From Solution to Biointerface: Graphene Self-Assemblies of Varying Lateral Sizes and Surface Properties for Biofilm Control and Osteodifferentiation

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Supporting Information

ABSTRACT: Bringing multifunctional graphene out of solution through facile self-assembly to form 2D surface nanostructures, with control over the lateral size and surface properties, would be an intriguing accomplishment, especially in biomedical fields where biointerfaces with functional diversity are in high demand. Guided by this goal, in this work, we built such graphene-based self-assemblies on orthopedic titanium, attempting to selectively regulate bacterial activities and osteoblastic functions, which are both crucial in bone regeneration. Briefly, large-area graphene oxide (GO) sheets and functionalized reduced GO (rGO) micro-/nanosheets were self-assembled spontaneously and controllably onto solid Ti, through an evaporation-assisted electrostatic assembly process and a mussel-inspired one-pot assembly process, respectively. The resultant layers were characterized in terms of topological structure, chemical composition, hydrophilicity, and protein adsorption properties. The antibacterial efficacies of the assemblies were examined by challenging them with pathogenic Staphylococcus aureus (S. aureus) bacteria that produce biofilms, whereby around 50% antiadhesion effects and considerable antibiofilm activities were observed for both layer types but through dissimilar modes of action. Their cytocompatibility and osteogenic potential were also investigated. Interfaced with MC3T3-E1 cells, the functionalized rGO sheets evoked better cell adhesion and growth than GO sheets, whereas the latter elicited higher osteodifferentiation activity throughout a 28-day in vitro culture. In this work, we showed that it is technically possible to construct graphene interface layers of varying lateral dimensions and surface properties and confirmed the concept of using the obtained assemblies to address the two major challenges facing orthopedic clinics. In addition, we determined fundamental implications for understanding the surface–biology relationship of graphene biomaterials, in efforts to better design and more safely use them for future biomedicine.

KEYWORDS: self-assembly, poly(dopamine), graphene nanomaterials, antibacterial, cytotoxicity, osteogenic

1. INTRODUCTION

The successful “scotch-tape” isolation of graphene, a honeycomb-structured monatomic nanosheet of sp²-hybridized carbon,1 has triggered a wave of development for next-generation 2D planar nanomaterials. Over the past decade, intensive research has been rapidly expanding nanoscale graphene materials from fundamental physics to a myriad of widespread applications, including in electronics, energy, water, and biomedicine.1−3 In particular, graphene and graphene derivatives are integrated with biological systems given that they are biocompatible, multifunctional (conductive, antibacterial, osteogenic, able to deliver drugs, etc.), inexpensive, and sustainably available.2,4 Of these materials, graphene oxide (GO), bearing a great deal of functional groups on its carbon plane, exhibits great promise because of its good hydrophilicity, chemical reactivity, and solution processability.5 Herein, graphene-based interfaces with antibacterial and osteogenic biofunctionalities are highlighted. On one hand, to date, intense studies have employed GO solutions (dispersions) for the purposes of bacteria inactivation, giving generally exciting results.6−8 In contrast, pure-graphene-presenting antimicrobial surfaces (not composite coatings, such as GO/hydroxyapatite/Ag) are far fewer,9−12 thus requiring more focus, especially on their antibacterial actions, which can vary from those observed in solution.11 On the other hand, GO has displayed great osteoconductive and osteoinductive abilities for regulating osteoblastic differentiation.13,14 Nevertheless, the nanotoxic danger has raised a lively safety discussion. For example, the rough edges of graphene sheets can slice through and penetrate the membrane and disrupt normal cell functioning.15 It is expected that graphene-on-substrate engineering can restrict graphene sheet mobility (thus avoiding unwanted interactions.
with cells) and tailor the sheet size and the levels of defects and surface functionalization, hence enabling control of the cytotoxicity below an acceptable level. Further, it allows concomitant chemical modification of the graphene to reinforce its physicochemical properties and perhaps add new functionalities such as cell- and bacteria-specific responses.

In orthopedics, Ti-based implants are frequently used for bone repair and replacement; however, their poor osteointegration and vulnerability to implant-centered infections remain two major clinical challenges. Dual-functional Ti surfaces that depress bacterial activities while enhancing osteoblastic functions are therefore in dire need of development. To date, considerable interest has been focused on constructing complicated Ti surfaces with multiple releasable components (normally, combining bactericides with osteogenic factors). However, these strategies could potentially suffer one or more limitations in real applications, such as bacterial resistance, low stability, decreasing efficiency with time, discrete sterilization, high costs, and tedious and intricate preparation and handling. Thus, it is better to find a simple, benign, inexpensive, yet unified biofunctionalization approach that is amenable to practical implementation. An alternative is to transform dispersed graphene in situ into biocompatible, multifunctional surface layers that can selectively influence biological activities of microbes and living cells; in turn, the established surfaces could serve as an alternative platform, other than solution-based, for better elucidating the roles of the available, diverse antibacterial/nanotoxicological mechanisms of graphene, as some of them (e.g., cell wrapping, membrane penetration, and cellular intake) can be excluded if the system dimension (complexity) is reduced from 3D (solution) to 2D (surface). Such an approach can also permit an isolated investigation of the wanted physical or chemical factors (through variable control), as long as the on-surface graphene is exquisitely designed. We believe that the results will be useful for both the theoretical understanding and the practical utilization of graphene in biomedical fields. This concept (also illustrated in Figure 1a), to the best of our knowledge, has not yet been tested. Indeed, it is technically difficult to implement, especially when the size and surface properties as well as cellular/bacterial behaviors of graphene all need to be considered.

To this end, the promising aqueous self-assembly technique is highlighted for its ability to form thin layers on selected targets, with simplicity, universality, and easy thickness control. Based on different assembly principles, it is possible to produce 2D graphene layers of varying lateral dimensions and surface properties that critically dictate their biological behaviors. Herein, two flexible, easy-to-use self-assembly strategies, depicted in Figure 1b, were developed to yield two different biofunctional graphene interfacial layers on metallic Ti: (1) macro-to-microscopic GO bulk sheets, as constructed through electrostatic self-assembly assisted by poly(ethyleneimine) (PEI) and promoted by evaporation, and (2) surface-modified submicroscopic sheets of chemically reduced/functionalyzed GO, assembled using the novel one-pot reactivity and versatility of dopamine (DA), as inspired by marine mussels. Catechol-bearing DA is shown to form substrate-independent, adhesive poly(dopamine) (PDA) layers through mild, pH-triggered oxidative self-polymerization, and PDA is multifunctional: It acts as a secondary platform to react further with various functional groups (quinone, thiol, amine, etc.) through dismutation or Michael addition or Schiff base

Figure 1. (a) Conceptual illustration of constructing multifunctional graphene biointerfaces through straightforward self-assembly for scientific/practical purposes. (b) Routes for the two assembly strategies: (1) evaporation-assisted, colloidal electrostatic assembly and (2) mussel-inspired, one-pot covalent assembly. Note: The graphene sheets extend farther than depicted, and the molecular structure of PDA is simplified.
for over 6 h to form brown colloidal dispersions. Commercially available pure titanium (cpTi) foils with dimensions of Φ 10 mm x 1 mm were polished using SiC paper up to 2000 grit; rinsed sequentially in acetone, alcohol, and deionized water (DI); and then dried at 60 °C before use.

2.2. Evaporation-Assisted Electrostatic Assembly of GO Bulk Sheets. In brief, Ti foils were first immersed into a PEI solution (5 mg/mL, pH 7.4) for 30 min and then withdrawn and rinsed thoroughly to dislodge excess PEI. Thereafter, a GO suspension (pH ~3.0) and PEI-treated Ti disks were coincubated without a cover and left shaking at 37 °C overnight. This induced evaporation-assisted electrostatic assembly and gave the so-called bulk GO.

2.3. One-Pot Assembly of rGO-PDA Micro-Nanosheets. The one-pot surface modification consisted of three components: GO, DA, and metal Ti. Typically, GO suspensions were first mixed with a Tris buffer solution (10 mM) of DA (2 mg/mL) at adjustable DA/GO ratios of 1:1, 2:1, 5:1, and 10:1. A pH value of 8.5 was achieved. Next, Ti foils were introduced, and the resulting mixtures were constantly shaken at 45 °C for 24 h. After that, samples were withdrawn, ultrasonically treated to detach unbound products, and further rinsed and dried under nitrogen. For clarity, samples prepared at DA/GO ratios of x are denoted as rGO-PDA, (x = 1, 2, 5, and 10).

2.4. Surface Characterization. Surface morphology and topology were observed by field-emission scanning electron microscopy (FE-SEM, S4800, Hitachi) and atomic force microscopy (AFM, Dimension ICON, Bruker) in contact mode. X-ray photoelectron spectroscopy (XPS) was performed on an AXIS Ultra spectrometer (Kratos Analytical, Manchester, U.K.) with Al Kα excitation radiation (1486.6 eV). Fourier transform infrared (FTIR, Nicolet, Madison, WI) spectra were collected in transmission mode in the range of 700–4000 cm⁻¹. Micro-Raman spectra were recorded on a confocal Raman microscope (Renishaw 1000) under an excitation laser of Ar⁺ at 514 nm.

2.5. Contact Angle (CA) Measurements. The surface hydrophobicity was determined using the sessile-drop water method, under ambient conditions, on an SL200B Contact Angle System (KINO, Norcross, GA) equipped with a high-resolution camera. Measurements were taken until droplets were well settled on samples and repeated in triplicate, at six different positions per substrate type.

2.6. Protein Adsorption. Bovine serum albumin (BSA) was used as a model protein. Aliquots of BSA solution (1 mg/mL, pH 7.4) were introduced carefully onto different surfaces and incubated at 37 °C for 1 h. Samples were collected and rinsed with phosphate-buffered saline (PBS), and the adsorbed proteins were eluted using 2% sodium dodecyl sulfate (SDS, Sigma) under shaking at 37 °C for 2 h. Subsequently, quantification was chieved with a Micro BCA Protein Assay Reagent Kit (Thermo Scientific) by measuring absorbance at 570 nm on a microplate reader (Bio-RAD, Hercules, CA). Alternatively, specimens adsorbed with fluorescein isothiocyanate- (FITC-) conjugated BSA under identical conditions were fixed in 4% paraformaldehyde (PFA, in PBS) for 15 min and then subjected to fluorescence imaging using confocal scanning laser microscopy (CLSM, Nikon ALR-SI) at an excitation wavelength of 488 nm.

2.7. Antimicrobial Activity Assays. 2.7.1. Bacteria Culture and Inoculation. S. aureus was used and incubated aseptically in Luria–Bertani (LB) broth at 37 °C. The overnight-grown bacteria (adjusted to 10⁶–10⁷ cells/mL) were seeded onto each surface (sterilized by autoclaving) and then incubated for predetermined time periods. All experiments and measurements were carried out in triplicate.

2.7.2. Microbial Viability Assay. To evaluate the performance of various surfaces in suppressing bacterial adherence, a convenient WST-8-based microbial viability assay (Dojindo, Kumamoto, Japan) was carried out according to the manufacturer’s instructions. Results were obtained by colorimetrically measuring the formazon dye released during microbial metabolism in the presence of WST-8 and an electron mediator for each sample, controls with materials only were set to exclude any background interference.

2.7.3. Characterization of Adherent Bacteria by SEM. For SEM observations, adhered bacterial cells were fixed with 2.5% (v/v) glutaraldehyde (GA) and dehydrated in serial ethanol (30–100%). After being dried in air, the constructs were sputtered with thin gold layers prior to analysis.

2.7.4. Live/Dead Staining and CLSM Investigation. The samples were first rinsed with 0.85% physiological saline twice, and a LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Invitrogen) was used to stain the specimens, as previously described. Image stacks were recorded successively by CLSM, and then 3D visualizations were reconstructed with aid of a NIS-Elements AR package, version 3.0.

2.7.5. Biofilm Formation Assay by Crystal Violet Staining. After samples had been cultured for 5 days, 400 μL of PBS was added twice to gently remove the loose bacteria. The biofilms on the back sides of the samples were cleaned carefully with a swab immersed in 70% (v/v) EtOH. To visualize biofilm development, the samples were fixed with 4% PFA, stained with 0.1% (w/v) crystal violet for 15 min, and washed three times with PBS to remove excess stains. After pictures were taken, the dyes were eluted with 95% (v/v) EtOH, and absorbance at 570 nm was determined.

2.7.6. Production of Reactive Oxygen Species (ROS). The formation of OS was measured using a sensitive 2′,7′-dichloro fluorescein diacetate (DCFH-DA) fluorescent stain method, in accordance with the manufacturer’s protocol (Jiangshen Biotech, Nanjing, China). Briefly, dilute DCFH-DA (nonfluorescent) was incubated at 37 °C with bacteria (on specimens that had been precultured for 24 h) for 2 h, and it could be transformed into 2′,7′-dichlorofluorescin (DCF, fluorescent) in the presence of ROS. CLSM fluorescence images were collected at 488 (Ex) and 535 (Em) nm in triplicate, and for each sample, five random data points were selected. ImageJ freeware was used for data analysis.

2.7.7. Bacterial Impacts on Materials. Ti foils with graphene assemblies were cultivated with S. aureus for 48 h. Thereafter, samples were collected, rinsed, and ultrasonically vibrated to dissociate adhered bacteria. The resulting surfaces were dried and characterized by SEM.

2.8. Cytocompatibility and Osteogenic Activity Assays. 2.8.1. Cell Culture and Seeding. The bone-forming MC3T3-E1 cells were cultivated in αMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen-strep) in a humidified incubator with 5% CO₂ at 37 °C. Subconfluent cells were harvested and seeded on autoclaved samples at a density of 5 x 10⁵ cells/mL in 48-well tissue culture plates (TCP5; negative control also). The medium was refreshed every 2–3 days.

2.8.2. Cell Adhesion. Cells were allowed to attach to surfaces for 4 and 8 h postseeding. To visualize cell attachment, polychrome immunofluorescence staining was performed in sequence using specific stains of focal adhesions, tubulin cytoskeleton, and nuclei, namely, Anti-FAK antibody kit (1:100, Abcam), Tubulin-Tracker Red Probe (1:250, Beyotime), and 4′,6-diamidino-2-phenylindole (DAPI, 1:1000; Sigma), respectively, in accordance with the manufacturers’ protocols. Prior to staining, samples were fixed with 4% PFA for 15 min and then subjected to 5 min of permeabilization using 0.1% Triton X-100 and 1 h of blocking in 1% BSA. For each of three parallel samples, images were recorded over five areas by CLSM in multichannel mode, and afterward, quantification was performed using ImageJ software.

2.8.3. Cell Proliferation and Morphology. Cell proliferation was quantified using Cell Counting Kits (CCK-8, Dojindo), as detailed elsewhere, based on the measurement of mitochondrial activity. In addition, the cell morphology was determined by CLSM study of the stained actin cytoskeleton. For staining, the samples were rinsed three times with PBS and fixed in 4% PFA. Fixed cells were permeabilized and counterstained with FITC-phalloidin (1:200, 40 min; Sigma) and DAPI (1:1000, 5 min).

2.8.4. Cell Apoptosis. To determine the degree of cell apoptosis, the extracellular release of lactate dehydrogenase (LDH) was measured at 3 days with an LDH kit (Abcam, Cambridge, MA), following the manufacturer’s protocol. LDH release at the single-cell level (i.e., cellular LDH activity) was determined by normalizing the total LDH activity to the number of cells.

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2.8.6. Intracellular ROS Levels. The levels of intracellular ROS for MC3T3-E1 cells were assayed using a DCFH-DA-based fluorescence test similar to that described in section 2.7.6 for bacteria.

2.8.7. Cellular Live/Dead Staining. At 7 days, the survival abilities of cells on different samples were assessed by staining living cells with 2 μM Calcein AM and the dead cells with 4 μM PI (Live/Dead Cell Stains, Dojindo, Kumamoto, Japan), after which confocal fluorescence images were recorded.

2.8.8. Alkaline Phosphatase (ALP) Activity. The ALP activity was assayed by measuring the transformation of substrate p-nitrophenylphosphate (pNPP; Jiancheng Biotech, Nanjing, China) into p-nitrophenol (pNP) after culture for 7 days. In short, cells were washed with PBS and lysed with 1% Triton X-100 for 40 min, and then the cell lysis was mixed to react with buffered pNPP solution at 37°C. Absorbance at 520 nm was read after colorimetric reaction. The ALP activity was normalized against total cellular proteins, as determined by BCA reaction (see section 2.6), and expressed in U per gram of protein.

2.8.9. Extracellular Matrix (ECM) Collagen and Calcium Assays. Histochemical dyes Sirius Red (SR, 0.1%; Sigma) and Alizarin Red S (ARS, 2%; Sigma), which bind specifically to ECM collagen and calcium salts, were employed following a 21-day and 28-day cultures, respectively. For staining, cells were fixed in 4% PFA and rinsed with PBS, and 500 μL of SR or ARS dye solution was added and incubated for 18 h or 15 min, respectively; after thorough washing, specimens were dried and photographed. Quantitatively, dyes were extracted using 50% 0.2 M NaOH/methanol (for SR) and 10% cetylpyridinium chloride (for ARS), and the samples were then read on a microplate at 570 or 562 nm.

2.9. Statistical Analysis. All data in this paper were analyzed using SPSS 19.0 software. Statistical significance was determined using one-way analysis of variance (ANOVA) or Student’s t test and defined as a p value of less than 0.05. Data values are expressed as mean ± standard deviation.

3. RESULTS AND DISCUSSION

3.1. Material Preparation and Characterization. In this study, we established two graphene-based layer types with varying sizes and chemical states in situ on the surface of solid Ti. First, GO bulk sheets were constructed readily by evaporation-assisted electrostatic assembly (Figure 1b; route 1). Actually, this strategy was motivated by combining the existing electrostatic assembly and evaporation-induced interfacial assembly of GO. The aqueous GO sheets were moderately charged (negative, because of ionized carboxyl groups), but they were metastable and tended to precipitate onto Ti foils that were precharged with PEI, a well-known cationic polyelectrolyte that is rich in amino groups. As a result, some GO sheets bound to the PEI-modified Ti, initiating the formation of a GO prelayer with incomplete coverage. Subsequently, the slowly evaporation of EtOH/water induced an intrinsically self-concentrating process of additional GO sheets at the liquid/prelayer interface, eventually leading to interlinked macroscopic GO films on the substrate. The major chemical interactions can include electrostatic interactions between PEI and GO and π−π stacking and hydrogen-bonding interactions among different GO sheets.

As an alternative, chemically modified rGO sheets were prepared according to the principle of catecholamine-assisted, facile, spontaneous coassembly (Figure 1b; route 2). To initiate this process, mixed solutions of DA and GO were...
placed in one pot with Ti foils at an alkaline pH of 8.5 and a temperature of 45 °C. Note, here, that the DA chemically modified and reduced GO into rGO and, in turn, the resultant rGO acted as a template for DA to autooxidize and self-polymerize, during which the brown color of the mixture darkened over time. In detail, the reaction might have proceeded as a three-step process (Figure S1a): (1) chemisorption (π−π stacking, aryl−aryl coupling) or covalent grafting of DA molecules onto GO and conversion of GO into rGO (partly reduced) by their catechol groups; (2) the pH-induced, graphene-templated formation of PDA precursors and cross-linking of adjacent graphene sheets; (3) further growth and aggregation of PDA clusters. Simultaneously, the settling and immobilization of PDA-functionalized rGO (rGO-PDA) onto Ti metal took place, probably in three steps as well (Figure S1b): First, PDA nanolayers formed easily on the Ti because of the strong bidentate coordination of DA toward TiO2 species, which are rich on passivated Ti. Second, the rGO-PDA sheets could be readily integrated with the PDA-modified Ti, possibly through covalent cross-links (mediated by free DA molecules) and aryl−aryl coupling. Additionally, at an earlier stage, the PDA-precursor-modified graphene sheets might also associate with the already-modified Ti through π−π stacking and covalent linkages. Third, as PDA grew further and new sheets were deposited, stronger interactions were likely established. Nonetheless, the exact reactions might be far more complex, so future work on this issue is needed.

Predictably, the DA/GO ratio is a critical factor needing consideration to obtain ideal layers. To this end, we prepared rGO-PDAx samples (x = 1, 2, 5, 10) and subjected them, together with bulk GO, to Raman spectroscopy, a powerful analytical tool for probing the crystal structure, disorder, and lattice defects of carbonaceous nanomaterials. The results are presented in Figure 2a. For GO materials, the Raman spectrum features the broadening of two associated bands: the G band at ca. 1580 cm−1 and the D band centered at ca. 1350 cm−1. Whereas the G peak is general in all sp2 graphitized structures, the D-mode peak is exclusively assigned to the defect level and crystallinity. Obviously, after modification, the intensity of the D peaks (relative to the G peaks) decreased markedly, indicating that the carbon defects were restored and that rGO was obtained. In addition, bulk GO showed the strongest G band, sequentially followed by rGO-PDA2, rGO-PDA5, and rGO-PDA10 (almost lost G bands), indicating that the DA/
GO ratio should be neither too large nor small. Figuratively, this resembles the process of building with bricks and glues: If the ratio is large, limited GO (“brick”) is available to be reduced and immobilized, whereas if the ratio is small, less PDA (“glue”) is available to stick rGO to Ti. In particular, at a ratio of 1:1, GO and DA reacted thoroughly to form stable 3D “hydrogels” other than to decorate the substrate (Figure S2). Similar phenomena were noted before for DA-GO two-component systems.33 Given these facts, we suggest a feasible DA/GO ratio of 2, which was fixed in all subsequent experiments (unless otherwise stated, rGO-PDA now refers to samples at x = 2). This ratio can allow both sufficient reduction of GO and effective fixation of rGO onto titanium. The resultant layers are expected to manifest strong interfacial bonding through the occurrence of PDA–rGO (e.g., covalent cross-linking, 34 π–π stacking36) and TiO2–PDA (covalent bonds35) interactions (Figure S1). Further, they can impart specific biological properties to the modified rGO, as detailed later, through the use of the biological adhesive “mussel power.”

Despite the flourishing development of graphene, the simple, economical, and scalable size fractionation of its sheets has remained challenging thus far.34 The sonication of GO is common in research works. However, the precursors obtained are inevitably split into pieces with wide size distributions. Herein, the lateral dimensions of graphene were easily refined through the PDA-mediated one-step aqueous selection. Notably, neither centrifugation nor filtration was performed to decrease these sizes.34,35 Instead, PDA reacted with GO and segregated the products naturally into two portions: The part with macro-/microscopic dimensions formed precipitates spontaneously, whereas the submicrometer part preferentially assembled onto the substrate. For the underlying kinetics, we postulated the “nano-effects” of submicrometer sheets might have provided them with energetically favorable affinity (reactivity) toward bulk objects. As evidence for this possibility, a fraction of these sheets (asterisks, Figure S3) were observed to bind identically to the surface of precipitates, essentially another type of “substrate.”

An overall understanding of the surface characteristics of biomaterials is crucial given that osteoblastic cells, as well as bacteria, respond to characteristics such as surface chemistry, topography, and hydrophilicity, which ultimately affects the success of the implants. The surface physicochemical properties of two assemblies were investigated synchronously. The chemical characteristics were revealed by means of FTIR spectroscopy and XPS. In the FTIR spectra of GO (Figure 2b), the peaks at 1046, 1216, 1592, and 1728 cm−1 correspond to the sp2 stretching vibrations of C−O, C−OH, C=C/C=C, and C≡O groups, respectively.17,35 For rGO-PDA0, the oxygen-associated peaks weakened or disappeared, indicating a significant degree of restoration within the graphene framework or at the edges. A peak emerged at 1250 cm−1, assigned to the phenolic C−OH vibrations of PDA. Nevertheless, amino signals were absent, implying that the polymer layer was thin. For the control rGO-PDA10 (with thicker PDA), an additional peak at 1516 cm−1 ascribed to N−H vibrations was noted.37 The degree of reduction was revealed by an XPS study of the C 1s core-level band, which can be generally fitted to three components, corresponding to carbon atoms in several chemical environments:34,35 in-plane sp2 carbon (C−C/C=C) at 284.8 eV, C−O-bound carbon at 286.9 eV, and carbonyl carbon (C=O) at 288.5 eV. For bulk GO (Figure 2c), the C−O and C≡O contents were 42.0% and 8.1%, respectively, signifying an appreciable degree of oxidation. For rGO-PDA (Figure 2d), the fractions of C−O and C≡O were only 17.6% and 4.0%, respectively.

The surface microstructure and topology were examined by SEM and AFM. As depicted in Figure 3a,b, bulk GO sheets were interconnected in continuous large pieces, similarly to films, and were characterized by a myriad of ridged protrusions, known as asperities. In addition, the ridges were micro- to macroscale (~1−100 μm; Figure 3e). No apparent edge or corner was observed. The AFM measurements in Figure 4a−c suggest a ridge width of several micrometers, a height of dozens of nanometers, and an average roughness (Ra) of 28.4 nm. By comparison, the mussel-inspired assembly approach gave rise to a very different graphene counterpart in rGO-PDA. The interface was constituted by sheet aggregates rather than films (Figure 3c,d). These micronanosheets ranged from <100 nm to 1 μm (Figure 3f), and a few of them overlapped. Moreover, intimate sheet–substrate contact and a capping layer were noticeable upon closer examination (inset, Figure 3c). Unlike bulk GO, the rGO-PDA was relatively smooth, having a
roughness of 4.6 nm according to AFM (Figure 4d–f). Of note, the rGO-PDA exhibited many irregular jagged edges, with nanoneedles protruding upward at the edge margins. These needles were atomically sharp, likely generated by long hours of fierce sonication that conferred an extremely high free energy to the edges and made them curl spontaneously at the ends.

3.2. Wettability Properties. Additionally, the sensitivity of contact angle (CA) measurements was utilized to assess the wettability of the resultant surfaces (Figure 5a). High CA values describe hydrophobicity, and low angles indicate hydrophilicity. Initially, the titanium was relatively hydrophobic (60.4°). DA-GO codeposition improved the surface wetting by 14.2°, probably because of the hydrophilic nature of PDA. The GO coverage dramatically decreased the water CAs by 20°, as a result of the presence of hydrophilic carboxylic ends on GO. Taken together, the data in sections 3.1 and 3.2 indicate the successful interfacial assembly of graphene with varying structural and compositional properties.

3.3. Protein Adsorption. The adsorption of BSA on different biomaterial surfaces was examined because the attachment of cells is, to some extent, governed by the interactions of membrane integrin with preadsorbed serum proteins. To our delight, both bulk GO and rGO-PDA preferentially facilitated the absorption of BSA compared to cpTi, as evidenced by BCA measurements and fluorescence visualization (Figure 5b). Factors that strongly influence protein adsorption briefly include surface chemistry, wettability, and topography (roughness). Here, both assemblies were more hydrophilic than cpTi, thus favoring the retention of the protein. Moreover, bulk GO had a rougher surface and perhaps a greater capacity for holding protein; it can also adsorb proteins through hydrophobic interactions, electrostatic forces, and hydrogen bonding. Regarding rGO-PDA, PDA is potent for covalently grafting BSA through o-benzoquinone–amine coupling.

3.4. Antimicrobial Effects of the Assemblies. 3.4.1. Anti-Adhesion and Anti-Biofilm Activities. Biofilms, sessile communities of microbial cells, clinically colonize on orthopedic implants and lead to the outbreak of catastrophic infections, such as osteomyelitis. The buildup involves several steps, starting with bacterial attachment on the surface, subsequent cell aggregation and accumulation into microcolonies, followed by biofilm maturation, and ending with the detachment of cells from the biofilms into planktonic states to initiate a new cycle of biofilm formation elsewhere. Once biofilms are established, they encase and defend bacteria from host immune responses or antibiotic attacks. It is generally accepted that inhibiting microbial anchoring is better than treating already-colonized biofilms. Hence, the impacts of bulk GO and rGO-PDA layers on the antiadhesion and antibiofilm abilities of Ti disks were assessed against S. aureus, a frequent pathogenic strain that is reported to be able to infect 95% of subcutaneous implants with only a 10²-CFU inoculum in vivo.

The antiadhesion ability of the obtained surfaces was investigated after 24 h in culture, by measuring the mitochondrial activity of surface-retained bacteria. As shown in Figure 6d, both forms of graphene assemblies impressively reduced the adherent bacterial cells relative to cpTi, by 45.0%
for rGO-PDA and by 55.1% for bulk GO. In addition, SEM was performed to obtain evidence of membrane disfigurations (Figure 6a). In this case, bacteria adhered to and proliferated readily on cpTi and formed aggregates of cells whose walls remained intact. Upon encountering rGO-PDA, however, the bacterial membrane typically became deformed. In particular, bulk GO resulted in generally wrinkled and damaged morphologies.

To visualize the viable state of the bacteria, in situ live/dead fluorescence imaging was performed, as depicted in Figure 6b. Many bacterial colonies were easily observed on cpTi, which were active and ready to form 3D mature biofilms. In contrast, much fewer microbes were anchored on the rGO-PDA. In addition, the majority of cells were individuals, and some were even killed (red fluorescence). Bulk GO retained many fewer bacteria than cpTi but slightly more than rGO-PDA. Nevertheless, the bacterial death was greatest on bulk GO.

The morphologies and structures of graphene materials are responsible, more or less, for their antibacterial abilities. However, they can be changed by the physiological milieu and/or by interactions with bacteria. Hence, the structural stability of the materials was investigated preliminarily in vitro. As shown in Figure 56, exposure of the samples to bacterial suspensions for relatively long times did not result in obvious de-adhesion of layers, nor did it signify in Figure 6c, large-area, dense biofilm patches were formed macroscopically on cpTi. In sharp contrast, all surfaces treated with graphene remained relatively clear, with only a few small, disperse zones of violet stains. Quantitatively, the S. aureus biofilm content was reduced by 30.5% and 40.7% by rGO-PDA and bulk GO, respectively (Figure 6e). Taken together, the results indicate that Ti implants with graphene self-assemblies alone, in the absence of any other bactericides, were potent for suppressing initial bacteria anchoring and delaying subsequent biofilm formation. In clinics, this could greatly enhance the chance for the remaining bacteria to be eradicated by host immunity or that for early infections, if any, to be diagnosed and treated in a timely manner, thereby avoiding serious infections.

3.4.2. Antibacterial Mechanisms. Graphene is a well-known antimicrobial agent that is able to inactivate or kill microbes by interacting with membranal and intracellular components (lipids, proteins, DNA/RNA, etc.) through π−π stacking, hydrogen bonds, and electrostatic adsorption. These physicochemical interactions have derived a group of antibacterial mechanisms (Figure 7a), briefly including nanoknives/nanoneedles through the physical action of sharp edges/corners, oxidative stress through ROS production or charge transfer, membrane wrapping/trapping originating from the flexibility of large graphene sheets, extraction of membrane lipids derived from overwhelming hydrophobic attraction between sp² carbons and lipid molecules (“nanoscale dewetting”), and spontaneous insertion that penetrates lipid bilayers. More generally, these actions fall into two categories: membrane damage and oxidative stress (Figure 7b). The former is more or less associated with physical or mechanical modes of interaction with the cell membrane, whereas the latter is typically accompanied by a series of metabolic events. However, most of the described actions rely heavily on dispersed graphene sheets (graphene solutions were used) that are able to assume dynamic exposure, fully penetrate the membrane, and interact actively with components inside bacteria. By contrast, the antimicrobial behaviors of on-surface graphene have rarely been studied (see the summary in Table 1). In these cases, three major mechanisms seem applicable, namely, nanoknives/nanoneedles, ROS production, and charge transfer. Using these mechanisms, we attempt to establish putative mechanistic links for the graphene interfaces in this work.

Principally, early research endeavors have ascribed nanographene-induced bacterial death to “nanoknives” effects, which highlight the role of nanosheets’ sharp edges that act like cutters to laterally incise bacterial membranes, causing
Intracellular substances to leak and, consequently, cells to die. However, micro-/nanosheets such as rGO-PDA are assembled quasiparallel rather than perpendicular to the substrate, making a near-orthogonal cut impossible. Recently, a study by Pham et al. expanded the “cutting” theory, by pointing out that graphene sheet surfaces, actually, induce the formation of pores within the bacterial membrane instead of simply cutting through it, which altered the osmotic pressure and then induced membrane collapse (here, called the nanoneedle effect). From a similar viewpoint, we speculate that the spherical S. aureus, once settled on the jagged rGO-PDA sheets (having defected, atomically sharpened edges, with needlelike nanostructures; Figure 4f), can encounter a constant, ultrastrong flow of mechanical pressure, imposed by the peripheral nanoneedles of various sheets, that is able to pierce the membrane to form localized nanopores. These local, multipoint pores, if sufficient, can presumably initiate “crack” propagation and trigger the efflux of cytoplasmic components such as proteins and DNA (Figure 7a). The needles could further retard bacteria from growing on them, thus delaying the onset of biofilms. Note also that the described effect is likely random and cumulative (not instantaneous) and should, therefore, be more inhibitory than lethal (Figure 6b).

Comparatively, the scenario for bulk GO sheets, large in area and entirely immobilized, interacting with bacteria is rather different; it would be difficult for such sheets to trap or wrap the bacteria as dispersed large films do, and edge effects similar to those of nanosheets would also not be possible. Indeed, something like a ridge, if tiny and sharp enough, could also impose mechanical pressure on bacteria. However, for GO sheets prepared by our method, the ridges’ lateral dimensions spanned across orders of magnitude, from ~1 to 100 μm, a scale much greater than that of S. aureus cells (typically, 500 nm–1 μm). Considering the large ridge width (mostly several micrometers) and rough surface landscape, they would better be described as blunt than sharp. Therefore, the chance that the bacteria perceive the mechanical feature and are further incised or punctured is fairly small, excluding so-called “physical damage” as a major bactericidal route here (if any, it could account for a small part). Given its superior bacteria-killing capacity (Figure 6b), other modes of action should exist for bulk GO. The first possibility is an oxidative mechanism involving ROS overproduction. Excess ROS is known to oxidize fatty acids and yield lipid peroxides that affect respiratory chain reactions, thereby disrupting membrane integrity and resulting in the loss of viability. ROS-mediated oxidative stress was corroborated through use of the DCFH-DA fluorescence probe (Figure S4). The stronger the fluorescence, the larger the amount of ROS. Clearly, interfering bacteria with both graphene assemblies, particularly with bulk GO, triggered elevated ROS levels. Another rationalization is the charge-transfer mechanism that interrupts the membrane respiratory chain and also gives rise to oxidative stress. The respiratory chain of bacteria is known to rely on electron transport to produce energy for bacterial survival and maintenance. In principle, for a bacteria/graphene/metal system, a circuit can be established through the "membrane@graphene@TiO₂" junctions that allows for electrons to be transferred from the microbial membrane to the graphene sheet and then to the underlying metal (Figure 7a).

### Table 1. Construction of Graphene-Based Antibacterial Interfaces and Their Toxicity Mechanisms

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Graphene (GO, rGO)</th>
<th>Major antibacterial findings</th>
<th>Antibacterial mechanism</th>
<th>Charge transfer by direct contact</th>
<th>Oxidative stress mediated by ROS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stainless steel</td>
<td>Large-area graphene</td>
<td>Membrane damage by sharp edges, charge transfer</td>
<td>Cut through membrane, inducing ROS and oxidative stress</td>
<td>Strong bacteria-killing, zone-dependent membrane damage</td>
<td>Oxidative stress and membrane collapse</td>
</tr>
<tr>
<td>Cu, Sn, Ti, CuO</td>
<td>Large-area graphene</td>
<td>Membrane damage by sharp edges, charge transfer</td>
<td>Cut through membrane, inducing ROS and oxidative stress</td>
<td>Strong bacteria-killing, zone-dependent membrane damage</td>
<td>Oxidative stress and membrane collapse</td>
</tr>
<tr>
<td>Poly(ethylene terephthalate)</td>
<td>Large-area graphene</td>
<td>Membrane damage by sharp edges, charge transfer</td>
<td>Cut through membrane, inducing ROS and oxidative stress</td>
<td>Strong bacteria-killing, zone-dependent membrane damage</td>
<td>Oxidative stress and membrane collapse</td>
</tr>
<tr>
<td>Polyethylene (PET)</td>
<td>Large-area graphene</td>
<td>Membrane damage by sharp edges, charge transfer</td>
<td>Cut through membrane, inducing ROS and oxidative stress</td>
<td>Strong bacteria-killing, zone-dependent membrane damage</td>
<td>Oxidative stress and membrane collapse</td>
</tr>
</tbody>
</table>

Note: The described effects are likely random and cumulative (not instantaneous) and should, therefore, be more inhibitory than lethal (Figure 6b).
membrane integrity, and eventual microbial death. Actually, this action can be extrapolated from an interesting, zone-dependent antibacterial phenomenon (Figure S5): the degree of membrane distortion for bacteria in planar zones was much greater than that near the ridges, which was validated by repetitive experiments and analysis. The reasoning is as follows: The graphene–substrate contact (prerequisite for a circuit) for the planar zones should be better than for the ridged surfaces where metal–film gaps are possible; as a result, the former should have easier, faster electron transfer and, thus, impose more severe membrane disruption.

Therefore, we suggest that membrane stress by nanoneedles and oxidative stress through charge transfer or ROS were possibly dominant mechanisms enabling the antibacterial activities of rGO-PDA and bulk GO, respectively. Nevertheless, for graphene-related antimicrobials, the destructive effects can be multiple. For example, Liu et al. systematically investigated the bacterial toxicity of four graphene types (graphite, graphite oxide, GO, and rGO) and found that both membrane and oxidation stress made contributions. In another study, rGO and GO nanowalls were used to kill S. aureus, and the former was revealed to be more toxic, due to both better charge transfer and more sharpened edges. In this regard, it is more reasonable that the aforementioned mechanisms are shared by both types of graphene assemblies, but in varying degrees, as schematically represented in Figure 7c. Further, we deduce that oxidative stress is a more active toxic mechanism for graphene materials, at least for bulk graphene at the interface. Because these antimicrobial activities exhibit excellent biological persistence, they can inactivate bacterial replication and retard biofilm formation in the long run. In addition, as-treated devices should have long shelf lives, and their efficacy is unlikely to be impaired by routine sterilization (here, all specimens were autoclaved in a standard manner, yet with good antibacterial effects), which is common in clinical practice. Further, because of the robust mechanical nature of graphene18 and the aforementioned substrate–sheet interactions (section 3.1), relatively good structural/chemical stability of the material in physiological milieu is possible (Figure S6). Therefore, on-the-surface graphene might offer a viable alternative to antibiotic- or silver-releasing surfaces whose effects deplete over time or are comprised by harsh sterilization.

A major aspect distinguishing graphene-based interfaces from graphene solutions is that, in addition to an intrinsic bacteria-killing ability (discussed above), their antibacterial activity can also stem from the surface properties that repel bacterial adhesion, alter the adhesion phase, and restrict cell growth (Figure 7d). For example, surface wettability and topography (roughness) are critical considerations in controlling microorganism retention. For wetting, principally bacteria with hydrophobic natures prefer hydrophobic surfaces, and vice versa. S. aureus cells are hydrophobic (CA ≈ 72°47), so exposing them to hydrophilic surfaces decreases the amounts adhered (Figure 6d). Concerning topological configuration, generally, a lower roughness mitigates fouling, as it provides fewer anchor sites for germ retention and lowers the membrane shear forces. Notably, for rGO-PDA, the needlelike nanotopography at the edge margins can impart, aside from pore-forming death, additional fungal-repelling capabilities to the surfaces. On one hand, we believe that bacteria tended to circumvent the harmful needles by instinct, so most of them were likely to be held planktonic. On the other, a nanofeatured topography can play an integral role in reducing initial bacterial attachment. For example, Liu et al. confirmed that nanoscale roughness (≈14 nm) alone hampered the surface adhesion and growth of both Escherichia coli (E. coli) and S. aureus for up to 2 days. However, the roughness should not be too large; otherwise, more bacteria can be retained, as is the case for bulk GO (Figure 6b). Additionally, the effects of other surface properties such as surface chemistry and electrostatic charges have also been noted. The functional groups in the PDA matrix include quinone, carboxy, amino, imine, and phenol groups. In particular, the amine groups are anticipated to endow the substrate with positive charges that disrupt membrane charges (from −20 to −200 mV) and to react with the functional groups (e.g., thiols) from bacteria membrane components (peptidoglycan, porins, lipopolysaccharides, phospholipid, etc.). This can lead to altered metabolic activity and cellular integrity, thereby influencing bacterial adherence and even survival. These surface-specific effects should be viewed as a whole, together with the intrinsic killing ability of graphene itself, to rationalize the observed variations. Although a better elaboration of the role of each part is still needed, we can roughly conclude that the intrinsic killing ability and surface physicochemical properties were the key contributors for bulk GO and rGO-PDA, respectively, to be antibacterial and to resist biofilm formation (also included in Table 1).

3.5. Cytocompatibility of the Assemblies. The abilities of cells to anchor to a substrate and to proliferate and further express destined functions critically dictate the long-term success of an implant and, in an equal sense, the fate of the regenerating tissue. In particular, in orthopedics, these interfacial responses are associated with biomaterials’ surface properties,7 and they are often examined by using bone-forming cells (e.g., MC3T3-E1) that synthesize extracellular matrix (ECM, primarily collagen) and control its mineralization. This ex vivo model makes it possible to gain a quick impression of whether a suggested surface modification is efficacious in improving implant biocompatibility and encouraging its integration with bone tissue.

First, the initial cell–material interactions were investigated, including (1) substrate attachment, (2) cell spreading, and (3) cytoskeleton development. Here, the cell attachment hinged on both surface conditions and cultivation time (Figure 8a,c). After 4 h of culture, the number of osteoblasts (nuclei, blue) on rGO-PDA was clearly the greatest, and their focal adhesions (white arrows) were notable. In addition, a well-organized cytoskeleton was visualized by red tubulin tracker, indicating fairly good, dynamic stretching. Bulk GO also evoked higher cell affinity and better morphology than cpTi, but they were inferior to those of rGO-PDA. This result is attributable to the unique role of PDA in mediating strong biological anchorage and superb stretching during cell adhesion.33,45,46 PDA not only helped adsorb more serum proteins (Figure 5b), it might also have provided them with better conformations, thus establishing a favorable matrix for rapid cell recruitment, stable adherence, and effective cytoskeleton construction. As the time was increased to 8 h, the remaining planktonic cells continued to adhere to each surface. Still, the rGO-PDA groups had the most cells, richest tubulin networks, and best spreading. Moreover, the F-actin development was evaluated after 1 and 3 days (Figure 8b). After 1 day, compared to those on cpTi, the osteoblasts on rGO-PDA exhibited a higher degree of cell extension and greater stress fiber formation; for bulk GO, however, some cells were better spread, but some were worse.
Graphene microsheets (multilayered, 0.5 μm) exhibit extremely large bilayers of three cell types by Brownian motion and lipid wrapping/encapsulation (e.g., rGO-PDA) or for the wrapping of host cells by large membranes (e.g., bulk GO) should be critically low. For rGO-PDA, its better cytocompatibility was attributed to the surface modification, as depicted in Figure 9d.) For biomaterials physically interacting with cell membranes, toxicity cascades will likely be triggered as first the disruption of the membrane integrity, then the leakage of intracellular enzymes (e.g., LDH), and finally apoptosis or even cell death. For simplicity, LDH was chosen as an indicator for evidence of these damages, given that it leaks immediately into the surrounding medium once membrane-associated apoptosis occurs and that it is stable in the extracellular environment for a long time. As presented in Figure 9c, bulk GO did induce the strongest production of LDH, indicative of serious membrane damage, whereas rGO-PDA had a mild impact on membrane structure. The trend is just the reverse of that observed for bacteria (section 3.4), yet it can also be interpreted in terms of the differences in size among rGO-PDA, bulk GO, and cells (bacteria). The simple fact is that osteoblasts (10−50 μm) are much larger than S. aureus (<1 μm) and rGO-PDA sheets (<100 nm to 1 μm) but are likely to be comparable to some bulk GO sheets (~1−100 μm). Therefore, the physical cues from bulk GO (e.g., roughness and topology) can be effectively perceived by cells, disrupting the membrane integrity and leading to leakage of intracellular materials (Figure 9d).

On the other hand, in light of the oxidative nature of graphene, the toxicity might also be mediated by oxidative stress through lipid peroxidation, for example. In particular, ROS-induced oxidative stress has been cited as a major toxicity paradigm of carbon nanomaterials such as graphene, which causes mitochondrial injury and cell apoptosis. For simplicity, the authors chose LDH as an indicator for evidence of these damages, and it leaks immediately into the surrounding medium once membrane-associated apoptosis occurs. The leakage of intracellular LDH, indicative of serious membrane damage, is shown in Figure 9c, whereas rGO-PDA had a mild impact on membrane structure. The trend is just the reverse of that observed for bacteria (section 3.4), yet it can also be interpreted in terms of the differences in size among rGO-PDA, bulk GO, and cells (bacteria). The simple fact is that osteoblasts (10−50 μm) are much larger than S. aureus (<1 μm) and rGO-PDA sheets (<100 nm to 1 μm) but are likely to be comparable to some bulk GO sheets (~1−100 μm). Therefore, the physical cues from bulk GO (e.g., roughness and topology) can be effectively perceived by cells, disrupting the membrane integrity and leading to leakage of intracellular materials (Figure 9d).

Figure 9. Cell–material interactions: (a,b) Intracellular production of ROS (*p < 0.05). (c) Level of LDH enzyme leakage (*p < 0.05). (d) Schematic of bulk GO inducing membrane and oxidative stress. (e) Cellular live (green)/dead (red) staining at day 7, displaying negligible cytotoxicity for all surfaces.
mitochondrial activity provided by bulk GO, as seen in Figure 8d.

Nevertheless, these negative impacts became less significant as cultivation went on, probably attributed to the fact that older cells began to get adapted while newer cells contacted less with substrate (e.g., through overlapping growth). Furthermore, we verified this by cellular live/dead staining at day 7. As shown in Figure 9e, around 100% confluence was reached for all groups; cell survival was basically satisfactory with few dead cells (red fluorescence). It should be admitted that GO materials had cell-assisting aspects. For example, their polar functional groups (—OH, —COOH) were revealed to interact with the polar components of cells and the culture medium.37 Also, the large surface area served as active sites available for cells to spread and grow.53 Therefore, once the negative effects weakened in late stage, improved cell compatibility and probably stronger osteodifferentiation instead of cytotoxicity shall be envisioned. Similarly, such staged cell behaviors were observed with nanosilver coatings.29 These suggest the term “biocompatibility” needs revision, or at least, more dialectical interpretation when adopted for risk assessment when developing novel nanobiomaterials.

3.6. Osteogenic Differentiation Properties. The level of ALP activity, widely used as an early hallmark of osteoblast differentiation, was examined at day 7. As shown in Figure 10a-4, the ALP expression for rGO-PDA and, especially, for bulk GO was notably improved in comparison to that for cpTi. The results were further confirmed by ALP staining (Figure 10a-1–a-3). The modified substrates elicited much darker blue stains (red arrows). In the long term, the differentiated osteoblasts will secrete collagen and assemble collagen fibers extracellularly, which later become mineralized, and ultimately, a highly complex and hierarchical architecture of bone matrix will be organized.47 To this end, matrix collagen secretion by MC3T3-E1 at day 21 was measured by SR staining and quantified (Figure 10b,d). Compared to cpTi, higher collagen content and denser patterns were observed for titanium decorated with graphene assemblies, especially for bulk GO. Furthermore, the ARS Ca²⁺ content at day 28 was measured, which corroborated the collagen assessment (Figure 10c). Notably, larger calcium nodules (arrows, Figure 10d) were deposited on the modified surfaces. During substrate-controlled biomineralization, the rate of compound Ca−P formation is a key index for defining the bioactivity of corresponding biomaterials. And this in turn is facilitated by the available functional groups on materials that affect the alternative electrostatic absorption of Ca²⁺ and PO₄³⁻ ions. For rGO-PDA, the catechol/amine groups of PDA, not only direct efficient interaction with the graphene sheets but also serve as active sites recruiting mineral ions, thereby enhancing apatite nucleation and growth.17 For bulk GO, the polar groups (—COOH, —OH) can have similar effects. In addition, the unique topographical ridges can be viewed as kind of “graphene patterns” providing biophysical cues for further enhancing the differentiation. Micropatterned geometries (5–50 μm) of graphene were shown to be effective at steering stem cell fate.13 Furthermore, the amphiphilicity, aromatic scaffold nature, and electrical conductivity (electrical stimuli) of graphene probably provided concerted efforts in both cases.13,54 All together, both kinds of sheets created an osteogenesis-enhancing microenvironment that is anticipated to benefit implant–tissue integration.

Figure 10. Osteodifferentiation properties of the graphene interfaces: (a) (a-1–a-3) ALP staining and (a-4) ALP activity, (b,c) quantification of (b) collagen and (c) calcium contents (*p < 0.05), and (d) optical images of collagen and calcium stains. Arrows indicate calcium nodules.
**3.7. Applications and Perspectives.** Undoubtedly, both large and small graphenes have respective merits, with which broader biomedical opportunities can readily be found. For instance, large-area GO is an ideal matrix for building versatile patterns, 3D networks, and scaffolds for stem cell cultures and bone tissue engineering.13 Also, the enlarged planar surface area provides abundant sites for efficient loading of aromatic drug molecules such as dexamethasone (osteogenic) and tetracycline (antibacterial) through π−π stacking for local drug delivery.14,55 rGO-PDA micro-/nanosheets, having more electrochemically active edging sites, hold promise in the modification of electrodes/devices for high-performance detection or biosensing.56 Taking advantage of the secondary reactive nature of PDA,24 one can further charge micro-/nanovehicles with inorganic particulates, bioactive minerals, and drugs/biomolecules for advanced multifunctional implants or other biomedical devices in future.15,17,27,57

Fundamentally speaking, the concept of applying graphene onto a surface is also appealing because it offers another viable route instead of dispersion to investigate the intricate nature of graphene materials,4 especially for the accurate determination of their bactericidal nature. On one hand, the surface properties of graphene, if well-devised, can allow for a focus on the effects from sole factor, such as the base plane, by lowering/excluding the effects from other factors, such as sheet size and edge density. On the other, a proper choice of substrate can make the identification of substrate-sensitive mechanisms, such as charge transfer, much easier. However, this area, with limited research attention, is still in its infancy (Table 1). Its development, as we see it, hinges greatly on progress in nanotechnology and methodologies that can engender a host of designer graphene-based interfaces, ideally with controllable physicochemical characteristics. As listed in Table 1, the currently available techniques/methods consist of chemical vapor deposition (CVD), vacuum filtration, Langmuir–Blodgett (LB), and electrophoretic deposition (EPD), with substrates including polymers, inorganics, and semiconductors and metals, yielding graphenes with dimensions ranging from nanometer to macroscopic scales. Indeed, each one is unique in regard to establishing a respective model for the demonstration of a specific theory. Yet, there is significant room for these theories to be improved, entailing research on other models by alternative methods. The self-assembly of graphene at interfaces, as exemplified here, is essentially easy, cheap, and general. By further careful engineering,23 diverse interfaces can be obtained, which could greatly help in allowing fundamental studies of graphene (Figure 1a).

To meet these extremely high expectations, ongoing efforts are warranted. For one example, the molecular assembly mechanisms, interlayer and layer–substrate interactions, and long-term physiological/mechanical stability of the assemblies should be further explored. In addition, the techniques/materials themselves require improvements to increase the materials’ interfacial strength (for bulk GO) and to maximize the antibacterial/osteodifferentiation efficiencies (for rGO-PDA) toward clinical applications. By utilizing the chemical versatility of GO/PDA, the former is possible through the introduction of strong covalent cross-links (Figure S7a),58 whereas the latter can be fulfilled by surface decorating nanosilver,27 antibiotics,55 and osteogenic molecules15,57 (Figure S7b). Furthermore, it remains an important task to exploit the efficiency and safety in vivo, as well as to deepen the understanding of the structure–function correlations of graphene sheet surfaces.

**4. CONCLUSIONS**

In this work, we demonstrated the possibility of imparting intrinsically dual-functional (i.e., antibacterial and osteogenic) properties of graphene to a metallic implant surface, to solve the pressing need for enhanced infection control and tissue integration in orthopedics. Two simple, straightforward self-assembly routes were established to form in situ on titanium substrate-anchored, 2D graphene structures with compositional and dimensional variations (i.e., macro-/microscale bulk GO vs submicroscale rGO-PDA). The former was based on an easy evaporation-assisted electrostatic assembly process, whereas the latter was rendered by exploiting a facile, mussel-inspired self-polymerization and the one-pot reactivity of PDA.

The resultant interface layers with differing structures and chemistries altered the hydrophilicity and protein adsorption of the substrate. Both modifications were confirmed to have potential to generate sterile implant surfaces with antiadhesion and antibiofilm activities, in the absence of extra antimicrobials, yet without much compromising their biocompatibility. In addition, by using the assemblies, we managed to achieve impressive osteogenic functions, which, coupled with the above antimicrobial properties and with the increasing availability and decreasing cost of graphene, bodes well for the further development of graphene-enabled biomedical care and therapeutic utility. This work should enrich the library of graphene-based nanotechnologies and facilitate the management of graphene-concerned health issues.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.6b05198.

Schemes of plausible reaction mechanisms, stability of postreaction solutions at various ratios, typical morphology of the reacted mixture for rGO-PDA, surface ROS levels with bacteria, zone-dependent antibacterial behavior of bulk GO sheets, structural stability of materials in bacterial tests, and proposed routes for further material improvements (PDF)

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Notes

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