnCl₂. Milli-Q water (resistivity 18.2 MΩ·cm) was used throughout the work.

2.2. Exfoliation and dispersion of graphite in water assisted by nucleotides

Although an array of dispersion tests was conducted by varying a number of experimental variables (e.g., initial graphite concentration, nucleotide nature and concentration, sonication time or centrifugation speed), an optimized preparation procedure to obtain the most stable aqueous graphene suspensions was the following. Natural graphite powder was added at a concentration of 30 mg mL⁻¹ to an aqueous 1 mg mL⁻¹ solution of a purine-based nucleotide (i.e., either AMP or GMP) that also contained an equimolar amount of the complementary pyrimidine nucleobase (i.e., ~0.33 mg mL⁻¹ of T in the case of AMP and ~0.28 mg mL⁻¹ of C in the case of GMP). The mixture was then subjected to ultrasonic treatment in an ultrasound bath cleaner (JP Selecta Ultrasons system, 40 kHz) for 5 h. To avoid an excessive heating of the aqueous suspensions and the bath cleaner, the treatment was carried out in 5 separate intervals of 60 min each, replenishing the bath cleaner with cool water after each interval. Furthermore, to allow meaningful comparisons between different nucleotides and/or nucleotide–nucleobase combinations in their role as graphene dispersants, batches of up to 6 different samples were sonicated at the same time in the same bath. In this case, the position of each sample vial in the bath was periodically changed during the ultrasonic treatment, so that after every 60 min period all the samples were made to reside in all the different possible locations for the same amount of time. Such a procedure was implemented to avoid the effect of possible spatial inhomogeneities of the ultrasound bath, which might cause samples in different locations of the bath to be exposed to different ultrasound intensities. Following the ultrasonic treatment, the resulting dispersions were left to stand undisturbed overnight to allow sufficient time for the nucleotide–nucleoside interactions to fully establish. Afterwards, they were centrifuged at 200 or 500 g for 20 min (Eppendorf 5424 microcentrifuge) to sediment non-exfoliated or poorly exfoliated material and the top ~75% of the supernatant volume,

which was the target graphene dispersion, was collected for further use. As reported elsewhere,²⁶ the dispersion concentration was estimated through measurement of its optical absorption at 660 nm using the Lambert-Beer law and an extinction coefficient of 2440 mL mg⁻¹ m⁻¹. To remove free, non-adsorbed nucleotide or nucleotide–nucleobase molecules from the aqueous graphene suspension, the latter was subjected to a washing protocol that consisted of 2-3 consecutive cycles of sedimentation via centrifugation (20000 g, 20 min), replacement of the top 2/3 of the supernatant solution by pure water and re-dispersion of the sedimented graphene material in the new solution through a brief sonication step. Typical concentration values for the as-obtained and washed graphene suspensions were around 0.1 mg mL⁻¹. When more concentrated samples were needed, the as-prepared or washed dispersions were sedimented via centrifugation (20000 g, 20 min) and re-dispersed in half the original solution volume. By repeating this procedure several times, homogeneous dispersions with concentrations of 1–2 mg mL⁻¹ were readily achieved. This allows checking if agglomeration occurs in each step and determining the maximum attainable concentration. However, we have checked that it is possible to prepare equally concentrated and colloidally stable dispersions in just one step, by re-dispersion in the appropriate volume.

2.3. Preparation of graphene–nucleotide supramolecular hydrogels and investigation of their uptake and release of dyes and drugs

Supramolecular hydrogels assembled through coordination of the AMP nucleotide to Zn²⁺ and incorporating different amounts of AMP–T-stabilized graphene flakes were prepared, and their ability to take up and release dyes (MB and RB) and drugs (TC) was tested. The hydrogels were obtained by first preparing mixed aqueous solutions of AMP (1.6 mM), ZnCl₂ (2 mM) and AMP–T-stabilized graphene (at variable concentrations up to ~0.8 mg mL⁻¹), which were then subjected to centrifugation (10000 g, 5 min) to trigger gelation. The graphene dispersions used for gelation were obtained through washing and concentration (up to ~1 mg mL⁻¹) of as-prepared suspensions using the protocols described above. Following the centrifugation step, the hydrogel appeared at the bottom of the centrifuge tube. The hydrogel was washed by removing the supernatant liquid and replacing it with pure water for several times. Dye- or drug-loaded hydrogels were prepared using the same protocol with the exception that the starting mixed aqueous solution contained a certain known concentration of the

dye/drug (typically, 0.5 mg mL^{-1} for MB and RB and 0.2 mg mL^{-1} for TC) in addition to AMP, ZnCl_2 and graphene. The amount of dye/drug loaded into the hydrogel was determined through UV-vis absorption spectroscopy by comparing the absorbance intensity of a characteristic peak of the molecule in the hydrogel solution (i.e., 664 nm for MB, 554 nm for RB and 357 nm for tetracycline) before and after the centrifugation step. To avoid the interference of graphene optical absorption in the determination of the dye/drug concentration, the absorbance before the centrifugation step was taken as that of the hydrogel prepared in the absence of graphene under exactly the same conditions. After the centrifugation step, all the graphene becomes part of the hydrogel, no interfering species being present in the analysed supernatant. Release tests of the loaded dye/drug molecules were also accomplished. To this end, a piece of dye- or drug-loaded hydrogel was transferred to a given volume (typically 10 mL in the case of RB and MB and 3 mL for TC) of 0.1 M PBS medium (pH ~ 7.4), and then the concentration of the dye/drug in this solution was monitored at different time intervals with UV-vis absorption spectroscopy.

2.4. L-929 cell proliferation tests for thin films of nucleotide-exfoliated graphene

The biocompatibility of thin graphene films obtained from aqueous dispersions stabilized by the AMP–T combination was evaluated on the basis of cell proliferation tests. To this end, the murine fibroblast L-929 cell line (ECAC No. 85011425) was chosen. This is a stable and fast-growing cell line that is commonly used in biocompatibility and cytotoxicity studies of biomedical materials.³⁴ Individual wells from a 48-well tissue culture plate made of hydrophilic polystyrene were pre-coated with either ~ 0.25 or 0.50 mg of AMP–T-exfoliated graphene flakes by drop-casting 0.5 or 1 mL of an aqueous graphene dispersion that had been previously washed and concentrated to $\sim 0.5 \text{ mg mL}^{-1}$ through the abovementioned procedure. For comparison purposes, graphene oxide (acquired from Graphenea) that had been previously reduced by vitamin C³⁵ was also assayed at ~ 0.25 and 0.50 mg of deposited mass. After seeding 5000 cells per well, the wells were filled with culture medium, which comprised Dulbecco's modified Eagle's medium (DMEM, from Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U mL^{-1}) and streptomycin (100 mg mL^{-1}), and then the culture plate was incubated at 37°C in a 5%

CO₂ atmosphere, replacing the medium every 2-3 days. Cell proliferation was assessed 2, 4, 7 and 10 days after cell seeding using the PrestoBlue (PB) reagent (Thermo Fisher Scientific). PB is a resazurin-based, membrane-permeable solution that does not require cell lysis and can be used to quantitatively analyze the proliferation of metabolically active cells by mitochondrial reduction of resazurin to a red fluorescent compound (resorufin) with both optical absorption and fluorescence profiles different to those of resazurin. To determine cell proliferation, a 10% aqueous PB solution was added to the seeded wells and incubated for 4 h at 37 °C in a 5% CO₂ atmosphere. The solution was then removed and its relative fluorescence was measured against a control (cells growing on bare, graphene-free wells from the tissue culture plate) by means of a Synergy MX microplate reader (Biotek Instruments) at excitation and emission wavelengths of 570 and 610 nm, respectively. To obtain statistically meaningful results, the experiments were carried out in hexaplicate. The statistical analysis was performed with the SPSS software. When data complied with the normality and homogeneity of variance requirements, their average values were compared by the parametric test ANOVA followed by Bonferroni's post hoc multiple t-test, setting the significance level to $p < 0.05$.

2.5 Characterization techniques

The different materials were characterized by UV-vis absorption spectroscopy, contact angle and zeta-potential measurements, Raman spectroscopy, X-ray photoelectron spectroscopy (XPS), transmission electron microscopy (TEM) and scanning transmission electron microscopy (STEM). UV-vis absorption spectra were recorded in a double-beam Helios α spectrophotometer (Thermo Spectronic). Contact angle measurement was performed at room temperature (~20 °C). Small volume (2 μ L) Milli-Q water droplets were used to render gravitational effects negligible.³⁶ The contact angle was determined by the tangent method using ImageJ software with the drop_analysis plugin.³⁷ Zeta-potential measurements were carried out at a temperature of 25 °C in a Malvern Zetasizer Nano ZSP apparatus from Malvern Instruments Ltd. using DTS-1070 cuvettes filled with 0.7 mL of graphene dispersion at a concentration of 0.5 mg mL⁻¹. Raman spectroscopy measurements were carried out with a Horiba Jobin-Yvon LabRam instrument at a laser excitation wavelength of 532 nm and an

incident laser power of 2.5 mW. XPS was accomplished in a SPECS apparatus under a pressure of 10^{-7} Pa with a non-monochromatic Mg K_{α} X-ray source operated at 11.81 kV and 100 W. Graphene samples for both Raman spectroscopy and XPS were prepared by drop-casting washed, nucleotide-stabilized aqueous graphene dispersions onto circular (12 mm in diameter) stainless steel disks pre-heated at ~ 50 -60 °C, which were then allowed to dry. A sufficient volume of suspension was drop-cast so as to ensure the formation of a uniform graphene film covering the whole disk. TEM images of the graphene flakes were recorded with a JEOL 2000 EX-II instrument operated at 160 kV. STEM imaging of the graphene-nucleotide supramolecular hydrogels was accomplished with a Quanta FEG 650 microscope, from FEI Company, operated at 30 kV. Graphene specimens for TEM were prepared by mixing equal volumes of nucleotide-stabilized aqueous graphene dispersion and ethanol. Subsequently, 40 μ L of the mixed water-ethanol solution were drop-cast onto a copper grid (200 square mesh) covered with a thin continuous film of amorphous carbon (Electron Microscopy Sciences) and allowed to dry with the assistance of a halogen lamp. For the hydrogels, a piece of hydrogel was first broken down into small fragments in water with the help of a spatula and some brief, gentle sonication. Then, 40 μ L of the resulting hydrogel suspension were drop-cast onto a carbon film-covered copper grid and allowed to dry under a halogen lamp. BET surface area of the hydrogels was measured in an Autosorb-1 volumetric analyzer (Quantachrome). Prior to the N_2 adsorption experiment, the wet hydrogel was freeze-dried (5×10^{-2} mbar, -50 °C, 5 days) and then degassed overnight at 70 °C under vacuum. The BET surface area was determined on the basis of the BET model from the adsorption branch of the N_2 adsorption isotherm in the relative pressure range between 0.04 and 0.1.

3. Results and discussion

3.1. Complementary nucleotide-nucleobase combinations as enhanced dispersants for graphene

Fig. 1 shows a schematic representation of the rationale behind the use of RNA- or DNA-based nucleotide–nucleobase combinations as graphene dispersants with enhanced performance. Indeed, extensive theoretical calculations have indicated that

RNA/DNA nucleobases adsorb on the graphene surface in a generally flat configuration with relatively weak binding energies (π - π stacking) in the order guanine (G) > adenine (A) > thymine (T) > cytosine (C) > uracil (U),³⁸⁻⁴¹ which is supported by experimental work on nucleobase adsorption onto graphene in aqueous medium.⁴²

Figure 1. Schematic representation of the interaction of (a) single nucleotides and (b) nucleotide-nucleobase combinations with graphene flakes towards aqueous colloidal dispersions. For a single nucleotide, e.g., adenosine 5'-monophosphate (chemical structure given in the top part of panel a), a relatively weak interaction with the graphene surface leads to a poor colloidal stability of the flakes in water. When a nucleotide is combined with its complementary nucleobase, e.g. adenosine 5'-monophosphate with thymine (shown as a hydrogen-bonded cyclic dimer in the top part of panel b), the resulting supramolecular entity is able to adsorb more strongly on the graphene surface (larger area of interaction), thus enhancing the colloidal stability of the flakes in aqueous medium.

However, for a given amphiphilic molecule to act as an efficient dispersant of graphene (or any other hydrophobic nanomaterial), a necessary condition is that its strength of adsorption (free energy of adsorption) be sufficiently high so that the flakes are covered at any given time by a sufficient number of stabilizing molecules.³² Owing to the small

size of their nucleobases (a single hexagonal ring for pyrimidines and a hexagon-pentagon pair for purines), such a condition does not appear to be met for RNA/DNA nucleotides (Fig. 1a), as very recent work by our group has suggested.³⁰ For example, even though sonication of graphite powder in aqueous solutions of these nucleotides initially affords graphene suspensions at concentrations of $\sim 0.1\text{--}0.2\text{ mg mL}^{-1}$ (or lower in the case of pyrimidine-based nucleotides), the suspensions are seen to readily agglomerate and precipitate with time (typically days).

To overcome this issue, we propose to use complementary nucleotide-nucleobase combinations (complementary in the sense of Watson-Crick base pairing) as graphene dispersants with enhanced stabilizing ability. Although in bulk water any two bases are believed to interact with each other mostly through $\pi\text{--}\pi$ stacking, with very little or even no contribution from hydrogen bonding (hydrogen bonds are mainly formed with nearby water molecules),³¹ there is indication that in the vicinity of graphite or graphene the two bases adsorb on the carbon surface ($\pi\text{--}\pi$ interaction) and bind to each other through hydrogen bonds.^{43,44} Due to its larger area of interaction with the graphite/graphene surface, the resulting supramolecular assembly (cyclic dimer) can be expected to be more strongly adsorbed than the single, stand-alone bases (Fig. 1b). As a consequence, such an assembly would be able to provide exfoliated graphene flakes with improved colloidal stability in aqueous medium. To test this idea, we conducted a series of benchmark experiments by which pristine graphite powder was sonicated in different aqueous solutions of single nucleotides, nucleotide-nucleobase combinations or nucleotide-nucleotide combinations. The nucleobases and nucleotides used in the experiments are shown in Fig. 2, and included A, G, T, C (nucleobases) and the nucleotides adenosine 5'-monophosphate disodium salt (AMP), guanosine 5'-monophosphate disodium salt (GMP), thymidine 5'-monophosphate disodium salt (TMP) and cytidine 5'-monophosphate disodium salt (CMP).

Figure 2. Chemical structures of the nucleobases adenine (A), guanine (G), thymine (T) and cytosine (C) as well as their ribose-based nucleotide counterparts in sodium salt form, namely, adenosine 5'-monophosphate disodium salt (AMP), guanosine 5'-monophosphate disodium salt (GMP), thymidine 5'-monophosphate disodium salt (TMP) and cytidine 5'-monophosphate disodium salt (CMP).

To allow comparisons on an equal footing between the different nucleotides and nucleotide-nucleobase combinations regarding their ability to disperse graphene, batches of samples were prepared through simultaneous sonication (in the same bath) of graphite in a number of different nucleotide/nucleobase solutions, with the position of all sample vials in the bath being regularly exchanged to ensure a uniform exposure to the ultrasound waves (see Experimental section for details). Prior to this, a set of experiments was carried out with single nucleotides (specifically AMP and GMP) to determine the optimal exfoliation/dispersion parameters that led to maximized concentrations of graphene flakes in the solution. We note that although the single nucleotides are at best mediocre stabilizers for graphene, the resulting dispersions served as reference systems for the subsequent investigations with the nucleotide-

nucleoside combinations. In brief, optimized graphene dispersions in either AMP or GMP were prepared by sonicating 30 mg mL⁻¹ of graphite powder in 1 mg mL⁻¹ aqueous solutions of the nucleotides for 5 h and then centrifuging the sonicated suspension at 200 or 500 g for 20 min. Initial graphite concentrations lower than 30 mg mL⁻¹ yielded correspondingly lower concentrations of suspended graphene, whereas the use of higher concentrations did not lead to similar increases in the final amount of graphene. Although longer sonication times afforded higher graphene concentrations, a 5 h treatment was found to be a reasonable compromise between final concentration and time required to prepare the samples. Centrifugation at 200 or 500 g sufficed to sediment the non-exfoliated graphite particles as well as thin graphitic platelets while maximizing the retention of graphene flakes in the supernatant with average thickness below 10 monolayers (see below). Maximum graphene concentrations were observed at nucleotide concentrations of ~1–2 mg mL⁻¹. The amount of dispersed graphene tended to decrease markedly at nucleotide concentrations above ~2 mg mL⁻¹, probably due to the higher ionic strength of the aqueous medium.³⁰ Finally, all the graphene dispersions obtained by this optimized procedure (using either single nucleotides or nucleotide-nucleobase combinations) were subjected to a washing step to remove a large fraction of the free, non-adsorbed nucleotide/nucleobase molecules from the aqueous medium (see Experimental section). This made their colloidal stability to be more critically dependent on the strength of the nucleotide/nucleobase-graphene interaction, so that differences in dispersing ability between the different nucleotides and nucleotide-nucleobase combinations could become more evident.³⁰

Fig. 3 compares the dispersing ability of different nucleotides and nucleotide-nucleobase (or nucleotide-nucleotide) combinations based on AMP (a) and GMP (b). Specifically, Fig. 3a shows the temporal evolution of the concentration of graphene suspensions prepared by the procedure described above (see also Experimental section) in aqueous solutions containing 1 mg mL⁻¹ (or ~2.55 mM) AMP (sample AMP), 2 mg mL⁻¹ AMP (AMP+AMP), 1 mg mL⁻¹ AMP with the same molar concentration of T (AMP+T), 1 mg mL⁻¹ AMP with the same molar concentration of TMP (AMP+TMP), and ~0.93 mg mL⁻¹ (~2.55 mM) TMP (sample TMP). Similarly, Fig. 3b shows results for samples GMP, GMP+GMP, GMP+C, GMP+CMP and CMP, where sample GMP stands for graphene dispersion prepared in 1 mg mL⁻¹ (~2.45 mM) of the GMP

nucleotide and the meaning of the other sample codes is equivalent to that of their corresponding AMP-based samples in Fig. 3a.

Figure 3. Colloidal stability of graphene dispersions prepared with the single nucleotides as well as the nucleotide-nucleobase (or nucleotide-nucleotide) combinations indicated in the different panels, namely: (a) AMP (cyan circles), AMP+AMP (blue circles), AMP+T (green circles), AMP+TMP (black circles), TMP (red circles); (b) GMP (cyan squares), GMP+GMP (blue squares), GMP+C (green squares), GMP+CMP (black squares), CMP (red squares). AMP+AMP and GMP+GMP refer to graphene samples prepared with a concentration of the corresponding nucleotide (2 mg mL^{-1}) twice as much than that of the AMP and GMP samples (1 mg mL^{-1}). (c,d) Digital photographs of the aqueous graphene dispersions described in panel a (c) and panel b (d) taken one year after their preparation.

It can be noticed that all the dispersions exhibited initial graphene concentrations in the $\sim 0.1\text{-}0.2 \text{ mg mL}^{-1}$ range except those prepared with TMP and CMP (concentrations around 0.05 mg mL^{-1}). The lower initial concentration attained with the pyrimidine-based nucleotides was consistent with their expected weaker adsorption onto the graphene surface.³⁰ However, the most significant result from Fig. 3a and b was the

observation that all the graphene suspensions tended to precipitate almost completely after standing for 6–8 weeks, except those obtained from the combination of AMP and GMP with their complementary nucleobases, i.e., T and C, respectively. For example, after standing undisturbed for 10 weeks the amount of graphene retained in the AMP+T and GMP+C dispersions was, respectively, ~20% and 40% of their corresponding initial concentrations, whereas it was below 3% for all the other samples. Moreover, following the initial 6-8 week sedimentation period the AMP+T and GMP+C dispersions were seen to remain colloidally stable in the long term, as evidenced by the digital photographs taken for the AMP (Fig. 3c) and GMP (Fig. 3d) series of samples one year after their preparation. There was virtually no suspended graphene material in any solutions other than those of the complementary nucleotide-nucleobase combinations. Zeta-potential measurements for AMP+T dispersions yielded an average value of $(-3 \pm 1) \times 10$ mV at a pH of 5.5. The negative sign of the zeta-potential confirms the stabilization of the graphene flakes in colloidal dispersion by electrostatic repulsion between the negative charges of the phosphate groups from the nucleotides adsorbed onto them. The magnitude of the potential, which is related to the strength of the stabilization, is similar to that previously reported for well stabilized colloidal dispersions of graphene materials, such as highly reduced graphene oxide.⁴⁵

The enhanced colloidal stability of graphene using the complementary nucleotide-nucleobase combinations reported here is interpreted to be a result of the relatively strong adsorption of cyclic dimers (i.e., AMP–T and GMP–C dimers), or even combined structures of higher order, formed on the carbon surface. Supramolecular entities of this type are generally expected to assemble from RNA/DNA nucleobases at the liquid-graphite/graphene interface and are stabilized by both base-surface (π – π stacking) and base-base (hydrogen bonding) interactions (Fig. 1b).⁴³⁻⁴⁸ We note that the well-known canonical Watson-Crick base pairing involves the formation of 2 and 3 hydrogen bonds between the A–T and G–C pairs, respectively.³¹ If the expected assembly of supramolecular entities (dimers) on the graphene surface was directed by such canonical base pairing, one would expect the GMP–C dimer to be more stable than its AMP–T counterpart,⁴³ and therefore graphene dispersions prepared with the former nucleotide-nucleobase combination should be more colloidally stable than those obtained with the latter. Such a prediction is indeed consistent with the results presented in Fig. 3a and b (larger fraction of graphene retained in the GMP-C dispersion after 10

weeks). However, we have to take into account that in addition to the canonical Watson-Crick base pairing, other associations between complementary nucleobases are also possible (e.g., reverse Watson-Crick, Hoogsteen or reverse Hoogsteen pairings), and all these involve the formation of only 2 hydrogen bonds.³¹ Furthermore, the presence of higher order entities adsorbed on the graphene flakes, such as base triples (e.g., T-AMP-T aggregates)²⁷ or even extended 2D networks based on the binding between complementary nucleotides and nucleobases,⁴⁶ should not be ruled out. Thus, although a clear stabilizing effect of using complementary nucleotide-nucleobase combinations was found here for the graphene dispersions, the actual assembly motifs responsible for such an effect remain currently unknown. The emergence of the different motifs is probably dictated by a number of experimental parameters (e.g., those of higher order should probably appear at high nucleotide/nucleobase concentrations), but elucidating this issue lies beyond the scope of the present work.

The fact that the strategy outlined here was effective for the complementary nucleotide-nucleobase combinations (AMP-T and GMP-C) but not for their nucleotide-nucleotide counterparts (i.e., AMP-TMP and GMP-CMP; see Fig. 3) is also worthy of mention. This result was not unexpected considering that the association between two complementary nucleotides should be hampered by the electrostatic barrier put in place by their respective, negatively charged phosphate groups, which is not present in nucleotide-nucleobase pairings. Hence, complementary nucleotide-nucleotide dimers will probably not form or at least will be much less stable than nucleotide-nucleobase dimers, and thus their stabilizing effect towards graphene dispersions will be much more limited. Moreover, it is well known that associations both between different, non-complementary nucleobases (e.g., A-C and G-T) and between identical nucleobases (homodimers; e.g., A-A) can also be established,^{27,31} so the corresponding nucleotide-nucleobase combinations could in principle be used as well to furnish graphene flakes with enhanced colloidal stability. However, the strength of the hydrogen bonding for these dimers in bulk solution is generally lower than that of complementary base dimers,³¹ and a similar trend has been predicted by molecular simulations when they are adsorbed on graphite from the aqueous phase.⁴³ This suggests that such non-canonical associations could be much less efficient at colloidally stabilizing graphene.

We conducted two sets of experiments that provided support to this hypothesis. In one of them, the stability of aqueous graphene dispersions prepared using the

complementary nucleotide-nucleobase combinations (i.e., samples AMP+T and GMP+C described above) was compared to that of dispersions obtained with the non-complementary combinations AMP–C and GMP–T (denoted as samples AMP+C and GMP+T). Even though all the four dispersions exhibited similar initial concentrations, those prepared with the non-complementary nucleotide-nucleobase combinations sedimented almost completely in a matter of weeks. Indeed, after standing undisturbed for one year there was virtually no suspended material in the AMP+C and GMP+T samples, which was in marked contrast with their AMP+T and GMP+C counterparts (Fig. 4a). In the second set of experiments, the stability of AMP and GMP suspensions was compared with that of dispersions obtained by combining these nucleotides with their own nucleobase, i.e., the AMP–A and GMP–G combinations were used to prepare graphene samples denoted as AMP+A and GMP+G. Nevertheless, no or very little suspended material was observed for these samples even immediately after their preparation (Fig. 4b), indicating that such combinations are very inefficient stabilizers.

Figure 4. Digital photographs of aqueous graphene dispersions obtained with (a) GMP–C, GMP–T, AMP–T and AMP–C nucleotide-nucleoside combinations, taken one year after their preparation, and (b) the single nucleotides AMP and GMP as well as the AMP-A and GMP-G combinations, taken immediately after their preparation.

We interpret that the strength of the AMP–A and GMP–G associations is far too low, at least compared to the AMP–T and GMP–C cases, to allow the formation of dimers with

sufficient stability on the graphene surface. Instead, the poor performance of these combinations is probably dictated by the competitive adsorption of individual AMP (GMP) and A (G) molecules on the graphene flakes. Thus, if a fraction of the negatively charged AMP or GMP molecules adsorbed on the graphene flakes in the AMP or GMP suspensions were replaced by their electrically neutral nucleobase, then the absolute net charge on the flakes would be reduced. As a result, the magnitude of the electrostatic repulsions between flakes would be reduced, thus compromising their colloidal stability. Competitive adsorption processes are also thought to be behind the loss of colloidal stability that was observed with the complementary nucleotide-nucleobase combinations (i.e., AMP–T and GMP–C) when the amount of nucleobase used was much higher than that of the nucleotide. In the experiments described above, equimolar quantities of nucleotides and nucleobases were employed. However, in the presence of a large excess of nucleobase (e.g., nucleotide:nucleobase molar ratios of 1:4) the resulting graphene dispersions were much more unstable. Again, the adsorption of electrically neutral nucleobase molecules that could not associate to any nucleotide molecules (due to the relative scarcity of the latter) was the probable reason for the observed behavior.

3.2. Physicochemical characterization of graphene obtained with nucleotide-nucleobase combinations as dispersants

To demonstrate that the aqueous dispersions of enhanced colloidal stability reported here based on the use of complementary nucleotide-nucleobase combinations are indeed made up of high quality graphene flakes, some general characterization of such dispersions was carried out. First, their UV-vis absorption spectra were dominated by a peak located at ~265 nm together with strong absorbance in the whole measured range of longer wavelengths, as illustrated in Fig. 5a for the AMP+T dispersion (subjected to 4 washing cycles to remove as much stabilizer as possible; see Experimental section). These features are consistent with the presence of a sp^2 -based carbon material having extended electronically conjugated domains and are very similar, if not identical, to those previously observed for, e.g., solvent-exfoliated pristine graphene flakes,⁴⁹ electrochemically exfoliated graphene with a low degree of oxidation,⁵⁰ or highly reduced graphene oxide nanosheets.⁴⁵ The small peak observed at about 210 nm arises from the stabilizer, which could not be completely eliminated. The chemical make-up of

the dispersed material was probed by XPS, which revealed its essentially pristine nature. The survey spectra (see Fig. S1 in Electronic Supplementary Material) indicated carbon to be, by far, the majority element, with oxygen, nitrogen and phosphorus also present to a very small extent. The amount of phosphorus was particularly low (~ 0.1 at%), but above the detection limit of the XPS technique, and its presence must be related to the phosphate groups from the adsorbed nucleotide molecules (the starting graphite did not show evidence of any phosphorus by XPS).

Figure 5. Spectroscopic and microscopic characterization of graphene prepared through aqueous exfoliation with complementary nucleotide-nucleobase combinations as dispersants: (a) UV-vis absorption spectrum of a typical graphene dispersion stabilized by the AMP-T combination; (b) high resolution C 1s core level X-ray photoelectron spectrum of exfoliated graphene flakes (black trace) and the starting graphite powder (red trace); (c) typical Raman spectrum of the exfoliated graphene material; (d-f) representative TEM images of the exfoliated graphene flakes; (g) histogram of lateral size of the graphene flakes measured on ~ 100 objects from STEM images.

The amount of nitrogen (~ 0.5 - 0.6 at%) was more or less commensurate with that expected to arise from the single nucleotide molecules or nucleotide-nucleobase dimers (~ 0.5 - 0.8 at% based on the measured amount of phosphorus). However, the same was

not true in the case of oxygen: the XPS data revealed the abundance of this element to be ~2.2 at%, compared with an expected amount of oxygen associated to the nucleotide/nucleobase molecules of only ~0.7-0.9 at%. Even if we assumed that all the remaining oxygen (i.e., ~1.3-1.5 at%) was present as oxidized species in the dispersed graphite/graphene material, the O/C atomic ratio of the latter would be in the 0.01-0.02 range, thus indicating its very low degree of oxidation (the O/C ratio of the starting graphite was 0.01). This conclusion was supported by the shape of the high resolution C 1s core level spectra of the dispersed materials (Fig. 5b, black trace), which was entirely dominated by the component at about 284.6 eV corresponding to sp²-hybridized carbon atoms in unaltered graphitic environments.^{50,51} The C 1s profile did not appear to incorporate any significant contribution from oxidized carbon atoms, which would have been observed in the ~286-289 eV binding energy range.⁵⁰ In point of fact, such a profile was virtually identical to that of the starting graphite sample (Fig. 5b, red trace).

The high structural quality of the dispersed materials was corroborated by Raman spectroscopy. As noticed from Fig. 5c, the spectra were dominated by three well-known bands: the defect-related D band (~1350 cm⁻¹), the G band characteristic of graphite/graphene materials (~1582 cm⁻¹) and the 2D band (overtone of the D band; ~2700 cm⁻¹).^{52,53} The D band was relatively weak compared with the G band. Indeed, calculation of their integrated intensity ratio (I_D/I_G ratio), which is frequently taken as a quantitative proxy of structural quality in graphitic materials,⁵² yielded values of ~0.2-0.3. These figures are comparable to, or even lower than, those commonly reported for solvent-exfoliated pristine graphene flakes, and their non-zero values are thought to arise mainly from the contribution of flake edges (the diameter of the Raman laser spot being larger than the typical flake size) and not from basal plane defects.^{49,54-56} Indeed, visualization of the dispersed material by TEM revealed it to be made up of more or less transparent flakes with typical lateral dimensions between a few and several hundreds of nanometers (Fig. 5d-f), which are smaller than the diameter of the laser spot (a few micrometers). The lateral size distribution of the graphene flakes measured from STEM images (not shown) is presented as a histogram in Fig. 5g. Typical values ranged between 100 nm and 1 μm (~90 % of the objects were below 500 nm).

The average thickness of the dispersed graphene flakes was determined by UV-vis absorption (extinction) spectroscopy, using the equation recently derived by Backes et

al relating mean thickness in number of monolayers, $\langle N \rangle$, and extinction of the graphene dispersion measured at the wavelengths of 325 (ϵ_{325}) and 550 nm (ϵ_{550}):⁵⁷

$$\langle N \rangle = 35.7 \times \epsilon_{550}/\epsilon_{325} - 14.8 \quad (1)$$

Such metrics is based on the empirical relation between the UV-vis extinction spectrum of graphene dispersions and the carefully measured average apparent thickness of the corresponding graphene flakes by AFM. This validated method is less time-consuming and more statistically meaningful than direct measurement of thickness by AFM measurements on a limited number of flakes. Using eq. (1), it was estimated that the graphene samples obtained with either the AMP-T or GMP-C combinations as stabilizers possessed flakes with an average number of monolayers of ~9–10 and ~8–9 for those dispersions prepared at a centrifugal force of 200 g and 500 g, respectively. The contact angle with water of a graphene film obtained with the AMP-T combination was $120 \pm 5^\circ$ (see Fig. S2 in Electronic Supplementary Material), which was similar to the values previously reported for films composed of several graphene flakes,⁵⁸ and indicative of high hydrophobicity.

3.3. Graphene-nucleotide hybrid hydrogels and their uptake/release of dyes and drugs

The ability to use RNA/DNA nucleotides as efficient dispersants for the preparation of high quality graphene flakes with significant colloidal stability by the strategy developed here also implies an improved ability to integrate the 2D carbon material with these relevant biomolecules, which in turn should facilitate access to different graphene-nucleotide hybrid materials of potential utility in a number of applications. To illustrate such a possibility, we have prepared AMP-based hydrogels containing graphene flakes that exhibit an enhanced uptake capacity and/or more sustained release behavior of dyes and drugs compared with their graphene-free counterpart. Molecular hydrogels are an important class of soft materials with a strong potential for application in, e.g., drug delivery, tissue engineering or photonic and electronic devices.^{59,60} In particular, a wide range of hydrogels using RNA/DNA nucleobases, nucleosides or nucleotides and their derivatives as gelators have been intensively investigated over the last decades.^{61,62} It has been recently demonstrated that AMP-based hydrogels can be generated through coordination of this nucleotide with Zn^{2+} ions.^{63,64} Capitalizing on the anticipated ability of AMP-T-stabilized graphene flakes to participate in the Zn^{2+} -AMP

coordination processes that induce formation of the hydrogel, we explored the possibility of producing functional graphene-AMP hybrid hydrogels with a uniform distribution of flakes within the gel.

The hydrogels were prepared through centrifugation of mixed aqueous solutions containing AMP (1.6 mM), ZnCl_2 (2 mM) and variable concentrations of AMP-T-dispersed graphene (see Experimental section for details). Fig. 6a shows a digital photograph of some starting mixed solutions before centrifugation in the absence (i) and presence (iii) of graphene flakes, as well as after gel formation via centrifugation, again in the absence (ii) and presence (iv) of graphene. The hydrogels can be seen suspended in the liquid (after detachment from the centrifuge tube bottom), and were almost colorless in the absence of graphene and uniformly black when graphene was incorporated.

Figure 6. (a) Digital photograph showing the formation of AMP-based hydrogels in the absence [(i), (ii)] and presence [(iii), (iv)] of graphene flakes. Tubes (i) and (iii) correspond to the precursor solution before gel formation (i.e., prior to centrifugation), whereas tubes (ii) and (iv) show the formed gels suspended in the liquid. The graphene-free hydrogel [tube (ii)] was almost completely translucent and could be hardly seen by the naked eye; its position in the tube is indicated by the yellow contour. (b-f)

Representative STEM images of AMP-based hydrogels prepared in the absence (b,c) and presence (d-f) of graphene flakes.

The hydrogels were seen to form in the whole range of graphene concentrations tested, i.e., between ~ 0.01 and 0.8 mg mL^{-1} , even though at the highest concentration the total weight of flakes incorporated in the gel was similar to that of the Zn^{2+} -AMP component (i.e., ~ 1.2 - 1.3 mg for a single centrifuge tube). On the micrometric scale, the graphene-free hydrogel exhibited a homogeneous appearance, as exemplified in the STEM image of Fig. 6b. However, detailed STEM inspection of thinner fragments of the gel clearly revealed the typical entangled nanofibrillar morphology expected for a supramolecular gel (Fig. 6c).^{62,63} Such morphology was preserved when the hydrogels were assembled in the presence of graphene flakes, the latter being uniformly distributed more or less as single entities throughout the gel, as can be noticed from the STEM images of Fig. 6d and e, which were taken from a hydrogel prepared at a graphene concentration of 0.1 mg mL^{-1} . The graphene flakes were seen as bright features in dark field images (Fig. 6d) and as objects with darker contrast in bright field images (Fig. 6e). Such an observation suggests a good interaction between the AMP-T-stabilized graphene flakes and the Zn^{2+} -AMP nanofibrils, which can be ascribed to the participation of the former in coordination processes with Zn^{2+} by way of the AMP molecules adsorbed on the flakes. Indeed, intimate intermixing between the nanofibrils and the flakes could be noticed from the STEM images (e.g., Fig. 6f). We also note that this type of uniform hybrid gels could be formed using AMP-T-stabilized graphene dispersions, but not dispersions prepared only with AMP. To prepare the hybrid gels, the graphene suspensions had to be pre-conditioned through washing and concentration steps (see Experimental section). While the AMP-T combination afforded washed and concentrated dispersions that were stable in a practicable timeframe, their AMP-only counterparts agglomerated immediately and failed to yield uniform gels. Our attempts to measure the BET specific surface area of the prepared hydrogels were unsuccessful due to the collapse of the organometallic component of the hydrogel during the drying process required before any physical absorption measurement. Neither substitution of the parent aqueous medium by a liquid with lower surface tension, such as acetone, nor freeze-drying of the hydrogel prevented the collapse of its three-dimensional structure. The low BET value

of the resulting powder ($\leq 2 \text{ m}^2 \text{ g}^{-1}$) was therefore not representative of the surface area of the wet, non-collapsed hydrogel.

The performance of the hybrid gels towards the uptake and release of some dyes and drugs was then investigated, which is a relevant application area of molecular gels.^{60,62} We reasoned that, due to favorable (e.g., hydrophobic or π - π) interactions between graphene and many dye/drug molecules, incorporation of this material in the AMP-based hydrogels could be advantageous in terms of increasing their dye/drug loading capacity and/or slowing down their release profile. We note that the high specific area and the presence of extensive sp^2 carbon domains that are believed to promote those interactions are not exclusive of graphene with high structural quality, such as the one prepared in this work, but associated to graphene materials in general. However, although low structural quality graphene, such as reduced graphene oxide, could also increase the dye/drug loading capacity of a given hydrogel, its preparation through the graphene oxide route is more complex and less environmentally friendly than the direct-liquid phase exfoliation of graphite employed here. Thus, even if high structural quality graphene is not necessarily needed for this application, its use is advantageous from the point of view of easiness of preparation. For the tests, we chose the antibiotic tetracycline (TC) as well as the dyes methylene blue (MB) and rhodamine B (RB), which can also be used as drugs (e.g., in photodynamic therapy processes).⁶⁵ To load the dyes/drugs into the hydrogels, the corresponding molecule was included in the precursor solution, and the loaded amounts were calculated by determining its change in concentration in the supernatant after the centrifugation step by means of UV-vis absorption spectroscopy (see Experimental section). For MB and RB it was observed that initial concentrations of 1 mg mL^{-1} and above prevented the formation of the hydrogel, possibly due to the emergence of some interactions that competed with the Zn^{2+} -AMP coordination. In the case of TC, gel formation was prevented at concentrations around 0.5 mg mL^{-1} and above. Therefore, for the uptake tests we used initial concentrations of 0.5 mg mL^{-1} for MB and RB and 0.2 mg mL^{-1} for TC. Fig. 7a-c shows the measured uptake of RB (a), MB (b) and TC (c) for hydrogels prepared at different graphene concentrations in the precursor solution. The loaded amounts are given in milligrams of dye/drug per gram of dried gel (i.e., mass of Zn^{2+} -AMP plus graphene).

Figure 7. (a-c) Uptake of RB (a), MB (b) and TC (c) into AMP-based hydrogels prepared in the presence of different concentrations of AMP-T-stabilized graphene flakes in the precursor solution. (d-f) Release profiles of RB (d), MB (e) and TC (f) from graphene-free, AMP-based hydrogels (colored traces in panels d-f) as well as from graphene-containing hydrogels (gray traces) prepared at a concentration of AMP-T-stabilized graphene in the precursor solution of 0.5 mg mL^{-1} . The larger than physically possible value of released MB is attributed to the experimental errors associated to the UV-vis absorption measurements.

In general terms, the uptake capacity tended to increase with the amount of graphene present in the hydrogel. This was especially the case with RB: whereas the graphene-free hydrogel was seen to load less than 5 mg g^{-1} , uptake values close to 50 mg g^{-1} were attained at the highest graphene concentration tested (0.8 mg mL^{-1}). For MB and TC, the presence of graphene led to more modest relative increases in the uptake capacity, e.g., from $\sim 19 \text{ mg g}^{-1}$ (no graphene) to $\sim 43 \text{ mg g}^{-1}$ (0.8 mg mL^{-1}).

graphene) with MB, and from $\sim 7 \text{ mg g}^{-1}$ (no graphene) to $\sim 16 \text{ mg g}^{-1}$ (0.3 mg mL^{-1} graphene) with TC. We interpret the well-known ability of graphite/graphene surfaces to adsorb molecules containing aromatic rings, which are present in RB, MB and TC in significant numbers, to be the main reason behind the beneficial effect of the graphene flakes on the uptake capacity of the hydrogels. The dye/drug release profiles of the graphene-free hydrogel and the hydrogel prepared with 0.5 mg mL^{-1} graphene were then recorded in phosphate buffer saline medium and compared (Fig. 7d-f). Broadly speaking, the graphene-free hydrogel was seen to release the dyes/drugs more rapidly than their graphene-containing counterparts did. For instance, the amount of RB released from the graphene-free hydrogel approached saturation after $\sim 2.5 \text{ h}$, compared with $\sim 5 \text{ h}$ for the graphene-containing hydrogel (Fig. 7d), and it was $\sim 2.5 \text{ h}$ vs $\sim 8 \text{ h}$ in the case of TC (Fig. 7f). For these two compounds, the maximum amounts released relative to the initial loaded amounts were seen to be much higher with the graphene-free hydrogel ($\sim 65\text{--}90\%$) than when the graphene-containing hydrogel was used ($\sim 25\%$). Again, such a result could be ascribed to the high affinity of graphene towards these aromatic molecules, to the extent that a large fraction of them appeared to be retained indefinitely in the hydrogel. The same did not hold true, however, in the case of MB, for which both hydrogels released $\sim 100\%$ of the originally loaded amount (although at a significantly slower rate with the graphene-containing one).

3.4. Biocompatibility of nucleotide-exfoliated graphene films

Finally, we carried out a preliminary biocompatibility survey of the nucleotide-exfoliated graphene flakes developed here. Graphene has demonstrated a strong application potential in biomedicine,^{3,66} including its use as scaffold or support for cell growth and differentiation in the context of, e.g., tissue engineering and regeneration.⁶⁷ To be safely used in this realm, an obvious pre-requisite that graphene or any material must fulfil is that of biocompatibility and non-cytotoxicity. Although it is commonly accepted that graphene scaffolds and films are in general biocompatible,^{67,68} such a characteristic must be evaluated on a case-by-case basis due to the possible influence of the specific physicochemical features of the graphene materials (e.g., nanosheet lateral size and thickness) and the (residual) presence of chemical compounds/reagents used for their preparation (surfactants, reducing agents, etc).⁶⁹ Because the present aqueous

graphene dispersions were obtained using innocuous biomolecules (RNA/DNA nucleotides and nucleobases) as stabilizers, they are expected to be good candidates for bio-related applications. To evaluate their biocompatibility, we investigated the in vitro proliferation of murine fibroblasts (L-929 cell lines) on thin graphene films prepared from the AMP–T-stabilized dispersions. The L-929 cell line is frequently employed as a benchmark for biocompatibility studies of materials. The recently developed fluorometric PrestoBlue (PB) assay was employed for the cell proliferation tests. The PB assay boasts some practical advantages over more conventional ones, such as the colorimetric MTT assay, including immediacy and simplicity of use.⁷⁰

Fig. 8 summarizes the results of the proliferation tests 2, 4, 7 and 10 days after seeding the fibroblasts onto tissue culture plates (hydrophilic polystyrene) at an initial density of 5000 cells per well, for wells that were covered with either ~0.25 or 0.50 mg of graphene flakes cast into films from an aqueous dispersion stabilized by the AMP–T combination (see Experimental section for details).

Figure 8. Proliferation of murine fibroblasts (L-929 cell line) seeded onto ~1 μm (0.25 mg; blue bars) and 2 μm (0.50 mg; cyan bars) thick films of graphene flakes obtained from AMP–T-stabilized dispersions in aqueous medium. For comparison, results for ~1 μm (red bars) and 2 μm (orange bars) thick films of vitamin C-reduced graphene oxide nanosheets are also presented. For each testing day, cell proliferation values are provided relative to the proliferation value measured the same day on the bare culture plate. Significant statistical differences between the different materials measured on the same day are indicated by the use of the different symbols *, † and •.

The thickness of the resulting graphene films was approximately 1 μm (0.25 mg) and 2 μm (0.50 mg). For comparison purposes, results obtained from vitamin C-reduced graphene oxide films (~ 1 and 2 μm thick) are also presented. In Fig. 8, cell proliferation values for a given day are provided relative (in percentage) to the proliferation measured on the bare culture plate, which is thus taken as the reference substrate. It can be noticed that cell proliferation rates on the AMP–T-exfoliated graphene films (either ~ 1 or 2 μm thick) were lower than those of their reduced graphene oxide-based counterparts for the shorter testing periods (i.e., 2, 4 and 7 days after cell seeding), whereas they tended to converge after 10 days. Compared with the bare culture plate, the proliferation rates on the AMP–T-based graphene films were initially very low ($\sim 25\%$ of the bare plate value after 2 days) but increased remarkably over time (up to $\sim 75\%$ after 7 and 10 days). Furthermore, the absolute proliferation values (data not shown) also revealed distinct and steady increases for these films from day 2 through day 10, suggesting that they are indeed biocompatible and non-cytotoxic. In the case of the reduced graphene oxide films, the absolute proliferation values (data not shown) tended to increase as well, although step-wise (from the value for 2 and 4 days after seeding to that for 7 and 10 days after seeding) rather than steadily. The initially much lower proliferation values noticed for the AMP–T-based graphene films compared with the bare culture plate and the reduced graphene oxide films is thought to be related to the more hydrophobic nature of the former. It is well known that the adhesion of cells, and consequently their growth and proliferation, is favored on moderately hydrophilic substrates compared with hydrophobic ones.⁷¹⁻⁷³ While the bare culture plate and the reduced graphene oxide films are relatively hydrophilic on account of their surface oxygen content, the essentially oxide-free, AMP–T-exfoliated graphene films are hydrophobic, as mentioned before. However, even though fibroblast attachment on the latter may take a relatively long time to occur, once the cells settle in, they exhibit a fast proliferation, which is indicative of a good compatibility with the substrate.

4. Conclusions

We have demonstrated that high quality graphene flakes can be exfoliated and colloidally stabilized in aqueous medium in an efficient manner using RNA/DNA nucleotides combined with proper nucleobases as the dispersing agent. Specifically,

while single RNA/DNA nucleotides are at best mediocre dispersants for graphene, combination of a nucleotide with its complementary nucleobase (e.g., AMP with T, or GMP with C) affords graphene suspensions with much improved colloidal stability. The formation of hydrogen-bonded supramolecular entities from the complementary nucleotide-nucleobase combinations, for example AMP–T or GMP–C cyclic dimers, was believed to be the main driving force responsible for the observed behavior. These supramolecular entities are expected to adsorb on the graphene flakes more strongly than their individual components, thus furnishing them with a higher colloidal stability in water. Notably, the use of complementary nucleotide-nucleotide combinations rather than nucleotide-nucleobase combinations (e.g., AMP–TMP instead of AMP–T) was ineffective towards the attainment of stable aqueous graphene dispersions, probably due to the strong electrostatic barrier being in place between the two nucleotides that hampers their supramolecular association. Non-complementary nucleotide-nucleobase combinations generally failed to give graphene suspensions with good colloidal stability, a result that can be ascribed to their weaker intermolecular interactions compared with those present in the complementary, canonical base pairings. The ability to attain tighter (although non-covalent) RNA/DNA nucleotide-graphene associations by the strategy developed here can be exploited not only to access stable graphene suspensions using these biomolecules as the dispersing agents, but also graphene-nucleotide hybrid materials with enhanced functionality. The latter possibility was demonstrated through preparation of graphene-containing, AMP-based hydrogels, where the graphene flakes were uniformly intermixed with the gel phase. Such hybrid hydrogels exhibited increased uptake and/or slower release profiles of dyes and drugs. Besides, cell proliferation tests carried out with murine fibroblasts seeded onto thin graphene films suggested that the nucleotide-exfoliated flakes are biocompatible. Interfacing graphene with biomolecules is highly relevant in many technological fields, such as biomedicine, sensing, or new materials for energy and environmental applications.^{74,75} Thus, we believe the present results open new opportunities for the development of hybrid materials where the attractive characteristics of graphene and RNA/DNA nucleotides, or even other (bio)molecules of interest to which this strategy could also be applied, can be concurrently used to good advantage.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.carbon.20xx.xx.xxx>.

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