Graphene-Based Nucleants for Protein Crystallization

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Protein crystallization remains a major bottleneck for the determination of high resolution structures. Nucleants can accelerate the process but should ideally be compatible with high throughput robotic screening. Polymethyl glycol grafted (PEGylated) graphenes can be stabilized in water providing dispensible, nucleant systems. Two graphitic feedstocks are exfoliated and functionalized with PEG using a non-destructive, scalable, chemical reduction method, delivering good water dispersibility (80 and 750 μg mL⁻¹ for large and small layers, respectively). The wide utility of these nucleants has been established across five proteins and three different screens, each of 96 conditions, demonstrating greater effectiveness of the dispersed PEGylated graphenes. Smaller numbers of larger, more crystalline flakes consistently act as better protein nucleants. The delivered nucleant concentration is optimized (0.1 mg mL⁻¹ in the condition), and the performance benchmarked against existing state of the art, molecularly imprinted polymer nucleants. Strikingly, graphene nucleants are effective even when decreasing both the nucleant and protein concentration to unusually low concentrations. The set-up to scale-up nucleant production to liter volumes can provide sufficient material for wide implementation. Together with the optimized crystallization conditions, the results are a step forward toward practical synthesis of a readily accessible "universal" nucleant.

1. Introduction

Despite recent progress in direct imaging methods, X-ray crystallography remains the dominant method for structural determination of proteins and other biomolecules, particularly at high resolution and for the whole range of molecular weights. Although cryogenic electron microscopy by-passes the need for crystals, its application has its own limitations, such as the lower resolution of the more flexible parts of the macromolecules (which are often the most interesting in terms of catalysis and protein–protein interactions) and the large molecular size that is usually required. High quality crystallographic data rely on successful crystallization methods; however, success remains severely limited. Attempts in structural genomics centers to run high-throughput protein structure determinations only succeeded in crystallizing about 20% of purified proteins.[1,2]

The crystallization process can be subdivided into a nucleation stage, during which a small number of macromolecules in solution overcome an energy barrier to assemble into stable (post-critical) crystal nuclei, followed by a crystal growth stage, during which the stable nuclei extend until they reach a useful (though generally still small) size.[3] The mechanisms of these two stages of protein crystallization are appreciably different. Nucleation is a first-order phase transition triggered by the aggregation of only a few protein molecules, due to stochastic fluctuations in the supersaturated solution or to the attractive or restraining effect of a heterogeneous nucleant.[4] These aggregates may initially have short-range order or be liquid droplets of highly concentrated protein which become fully ordered at a later stage.[5] Growth occurs mainly via two mechanisms, so-called “2D nucleation” on already formed crystal facets and the deposition of molecules on step edges created by screw dislocations in the crystal.[6] Depending on the supersaturation of the crystallization solution, some conditions can sustain crystal growth...
without the ability to initiate nucleation.[13] Indeed, the nucleation stage requires either an appreciably higher supersaturation or the introduction of nuclei, which can be either seeds (i.e., tiny fragments of already existing crystals or microcrystalline material[10]) or heterogeneous nucleants. The latter are substances or surfaces with properties that promote the close approach of the macromolecules, and lower the energy barrier associated with nucleation. Heterogeneous nucleants can, therefore, induce nucleation (and allow subsequent growth of the crystals) at the lower supersaturations that are sufficient for crystal growth but not for spontaneous nucleation.[7–10]

The control of nucleation afforded by heterogeneous nucleants has a twofold advantage: First, it can increase the number of “hits” in crystallization screening. Since crystallization is unpredictable, the first step in crystallizing a previously uncrystallized protein is to subject it to tens or hundreds of conditions (various buffers, precipitating agents and chemical additives mixtures, possibly at various temperatures and protein concentrations), to identify some that lead to crystalline material (a “hit”).[12,13] Such screens are now standardized, providing consistent sets of commercially available conditions. These conditions may often be in the metastable zone for a given protein, that is, they are suitable for crystal growth, but not for spontaneous nucleation.[12,13] In these cases, the addition of a nucleant may produce crystals where none would normally be expected. Second, lowering the supersaturation at known crystallization conditions, in order to deliberately reach the metastable zone, can suppress unwanted nucleation. The addition of a nucleant can control the number of nuclei, which then grow more slowly under lower supersaturation, yielding larger and/or better-ordered crystals, more suited to structural determination.[3,7,9,10,14] Heterogeneous nucleation is, therefore, useful for both screening and crystal optimization.

The search for effective heterogeneous nucleants is an ongoing effort by many laboratories throughout the world; several nucleants have been successfully tested, some of which are easy to obtain or otherwise commercially available. Most of these nucleants, however, are solid, meaning that a chip or grain has to be introduced manually into the crystallization solution, which is usually a droplet of size ranging from ten nanoliters to a microliter.[8,9,13] The use of such nucleants is thus a time consuming and exacting procedure requiring dedication and experience. Nucleants in liquid/suspension form, on the other hand, can be dispensed with a crystallization robot at the same time as the protein and screen conditions themselves. When used for optimization, they can be pipetted manually in the same manner as the other solutions of the crystallization mixture.[12] The use of liquid nucleants, therefore, has the potential to become a routine, high-throughput part of the crystallization process.

Carbon nanomaterials show interesting properties as nucleants for protein crystallization due to their high accessible surface area, ability to form porous networks, and versatile chemistry. Among different carbon nanomaterials, the flat geometry of graphene has demonstrated the most efficient protein nucleation behavior. Initial trials showed that grafting polyethylene glycol (PEG) to the graphene surface in a space-controlled manner improved hydrophilicity of the graphenes whilst allowing the protein to nucleate and form crystals mainly on edges and folds of the graphene layers.[15] Here, we systematically explore the effectiveness of graphene-related materials, as liquid-based nucleants, delivered robotically, across a wide range of proteins and screens, and benchmark the performance against the state of the art. A consistent demonstration of efficacy with a wide range of proteins and screens, as well as the identification of appropriate parameters, is an essential step to the wider uptake of the technology.

2. Results

2.1. PEGylation of Graphenes Using Reductive Chemistry

Two different graphite starting materials were exfoliated and functionalized following a reductive methodology developed for grafting short alkyl groups and extended to PEG chains.[15,16] The two graphitic sources were selected to assess whether layer size and stacking might influence the resulting protein nucleation abilities. For liquid phase applications, bulk graphene-related materials are required, rather than mechanically exfoliated or other single-layer substrate bound samples. One feedstock was a chemical vapor deposition (CVD)-derived few-layer graphene (FLG). The CVD approach converts volatile carbonaceous precursors into single- and few-layer graphene materials, often on a metallic substrate, though bulk products are available via plasma reactors.[17] The selected, bulk FLG material displays a relatively low crystallinity and consists of small, connected stacks of graphenes, providing a highly porous material with a moderate number of edges, which encourage the accommodation of proteins. The other feedstock was an exfoliated natural graphite (XG), which retains a higher degree of graphite crystallinity but with a moderate degree of exfoliation; the larger, flatter layers may promote the growth of protein crystal facets. Both starting materials were exfoliated using a reductive process, using Coulombic charge to introduce repulsion between anionic “graphenide” layers:[18,19] the charge was then used to initiate polymer grafting reactions on the exposed surfaces. Sodium and naphthalene were used as the reducing agent and transfer reagent, respectively. Both graphite starting materials were exfoliated using a C/sodium ratio of 12, previously found to be an optimum for graphite exfoliation/functionalization.[16] Each material was treated with sodium naphthalide in dimethylacetamide (DMAc) and functionalized with a methoxy polyethylene glycol (mPEG) monobromide (MW 5000 g mol$^{-1}$) (Scheme 1), as this polymer has shown good protein crystallization behavior on its own[20] and when grafted to other carbon nanomaterials.[15]

After polymer functionalization, the PEGylated graphenes were left to settle over 2 h and then separated into two fractions: the supernatant (S), corresponding to the most stable PEGylated graphene layers in suspension, and the deposit (D), corresponding to thicker aggregates, and less functionalized graphenes. Under scanning electron microscope (SEM) (Figure 1A), the as-received FLG layers have a small lateral size ($\approx 0.15$ μm), forming consistent globular aggregates (Figure S1, Supporting Information); after PEGylation there were no significant differences observed in the structure of the aggregates, either in the supernatant or deposit (Figure 1A, bottom panel). On the other hand, the XG starting material forms larger

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**Figure 1A**: SEM images of FLG and PEGylated FLG layers. (A) As-received FLG layers have a small lateral size ($\approx 0.15$ μm) and form consistent globular aggregates. After PEGylation, there are no significant differences observed in the structure of the aggregates, either in the supernatant or deposit (bottom panel). On the other hand, XG starting material forms larger
Scheme 1. Reaction scheme for the exfoliation and PEGylation of XG and FLG starting materials.

Figure 1. A) SEM images of XG-PEG deposit (D) and supernatant (S) (top panel) and FLG-PEG deposit (D) and supernatant (S) (bottom panel) after reductive functionalization. B) Raman and XRD spectra of the different PEGylated graphenes. C) TGA-MS profiles of the as-received and PEGylated graphenes. PEG fragments $m/z$: 15 (CH$_3^+$) and $m/z$: 31 (CH$_3$-O$^-$).
aggregates with sizes in the range of 29.1 ± 14.5 µm (Figure S1, Supporting Information); after PEGylation and sedimentation, the deposit still shows the presence of large aggregates; however, the supernatant fraction consists of smaller, more isolated layers (Figure 1B, top panel). The layer lateral size is significantly lower than the starting material (9.5 ± 2.4 µm), suggesting that the smaller, more individualized layers remain selectively dispersed after PEGylation.

Thermogravimetric analysis coupled with mass spectrometry (TGA-MS) is a well-established method to quantify the degree of grafting (Figure 1B). As-received XG and FLG show a small mass loss in the range from 100 to 800 °C, (2.8 wt% for FLG and 1.2 wt% for XG), probably due to the decomposition of oxygen functionalities. After PEGylation, the typical PEG mass fragments (m/z: 15 and 31) were observed in the range between 200 and 600 °C, confirming the presence of PEG attached to the graphene layers. The grafting ratio (grafted weight fraction) and grafting stoichiometry (graphitic carbons per chain, C/R) were estimated from analysis of the PEG mass fragments and the accompanying mass loss in the corresponding temperature range following calculations reported previously (see Supporting Information).[18] Grafting ratios of 16.8 and 29.6 wt% were observed for PEGylated XG and FLG, respectively (Table 1). The larger grafting ratio for smaller FLG compared to larger XG flakes follow trends recently described for other reduction reactions on a range of graphene-related materials.[18] The absolute C/R values of 2467 and 1404 for XG and FLG, respectively, are consistent with steric predictions for polymers grafted to nanocarbons.[19]

These grafting trends are in good agreement with quantitative Raman data obtained from the ratios of the defect, D, and graphitic, G, bands from statistical mapping experiments (I_D/I_G) (Figure 1C and Table 1). Histograms of the (I_D/I_G) ratio across a large number of independent locations, allow the effect of the grafting chemistry to be evaluated consistently (example spectra in Figure 1C, histograms in Figure S2, Supporting Information). The XG starting material showed a well-defined G band in Figure 1C, histograms in Figure S2, Supporting Information), indicating a degree of functionalization. The PEGylated FLG-PEG D also shows a shifted I_D/I_G distribution, and an even clearer, significant increase in the I_D/I_G ratios from 0.31 ± 0.021 for the as-received material up to 0.52 ± 0.024 for the PEGylated FLG. The overall increases in I_D/I_G ratio are consistent with the expected increase in the number of sp³ atoms due to the grafted PEG chains.[18,21] X-ray diffraction analysis (CuKα = 1.542 Å) of both the XG and FLG starting materials displayed broad graphitic (002) layer peaks (θ = 26.6°), indicative of short stacks; as expected, the XG was more graphitic compared to FLG. After reductive PEGylation, both materials were more exfoliated (Table 1 and Figure S3, Supporting Information). The broad weak signal at 26.5°, in the ar-FLG material corresponding to a layer spacing of 3.4 Å, became broader after reductive PEGylation. The layer peaks for XG-PEG also broadened significantly, although new peaks for kinetically trapped graphite intercalation compounds (GICs) also appeared, as reported previously for graphites exfoliated with Na-DMAc.[24] Since GICs are highly reactive, these regions must be very effectively isolated from the environment, and likely play little role in the nucleation behavior.

The dispersibility of the functionalized graphenes in water is critical for their use as robot dispensed nucleants. PEGylation dramatically improved the stability of the grafted graphenes in water (Figure S4, Supporting Information, and Table 1). As-received XG showed negligible dispersibility in water, whereas as-received FLG showed a slight dispersibility (4 µg mL⁻¹), most likely due to the larger defect concentration observed from Raman measurements (Figure 1B). After PEG grafting and separation of the supernatant, the PEGylated graphenes show an improved dispersibility in water of 80 and 750 µg mL⁻¹, for XG and FLG, respectively. Reductive processing is a suitable technique to produce stable aqueous dispersions of different size, functionalized, graphene-based flakes, suitable for robot crystalization trials (Figure 2).

### 2.2. Protein Crystallization Experiments

To assess the effectiveness of these candidate nucleants, the outcomes of crystallization trials were compared across hundreds
of different screening conditions, with and without nucleant, for numerous exemplar proteins. The criterion of effectiveness was the number of "hits" in the presence of nucleant compared to the controls. A "hit" refers to a crystallization trial at given conditions that gives rise to identifiable crystalline material. That hit condition can then be optimized to produce useful crystals for diffraction studies. The synthesized nucleants were tested with five different proteins (ferritin, trypsin, glulisine, proteinase K, and alpha-crustacyanin) against three different, 96-condition, commercially available, standard crystallization screens (Structure Screen, poly-γ-glutamic acid [PGA], and Index). The selected proteins represent a wide range of molecular weights, ranging from \( \approx 5.8 \) kDa for the small peptide hormone glulisine to \( \approx 474 \) kDa for the hollow multimeric ferritin that reaches \( \approx 12 \) nm in diameter. Their isoelectric points also cover a wide range, from \( \approx 4.4 \) for ferritin to 10.5 for trypsin. This diversity is important to assess how generally effective the nucleants are, or whether their efficacy may correlate with protein size or charge. Glulisine and alpha-crustacyanin are actively pursued target proteins, the structure of the former having been published only recently;\(^{[25]}\) a high-resolution structure of the complete alpha-crustacyanin has not yet been determined. The other proteins, in this study, are regularly used as benchmarks (model proteins) for understanding crystallization and developing new methodology.

The selected screens are also diverse. Structure Screen is a typical sparse-matrix screen, that is, one that aims to cover as widely as possible the chemical space of crystallization conditions, based on previously successful combinations of reagents.\(^{[11]}\) Index is a more systematic screen, aiming to test

![Figure 2](image-url)
some of the most commonly used precipitating agents against a range of pH and additives. PGA is a newer systematic screen where all conditions contain a common additive, PGA, a low molecular weight polymer.\cite{16} Taken together, these screens represent 288 distinct crystallization conditions, with very little overlap. Outcomes of the trials were observed regularly for 14 days. For concise presentation, the proportion of crystallization drops delivering hits, by day, has been consolidated across all 288 conditions, for each protein (Figure 2); however, it is important to note that the separate screens all show the same positive trends, individually, as presented in full in Figures S5–S7 and S9, Supporting Information. The trends are consistent both across screens, and with time, through are clearest at the end of the experiments; numerical statistical significance is very strong (see Experimental Section).

In all cases, for all proteins and all screens, the introdution of the graphene-based nucleants (dosed at 0.1 mg mL$^{-1}$ in the crystallization drop) either matched or increased the proportions of “hits” observed as a function of time, compared to controls with no added nucleant (Figure 2, and Figures S5–S7 and S9, Supporting Information). The supernatant fractions (shortened hereafter to XGS and FLGS for simplicity) systematically outperformed the deposit fractions (shortened hereafter to XGD and FLGD), and XG systematically outperformed FLG. Overall, therefore, XGS was the most efficient nucleant for all tested proteins. After 14 days, the addition of XGS more than doubled the aggregated number of hits for alpha-crustacycin (Figure 2A); the hit rate for glusline increased +64% (Figure 2B) while the rates of the remaining proteins increased by about a third (Figure 2C–E and Figures S3–S9, Supporting Information). FLGS displayed an increased number of hits over all the proteins tested, but less pronounced than for XGS, ranging from a marginal +3% for proteinase K to +83% for alpha-crustacycin (Figure 2A). XGD had a detectable but mostly marginal effect and FLGD was mostly ineffective (Figure 2 and Figures S5–S7 and S9, Supporting Information).

The different behavior between supernatant and deposit fractions relates to the different character of the materials fractionated after PEGylation. The larger, more aggregated graphene layers are heavier compared to thinner FLGs; thus, large aggregates will deposit and the thinner layers will remain in the supernatant fraction (see SEM, Figure 1), providing an increased surface area for more effective protein nucleation. The improved nucleation behavior for XG compared to FLG is more interesting, since the smaller ($\approx 3400 \mu m^2$) and much lower number density. Simple geometric calculations indicate that the 20 mL nucleant suspension, introduced into each well, contained around 106 XG platelets, but around 3375885 FLG platelets; on the other hand, the total surface area per well is more similar, but still lower for the XG ($\approx 3400 \mu m^2$) than for the FLG ($\approx 200000 \mu m^2$). Increasing the XG concentration to match the FLG number or area density is not feasible; however, decreasing the FLG concentration (Figure S11, Supporting Information) toward the XG densities shows that efficacy is lost. One explanation for the greater effectiveness of the XG nucleant is that the larger number of PEG chains across the FLG layers may prevent the protein from adsorbing to the surface, as previously reported on PLL-PEG modified surfaces.\cite{17} Recent STEM studies have shown that smaller layers, like FLG, have a homogeneous functional group distribution,\cite{18} assuming a uniform distribution, the grafting ratio implies a spacing, D, between grafted PEG chains around 6.8 nm (Table 1) compared to 9.1 nm for XG, closer to the denser PEG surfaces used to suppress protein adsorption.\cite{28} Initial experiments with shorter grafted PEG chains produced a denser grafted layer, but the PEGylated graphenes were less effective as protein nucleants (Figure S8, Supporting Information). The optimum pore size for nucleation is reported to be around 10 nm, closer to the XG case,\cite{16} if the bare “patches” on the graphene surface are considered to be analogous to pores. In fact, the STEM measurements show that large flakes, such as XG, have a more heterogeneous distribution of functional groups; thus, there may be a range of patch sizes, including some larger areas for protein adsorption and organization. The flatter XG layers may favor the accumulation of the protein in such regions and subsequent nucleation as a crystal facet. Folds in the graphene layers may define an edge of the facet, providing further organization of the protein molecules.\cite{15}

The two most effective candidate nucleants (XGS and FLGS) were subjected to further testing with trypsin, the most commonly used model protein of the set, to explore the effect of concentration. When diluted by half to 0.05 mg mL$^{-1}$, both nucleants became only marginally effective. When diluted to 0.025 mg mL$^{-1}$, the efficacy even dropped below the controls (Figure S11, Supporting Information), most likely due to the slight dilution of the crystallization solution by the added nucleant solution. Adding a larger volume of nucleant, rather than a higher concentration is not attractive for the same reason; however, higher dosing concentrations were not stable. Therefore, dosing the nucleant at 0.1 mg mL$^{-1}$ in the crystallization drop appears to be the optimal concentration for these nucleants.

The most successful nucleant (XGS) at the optimized concentration (0.1 mg mL$^{-1}$ in the crystallization drop) was benchmarked against a state-of-the-art trypsin-imprinted molecularly imprinted polymer (MIP). Molecular imprinting is an established method to create binding sites for large, complex molecules like proteins or DNA, although synthesis and specificity remain challenging.\cite{29} Such MIPs are known to be particularly effective as nucleants but are ideally prepared for each specific protein of interest to create matched cavities. MIPs work by a different mechanism to most nucleants, as they rely, not on porosity or some other microstructural feature of the nucleant, but on shape and charge complementarity with the protein under crystallization. MIPs are polymerized in the presence of protein which is later extracted, leaving complementary (“ghost”) sites behind, which can be highly specific for re-binding that same protein in the early stages of nucleation.\cite{9} Significantly, MIPs can be deployed in dispersion, and are currently the preferred nucleant for automated use, representing a good reference for the new materials. The Trp-MIP and XGS nucleants were compared at two different trypsin concentrations, 30 and 15 mg mL$^{-1}$ (Figure 3 and Figure S11, Supporting Information). As expected, the hit rate in the controls
was halved at lower protein concentrations, and in both cases, the presence of MIP was effective, approximately doubling the proportion of hits. Strikingly, in the presence of XGS, however, the number of hits was the same for both protein concentrations, with no protein dilution effect observed. In other words, although the increase in hit rate in the presence of XGS for 30 mg mL$^{-1}$ trypsin was $\approx 40\%$, making XGS a little less effective than MIP at that concentration, the increase at 15 mg mL$^{-1}$ trypsin was 165%, making XGS substantially more effective than MIP. The concentration of MIP in each drop was also much higher at around 2.7 mg mL$^{-1}$, as already optimized previously. The fact that lower loadings of the generic XGS nucleant outperform the optimized, customized Trp-MIP nucleant, is particularly promising. Activity for crystallization at low protein concentration is desirable as protein samples are typically valuable and in short supply, due to the challenges of isolation.

Alpha-crustacyanin, the protein most responsive to the nucleants, was also tested against trypsin-imprinted MIP at a lower concentration (5 mg mL$^{-1}$ as opposed to 8 mg mL$^{-1}$ in the original experiments). No alpha-crustacyanin-customized MIP was available, so the trial was completed with Trp-MIP; unmatched MIPs can act as nucleants but effects may be less pronounced. In this case, the hit rate, both in controls (2.1% of trials) and with nucleants, were very low, but the same trend was again observed, with the XGS (4.7%) outperforming the MIP (3.1%) (Figure S10, Supporting Information).

The general reductive procedure to synthesize PEGylated graphenes as effective protein nucleants has been further optimized to scale-up its production. The reductive chemistry has been transferred from small scale lab-based approaches, in a glove box, to reactors able to produce 5–10 g of PEGylated material in 1–5 L vessels (Figure 4). This process takes place in one single-pot, thus minimizing the overall process time. Such a scale of production is sufficient to provide for worldwide implementation of the technology, since 1 g of nucleant would provide for 50 million droplet trials, if there were no other wastage.

3. Conclusions

This work reports the PEGylation of two types of graphenes with varying sizes and morphologies using reductive chemistry, to produce aqueous suspensions suitable for automated crystallization experiments using liquid dispensing robots. Polymer grafting was confirmed by TGA-MS and further supported by Raman measurements. An increase in water dispersibility of 150 times was observed for FLG material after PEGylation and fractionation. The increase in water dispersibility of 80 times, observed for the XG material, is especially notable, as these layers are considerably larger than the FLG material. This work has shown that both supernatant fractions of the PEGylated graphene materials are effective nucleants for a wide range of proteins (with respect to size, charge, function, and ease of crystallization), with optimized condition concentration of 0.1 mg mL$^{-1}$.

Surprisingly, the larger graphene layers (XGS) were more effective than the higher surface area FLG materials, a result attributed to their characteristic flat geometry, and less dense PEG grafting. These XGS nucleants were found to be as efficient as the protein-imprinted MIP, a well-known, patented,
and commercially available class of nucleant. Significantly, XGS was found to be even more effective than bespoke MIPs for nucleating lower protein concentrations.

The nucleants tested here are chemically and mechanically stable, and they can be stored as stable aqueous suspensions, remaining effective for at least many months. They can, therefore, be easily shipped, stored, and most importantly, dispensed within a high-throughput setup. They are, therefore, superior in ease of use to one of the most successful heterogeneous nucleants, a specially designed porous Bioglass that is commercially available as Naomi’s Nucleant (Molecular Dimensions Ltd.).[14] The materials used here also have a potentially longer shelf life than another successful type of nucleant, the MIPs, better generality, and applicability at low protein concentration.

The discovery and development of new nucleants for protein crystallization is a very active field, as the control of nucleation is a key element both in uncovering new crystallization conditions and in optimizing known ones, to prepare better diffracting crystals for high resolution macromolecular crystallographic structure determination.[7–10,12,14] The most efficient nucleant tested in this work has been found to be consistently highly effective amongst the wide range of proteins tested. Further optimization of PEG grafting length, density, and flake size can be expected to yield further improvements in performance. However, this promising material is ready for initial deployment, since it can be readily prepared in suitable quantities for widespread use in the structural biology community. These PEGylated graphene-based materials may also be relevant to many other applications such as drug and/or gene delivery[10] or conductive inks.[31]

4. Experimental Section

Materials: XG graphite was purchased from XG Sciences. FLG G3 was obtained from CamGraph nanosystems UK, with a carbon purity >99.5% and an average lateral size of 0.15 µm. Both graphite starting materials were used without any further purification, though dried thoroughly as described below. Anhydrous DMAC (Sigma-Aldrich) was dried over 20 vol% 4 Å activated molecular sieves. Naphthalene (99%, Sigma-Aldrich) was dried under vacuum in the presence of phosphorus pentoxide before use. Sodium (99.95%, ingot) and PEG (MW 5000 g mol⁻¹) were purchased from Sigma-Aldrich and used as received. Brominated 5 kDa mPEG was prepared via a method described in literature.[32] The polymer was dried under vacuum over phosphorus pentoxide.

Preparation of Sodium-Naphthalide Solution: A stock sodium-naphthalide solution was prepared to allow for accurate, simple addition of sodium to the corresponding graphite starting material. 23 mg (1 mmol) sodium and 128 mg (1 mmol) dried naphthalene were added to 10 mL anhydrous DMAC in a N₂-filled glove box, and stirred for 1 day until all sodium had dissolved, forming a dark-green solution.

Synthesis of Graphite-PEG: A Young’s tube containing graphitic starting material (15 mg, 1.25 mmol carbon) and a magnetic stirrer bar was heated at 400 °C for 1 h under vacuum, and then kept under vacuum for 16 h at room temperature, before placing in a glove box. 1.04 mL of sodium-naphthalide solution was added to the Young’s tube and the concentration of graphite in DMAC was adjusted to 0.1 M by addition of 11.46 mL of DMAC (C/Na = 12, [Na] = 0.008 M). 5 kDa mPEG monobromide (0.31 mmol, 1:1 ratio Na:mPEG) was subsequently added to the suspension and the reaction was stirred for 1 day at room temperature. To quench the product, dry O₂/N₂ (20/80%, ~1 L) was bubbled into the solution for 15 min, and then stirred overnight under dry O₂/N₂ to quench any remaining charges. The mixture was filtered through a 0.1 µm PTFE membrane and washed thoroughly with DMAC, ethanol, and water to remove any residual naphthalene, unreacted mPEG, bromide, and sodium salts formed during the reaction. The PEGylated graphites were resuspended in 10 mL of DI water and sonicated for 5 min. The solutions were allowed to sediment for 2 h and the supernatants (XGS and FLGS) were separated from the deposit fractions (XGD and FLGD). Graphene concentration in solution was estimated by evaporating the solvent in the TGA instrument for more accurate measurement of the remaining solid.

Figure 4. A) Scheme representing the protein nucleation process using PEGylated graphene. B) Scale-up process to produce PEGylated graphenes from graphites and PEG polymer.
Protein Experimental Procedures: Trypsin (T9201) from porcine pancreas was prepared in 10 mg mL⁻¹ benzamidine hydrochloride, 20 mM calcium chloride, and 20 mM HEPES (pH 7.0) at 15 and 30 mg mL⁻¹; Ferritin solution from equine spleen Sigma (F4503) was diluted using deionized water to 10 mg mL⁻¹; Proteinase K (P2308) from Trichatrichum album in 50 mM HEPES pH 7.0 at 15 and 20 mg mL⁻¹. All the above proteins were purchased from Sigma Aldrich as lyophilized powders and were used without additional purification. Pharmaceutical grade Glulisine/Apidra (Sanofi S.A., Paris, France) (5F337A) was obtained in solution at ≈3.49 mg mL⁻¹; α-crucstacycin at 8 mg mL⁻¹ in 0.1 M Tris–HCl pH 7.0, 1 mM EDTA, and 10 mM NaCl was provided by Dr. Peter Zagalsky, Royal Holloway University of London. Both the insulin analogue Glulisine and α-crucstacycin were already purified to a degree suitable for crystallographic work.

Screens: HR2-134 Index HT from Hampton Research, MD1-30 Structure Screen 1 = 2 HT-96 and MD1-51, the PGA Screen HT-96 from Molecular Dimensions, UK. Trypsin-imprinted MIP (“Chayen Reddy MIP”) was dispensed at a final concentration of ≈2.7 mg mL⁻¹.

All screening experiments with the abovementioned screens were performed with a Mosquito robot (TTP Labtech, UK) in 96-well MRC plates (Molecular Dimensions, UK). 100 nL each of the protein and screen solutions in a 1:1 ratio were set up in sitting vapor drop crystallization drops for the control experiments. An additional 20 nL (10% of the total drop volume) of the graphene nucleant dispersions (concentrated to 1 mg mL⁻¹) or MIP control (at a standard ≈27 mg mL⁻¹) were dispensed into the crystallization drops. MIPs were used as positive controls, as described in the results section. All incubations were carried out at 20 °C and observed daily for a 14 day period. Crystallization drops were observed with a Leica stereoscope.

Statistical Analysis: Each of the three 96-condition screens was tested at two different concentrations, treated as separate experiments. In one case (ferritin), the full screens were performed in duplicate, demonstrating very high reproducibility of the results after 14 days of incubation as can be seen from the Table 2: Ferritin trials run in duplicate: number of “hits” in each run at t = 14 days.

For the two most effective nucleants (XGS and FLGS), paired-samples t-tests were performed as follows, to assess the statistical significance of the results. The numbers of “hits” with and without nucleant after 14 days for every protein/screen combination were paired. Since six proteins were tested (trypsin being counted twice, at two different concentrations) versus three screens; there were 18 sets of paired data for each nucleant. The results (two-tailed test, df = 17, critical value 2.11) were t = 6.71, p = 0.000004 for XGS, and t = 3.55, p = 0.002 for FLGS, thus rejecting the null hypothesis in both cases and confirming very strong statistical significance of the effect of the nucleants. The calculations were performed with the on-line statistical calculator Statology (https://www.statology.org/paired-samples-t-test-calculator/).

Equipment and Characterization: TGA-MS was performed using a Mettler Toledo TGA/DSC 1 instrument integrated with a Hiden HPR-20 QIC EGA mass spectrometer under nitrogen atmosphere.

Samples were held at 100 °C for 30 min, then heated from 100 to 850 °C at 10 °C min⁻¹ (N₂ flow rate = 60 mL min⁻¹). Raman spectra were collected on a Renishaw inVia micro-Raman (1000–3000 cm⁻¹); using a 50 mW 532 nm laser at 10% laser power. Statistical Raman data were obtained from measurements carried out in Streamline mode of at least 500 areas per sample. Samples were prepared by drop casting dispersions on a glass slide or silicon wafer. SEM images were taken using a Leo Gemini 1525 field emission gun scanning electron microscope with SmartSEM software, at an accelerating voltage of 5 keV, working distance of ≈7 mm, and a 30 µm aperture. Powder samples were fixed onto aluminum stubs using carbon tabs (Agar Scientific Ltd.). Protein crystals were observed using M165 C microscope and images were captured with the Leica DFC295 camera and processed with Leica Application Suite software.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest. N.C., M.S.P.S., H.S.L., and L.G. filed an application under the Patent Cooperation Treaty (PCT) WO2015150775A1.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

graphene, PEGylation, polymers, protein crystallization, protein nucleants

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