In Vitro and in Vivo Antimicrobial Evaluation of Graphene–Polyindole (Gr@PIn) Nanocomposite against Methicillin-Resistant Staphylococcus aureus Pathogen

Mohd Shoeb,†‡ Mohammad Mobin,*† Mohd. Ahmar Rauf,§ Mohammad Owais,§ and Alim H. Naqvi‡

†Department of Applied Chemistry, Z. H. College of Engg. & Tech., and ‡Interdisciplinary Nanotechnology Centre (INC), Z. H. College of Engg. & Tech., Aligarh Muslim University, Aligarh 202002 Uttar Pradesh, India
§Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202 002, India

ABSTRACT: Nowadays, the infection caused by the methicillin-resistant Staphylococcus aureus (MRSA) and countless different types of bacterial infection cause the death of millions of people worldwide. Therefore, several strategies have been explored for the advancement of better and active antimicrobial agents; one of these lies in the form of two-dimensional carbon-based nanocomposites. Herein, we demonstrate the synthesis of the graphene–polyindole (Gr@PIn) nanocomposite and polyindole (PIn) and significantly enhance the proficiency against MRSA strains which are immune to most antibiotics. The synthesized Gr@PIn and PIn have been characterized by the various physical techniques, especially X-ray diffraction (XRD), electron microscopy [scanning electron microscopy (SEM) and transmission electron microscopy (TEM)], Fourier transform infrared, Raman, UV–vis spectroscopy, and thermogravimetric analysis. Electron microscopic investigations revealed the disintegration of bacterial cell wall upon interaction with Gr@PIn. Significantly, the Gr@PIn found to be very potent in the eradication of the MRSA strain with minimal toxicity to the mammalian cells. Assessment of the antibacterial mechanism revealed that the Gr@PIn adhered toward the bacterial surface, irreversibly interrupted the membrane layer structure of the bacteria, eventually penetrated cells, and efficiently impeded protein activity, which inherently turns into bacterial apoptosis in vitro. Moreover, last, the synthesized Gr@PIn efficiently treated the S. aureus-mediated experimental skin infection in BALB/c mice as well. This work magnifies our comprehending antibacterial mechanism of nonmetallic graphene-based PIn nanocomposite and provides the support to activity anticipation.

INTRODUCTION

Bacterial contamination will continue to provide a significant threat to public health, notably with rising levels of antimicrobial resistance in previous years. ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species) are responsible for most of the hospital-possessed infections and one of the principal threats to healthcare as the consequences of their multidrug resistance. This, therefore, the pathogen in the ESKAPE group, particularly the methicillin-resistant S. aureus, has appropriately attracted interest over the earlier years. In addition to the characteristics mentioned earlier, polymers can also show antimicrobial properties. Among the various polymers, polyindole (PIn) has attained a tremendous attention in the past couple of years. Indole contains benzene and pyrrole rings simultaneously; indeed, PIn might have the attributes of both poly(para-phenylene) and polypyrrole. The advantages of PIn are the significant redox activity, sufficient thermal reliability, and slow degradation rate in comparability with those of polyaniline and polypyrrole. Some nanocomposites of polymers have reported with metal oxides, metal nanoparticles, and graphene and carbon nanotubes widely used in antimicrobial applications. A variety of materials such as carbon nanomaterials, inorganic nanomaterials, and conjugated polymers have been explored to synthesize antimicrobial agents. Among them, graphene, a 2D nanomaterial, has also revealed various applications in different

Supporting Information

DOI: 10.1021/acsomega.8b00326

© 2018 American Chemical Society

Cite This: ACS Omega 2018, 3, 9431−9440

http://pubs.acs.org/journal/acsodf

Published: August 1, 2018
Received: February 23, 2018
Accepted: August 1, 2018

ACS Publications
© 2018 American Chemical Society
9431

ACS Omega 2018, 3, 9431−9440
fields such as energy storage, nanoelectronics, nanocatalysis, and antimicrobial properties.\textsuperscript{16–19} Recently, among these applications, the biomedical potentiality of graphene and its own functionalized derivatives have received much more attention from a medicinal chemist for drug discovery.\textsuperscript{15} Hereinafter, graphene-based nanomaterial, such as graphene oxide (GO), reduced GO (RGO), graphene quantum dots, and graphene-based nanocomposites, being implemented to biosensors, bioimaging, drug delivery, and photothermal therapy.\textsuperscript{20,21} Also, they reveal in vitro and in vivo antibacterial activity.\textsuperscript{20} Liu et al. proposed antimicrobial mechanism for graphene-based nanomaterials, which include cellular trauma with graphene nanomaterials, wrapping the bacteria and membrane disruption through graphene nanosheets, and oxidative stress because of superoxide anion generation with the approach of graphene nanocomposite, limiting the metabolism of bacteria.\textsuperscript{22} Moreover, the graphene-based nanocomposite in animal’s models in the presence of methicillin-resistant \textit{Staphylococcus aureus} (MRSA) pathogen would probably generate the in-depth understanding of the antimicrobial activity of graphene-based nanocomposite. Besides, in vivo models would also provide a possibility to investigate potential toxicity of graphene-based nanocomposite.

In this research work, we address the issues to establish the effect of PIn polymer and graphene PIn nanocomposite (Gr@PIn and Gr$_3$@PIn = Gr@PIn) on MRSA microbes. The motivation of the current study was to develop a simple, excellent, economical, and eco-friendly strategy for the synthesis of Gr@PIn nanocomposites as excellent antibacterial agents. A series of PIn and graphene nanocomposite (Gr@PIn) materials were synthesized through aqueous medium polymerization method to disperse the modified graphene which reduced via eco-friendly strategies throughout the PIn matrix. Gr@PIn nanocomposites for the antimicrobial agent have the following advantages, most notably solubility, inexpensive, adequate mechanical strength, eco-friendly, biocompatible, and processability, for the application. The strategy in this research work successfully verified that the chemical bonding of the PIn modified on the graphene-based polymer matrix, and the π–π interaction among the aromatic system of the modified graphene and PIn might have them both drastically improve the dispersion of graphene in conjugated PIn via eco-friendly aqueous polymerization. Because of the graphene layers exhibiting high surface area, flexible nanoscale films with high aspect ratios were dispersed in the polymeric matrix; this strategy might improve the antibacterial properties drastically. The synthesized graphene-based nanocomposites characterized by using X-ray diffraction (XRD), Fourier transform infrared (FTIR), Raman, UV, thermogravimetric analysis (TGA), and electron microscopy [scanning electron microscopy (SEM) and transmission electron microscopy (TEM)].

Additionally, we have discussed these considerations through systematically exploring the contiguous ability of methicillin-resistant bacteria in the presence of Gr@PIn under in vitro conditions as well as in vivo employing Balb/c model-based skin infection. Subsequently, treatment with the Gr@PIn composite formulation, the invasive potency of MRSA was reduced to 60% and further the electron microscopic studies showed that Gr@PIn nanocomposite formulation can potentially damage the cell wall of MRSA and successfully inhibit the biofilm formation and further inhibit MRSA growth in a concentration-dependent manner.

\section*{RESULTS AND DISCUSSION}

\textbf{PIn and Gr@PIn Structural Characteristics.} XRD technique was used to analyze the structure and phase of the synthesized samples. The XRD pattern of PIn, Gr$_1$@PIn, and Gr$_3$@PIn is shown in Figure 1. Pristine PIn shows 2\(\theta\) = 22.368° with a d spacing of 3.629 Å. As the RGO concentration increases in the PIn, a typical enhancement in the gallery spacing of the synthesized PIn in Gr$_1$@PIn and Gr$_3$@PIn, respectively, followed the ranging from 3.744 to 3.773 Å with a redshift in 2\(\theta\) from 20.368° to 21.552°. This enhancement within the gallery spacing of the synthesized PIn in the respective RGO could anticipate to the lamination all over the PIn (inset Figure 1 clearly showed). Moreover, PIn has depicted distinctive diffused XRD spectra signifying their amorphous character and a broad peak at 2\(\theta\) between 20° and 30°, which reveals the presence of weak π–π stacking between the RGO sheets attributable to the creased and improperly ordered structure.\textsuperscript{23,24} Consequently, XRD data distinctly reveal the successful synthesis of PIn, Gr$_1$@PIn, and Gr$_3$@PIn in this study.

Furthermore, the morphology of the synthesized PIn, Gr$_1$@PIn, and Gr$_3$@PIn nanocomposites is determined through SEM and high-resolution (HR) TEM. As shown in Figure 2A–C, SEM images of PIn, Gr$_1$@PIn, and Gr$_3$@PIn were distinctly hierarchical microstructures on the scale of 5 μm. In Figure 2A, the PIn consists of homogeneously distributed spheres. As RGO concentration increases, the surface morphology continuously modified is observed in the Gr$_1$@PIn and Gr$_3$@PIn nanocomposite (Figure 2B,C) with hierarchical microstructure constructed from a framework of graphene sheets, on which PIn is attached. Complimenting energy-dispersive X-ray spectrum (EDAX) is revealed by the presence of C (carbon), N (nitrogen), and O (oxygen) in Gr$_3$@PIn nanocomposite. Additionally, Figure 2D shows that elemental mapping analysis will offer an instantaneous elemental distribution of the samples, which ensure the homogeneous distribution of C (carbon), N (nitrogen), and O (oxygen) atoms in Gr@PIn nanocomposites. In addition to that, PIn
reveals the formation of a single-phase system with homogeneous discrete nanoparticle regime, as shown in TEM images (Figure 3A–C). Because the RGO was incorporated with the PIn at lower concentration (Gr1@PIn), the morphology of the nanocomposite exhibited lateral RGO sheets looking similar to nanorods probably because of strong intrachain interactions of indole units which favored the formation of lateral RGO sheets. Furthermore, as the concentration of graphene increases in the sample Gr3@PIn, RGO sheet is distinctly observed. Moreover, the morphology of the PIn in the nanocomposite (Gr3@PIn) was a hollow nanosphere, which is confirmed by the sharp distinction within the dark edge and the pallid center in the TEM images (Figure 3C). Moreover, synthesized pristine RGO nanosheet at 100 nm scale TEM image was shown in Figure S1 of the Supporting Information, which apparently can be seen as in a crumbled-shape nanosheet. Herein, polymerization of indole is taking place in the presence of RGO, and with the polymerization of polymer, RGO covalently bonded with PIn. With a known constant concentration of indole, the optimized quantity of Gr1@PIn shown rod-shaped morphology. Increasing the quantity of RGO, that is, Gr3@PIn cannot provide rod-shaped morphology in the nanocomposite. As the optimal quantity of RGO is required through the polymerization to attain rod-shaped morphology, the optimum quantity might reduce the interfacial energy barrier between the solid surface and bulk monomer solution, significant to the subsequent propagation of the indole polymerization producing the RGO rod-shaped morphology.25 Furthermore, indolium ion is hydrophilic, unlike, RGO functions as a hydrophobic group. Therefore, RGO and indolium may yield micelles acting as a template to form the rod-shaped nanostructures. Furthermore, at a low ratio of RGO–indolium (Gr1@PIn), the additional indolium ions may produce vesicles, which in turn may develop toward a low curvature rod-shaped vesicle caused by extended carbon chain of RGO. The self-assembled indolium–
RGO vesicles become diluted because of the strong intrachain interactions of indolium ions which favored the formation of lateral RGO sheets. At a high ratio of RGO-indolium, vesicles are overloaded in the reaction and polymer chains do not develop to the same magnitude in every vesicle. Consequently, we acquire a different type of morphology. The rod-shaped morphology developed through the emergence of vesicles within a longitudinal direction and the graphene sheets is possible through the agglomeration of unused a-RGO sheets, which is shown in the TEM image. The selected area electron diffraction (SAED) patterns for PIn (Figure S3A) consists of the continuous ringlike patterns revealing the amorphous nature of PIn. However, the SAED pattern of Gr1@PIn and Gr3@PIn shows some distinct ringlike patterns (Figure 3B, C) besides the continuous ring, revealing the appearance of crystallinity attributable to the presence of RGO because RGO concentrations increase crystallinity in polymer nanocomposite.

The FTIR spectrum of PIn, Gr1@PIn, and Gr3@PIn is shown in Figure 4. The major peak at ~3350 cm−1 has shown N–H stretching; the peak at 1600 cm−1 revealed the C–C stretching of the benzenoid ring of indole; peaks at 1450 and 1383 cm−1 revealed the C–N and C≡N stretching, respectively, out of plane deformation of benzene, indicated by the peak at 735 cm−1.24,26 Furthermore, peak at ~3350 cm−1 confirms that no polymerization has occurred at nitrogen.26,27 The spectrum of the nanocomposite, that is, Gr@PIn (Gr1@PIn and Gr3@PIn) displayed the same but weak peaks as PIn at 619, 1118, 1383, 1608, and 3350 cm−1, which correspond to −C–H, −C–N, −C–C, −C≡C, and −N–H stretching vibrations, respectively (all functional group vibrations incorporated in Table 1), and synthesized RGO, FTIR are incorporated into Figure S2 of the Supporting Information. In Figure S2, the spectrum of RGO exhibits the existence of the basal plane of graphene sheet at 1690 cm−1 and a broad peak at 3400 cm−1, associated with O–H vibration. This is worth noting that most of the significant FTIR peaks of PIn shifted, with regard to the lesser value of wavelength in the Gr@PIn nanocomposite which has correlated with the confined growth and restricted modes of vibrations in the PIn, in the presence of RGO on account of π−π interactions between RGO layers and aromatic PIn rings.24,26,28

Raman spectrographic analysis had become one among the foremost widespread techniques for the characterization of disordered and amorphous carbons. In Figure S, the PIn, Gr1@PIn, and Gr3@PIn nanocomposite were studied by Raman spectroscopy. Figure S3 (Supporting Information) revealed that two prominent peaks at around 1340 and 1592 cm−1 were related to the verified G band (Eg symmetry of sp2 carbon atoms) and D band (breathing mode of A1g symmetry and relevant to structural anomalies and disorder) with a D/G ratio of 1.42, respectively. The spectrum for the nanocomposite (Gr@PIn) showed that two strong peaks have shown for all of these samples at 1355 and 1587 cm−1, significant to D and G bands which represent different atomic ratios of sp3/sp2 carbons.24 The D/G intensity ratios of Gr1@PIn nanocomposite and Gr3@PIn nanocomposite are 1.32 and 1.36, respectively. From Figure S, it clearly shows that PIn polymerization does not influence the structure of RGO (Figure S3), and the intensity ratio of graphene and Gr@PIn nanocomposite revealed more sp3 carbon owing to the efficient relationship between PIn and RGO with a D band ratio to G band varying from 1.26 to 1.36 for graphene and Gr@PIn nanocomposite, respectively.29

Table 1. FTIR Functional Group Vibrations

| band (cm−1) | PIn | band (cm−1) | Gr@PIn (Gr1@PIn and Gr3@PIn) |
|-------------|-----|-------------|---------------------------------|
| 627         | −C–H (bending) | 744       | −C–H (bending) |
| 1111        | −C–N (bending) | 1118      | −C–N (bending) |
| 1383        | −C≡N (stretching) | 1375     | −C≡N (stretching) |
| 1450        | −C–N (stretching) | 1455     | −C–N (stretching) |
| 1616        | −C≡C (stretching) | 1604     | −C≡C (stretching) |
| 3350        | −N–H (stretching) | 3350     | −N–H (stretching) |

Figure 4. FTIR spectra of the PIn, Gr1@PIn, and Gr3@PIn.

Figure 5. Raman spectrum of PIn, Gr1@PIn, and Gr3@PIn.

Table 1. FTIR Functional Group Vibrations

| band (cm−1) | PIn | band (cm−1) | Gr@PIn (Gr1@PIn and Gr3@PIn) |
|-------------|-----|-------------|---------------------------------|
| 627         | −C–H (bending) | 744       | −C–H (bending) |
| 1111        | −C–N (bending) | 1118      | −C–N (bending) |
| 1383        | −C≡N (stretching) | 1375     | −C≡N (stretching) |
| 1450        | −C–N (stretching) | 1455     | −C–N (stretching) |
| 1616        | −C≡C (stretching) | 1604     | −C≡C (stretching) |
| 3350        | −N–H (stretching) | 3350     | −N–H (stretching) |

Figure 5. Raman spectrum of PIn, Gr1@PIn, and Gr3@PIn.
polaron in the nanocomposite. This effect attributes to the increase in adsorption coefficient. However, Gr@PIn nanocomposites have given rise to the gradual decrease in the peak intensity at around 280 nm and other peak formations between 350 and 400 nm with a redshift because of the incorporation of RGO as well as the increase in the concentration of the nanocomposite (Gr1@PIn and Gr3@PIn). The 610 nm peak could be allotted because of the polaronic excitation peak and its position around minimum absorption sides. The absorption spectra of Gr@PIn reveal the distinctive feature in contrast to pristine PIn. Mainly n to π* transition would exist in nanocomposite, whereas the π−π* transition seemed to be altered to higher energy region with a lower intensity of polaronic peak (610 nm), that unveiled the interactions between graphene and PIn.

**PIn and Gr@PIn Thermal Characteristics.** The composition and structure of the PIn, Gr1@PIn, and Gr3@PIn have analyzed by TGA. As demonstrated in Figure 7, all of the samples reveal slight mass depletion around 100 °C caused by the deintercalation of H2O. PIn started off to disintegrate at a quite low temperature below 250 °C and comprehensively oxidized at around 600 °C. The carbonized fragments remained even at a higher temperature. The outcome revealed the covalent bond lead in the best thermal stability within Gr1@PIn and Gr3@PIn nanocomposites. Noticeably, there was rarely any mass loss at 100−300 °C for nanocomposite, that is, Gr@PIn, which revealed that the oxygen-functionalized groups were very scarce. It also finally demonstrated a mass loss of 37 and 28% between 170 and 600 °C for Gr1@PIn and Gr3@PIn nanocomposites, respectively, which primarily attributed to the decomposition of the PIn. The outcome mentioned above proposed the robust covalent bond interaction between PIn and RGO and performed a crucial role in the enhancement of thermal stability of the nanocomposite.

**Antibacterial Assessment of PIn and Gr@PIn Nanocomposites.** In light of the convincing evidence, the expanded growth of high resistance in the clinical isolates of MRSA and newly evolved families of antimicrobial agents have a short life span; thus, the need to explore new antibacterial drug is of superior importance. Researchers are nowadays drawing their attention to graphene-based nanocomposite, trying to explore the emerging prospects and to evolve superior graphene-based antimicrobial drugs against MRSA. The antibacterial potency of the in situ Gr@PIn nanocomposite was calculated against resistant SA isolates. Standard vancomycin was taken as a controlled standard antibiotic. The minimum inhibitory concentration (MIC) value for the graphene-based nano-clusters (NCs) was found to be around for Gr1@PIn nanocomposite 128 μg/mL against MRSA ATCC 43300 and 64 μg/mL against MRSA ATCC BAA-1708, whereas the other Gr1@PIn nanocomposite had an MIC of 256 μg/mL for both of the strains. The PIn alone had an MIC of 512 μg/mL for both of the MRSA strains, whereas the vancomycin had an MIC of 16 and 8 μg/mL for different MRSA strains, respectively. Further, the antibacterial potential of our synthesized NCs was evaluated by agar diffusion assay (Figure 8A). The zone of inhibition assay suggested the active bactericidal activity of nanocomposite against the two tested MRSA bacteria. The ROS produced by internalized nanocomposite damages DNA and other cellular machinery components of bacteria. Live-dead assay employing fluorescence microscopy also established the antimicrobial potential of nanocomposite against MRSA strains. The bacterial cells in their log phase were treated with different Gr@PIn nanocomposite formulations for 3 h, followed by staining with the propidium iodide (PI) and SYTO-9 dyes. In general, PI penetrates cells with disrupted and damaged membrane lesions. The NC treatment resulted in profound staining of cells with PI probe. The control group (live cells) showed green fluorescence, whereas the experimental samples treated with various formulations for 3 h showed bright red fluorescence. Because of the binding of dye with DNA of dead bacteria, dead cells appeared sharp red in points because of acquired PI. The PI bound to the double-stranded DNA is an indicator of NC-mediated damage of bacterial cell wall (Figure 8B). [Data are not shown for MRSA ATCC 44300]. The Gr@PIn nanocomposite showed a prominent antibacterial effect against both of the MRSA strains, as suggested by agar diffusion results (Table 2). Among various treatment groups, Gr1@PIn nanocomposite-treated group showed profound bacterial inhibition.

To study the antibiofilm activity of graphene-based PIn nanocomposite, the XTT assay was performed. The data obtained exhibited the dose-dependent antibiofilm activity of graphene-based nanocomposites (Figure 9) (data are not shown for MRSA ATCC 44300). There was a significant decrease in the bacterial count [p-value < 0.01 (**)] of both of the MRSA strains in the group treated with Gr3@PIn nanocomposite as compared with the vancomycin treatment. No significant difference was found between the Gr1@PIn
nanocomposite and vancomycin-mediated killing of MRSA. The observation concords with the zone of inhibition assay. However, PIn-treated groups showed shallow inhibitory activity as compared to the Gr3 and Gr1 groups; thus, the higher activity is due to the presence of Gr groups that are responsible for enhanced ROS production, thus assisting the likely antibiofilm effect. Nevertheless, it is worth mentioning that significant reduction in CFUs was evident in both of the cases when compared to positive control group (Figure 10) (MRSA ATCC 43300 also responded in the same manner upon treatment with Gr1@PIn nanocomposite; however, data are not shown with regard to simplicity).

**Effect of Gr3@PIn Combination on Bacterial Cell Wall as Revealed by EM Analysis.** SEM microscopy was employed to analyze the surface morphology variations of the control and treated MRSA ATCC BAA 1708 bacterium

Table 2. Agar Diffusion Assay for the Zone of Inhibition Observed (in mm units) against Result-Tested MRSA Strains

| Strains            | PIn     | Gr1@PIn  | Gr3@PIn  | Van (10 μg/disk) |
|--------------------|---------|----------|----------|------------------|
| ATCC 43300         | 5.667 ± 1.2 | 11.667 ± 2.082 | 20.667 ± 3.214 | 12.67 ± 2.309   |
| ATCC BAA-1708      | 5.33 ± 1.2  | 10.33 ± 2           | 17.667 ± 2.082 | 10.67 ± 2.5    |

Figure 8. Antibacterial activity of as-synthesized Gr@PIn formulations. (A) Zone of inhibition as a measure to establish the antibacterial potential of various Gr@PIn NC formulations against MRSA strains. (B) Fluorescence microscopic images showing MRSA ATCC BAA-1708 cells upon their treatment with various Gr@PIn formulations.

Figure 9. Antibiofilm activity of different Gr@PIn formulations (A). Effect of Gr@PIn nanocomposite against biofilm development in MRSA ATCC BAA-1708 strain. Growth inhibition was assessed by comparing relative metabolic activity determined using XTT assay; untreated control was considered showing 100% activities. Experiments were performed in triplicates; results are shown as mean ± standard deviation (SD); **P ≤ 0.01; ***P ≤ 0.001.

Figure 10. In vitro CFU counts assay. CFU counts as residual MRSA ATCC BAA-1708 surviving after exposure to various forms of Gr@PIn nanocomposite.
with synthesized Gr@PIn and Gr3@PIn nanocomposite. As demonstrated in Figure 11A, control MRSA ATCC BAA 1708 bacterium was mostly round with smooth morphology and intact cell surfaces. To treat with Gr3@PIn NCs, cell walls of MRSA ATCC BAA 1708 bacterium turned into wrinkled and disrupted morphology with the impaired surface. Further, bacterial cell lysis resulted in the release of cytosolic content upon their exposure to the Gr3@PIn formulation (Figure 11B) (data are not shown for MRSA ATCC 44300). Additionally, bacterial cell lysis was confirmed through TEM in Figure 11B. Herein, apoptosis has occurred through the cytosolic content release upon treatment with Gr3@PIn nanocomposite.

**Hemolysis Assay.** The essential leading function of every preferred pharmaceutical drug candidate is that the product should be biocompatible, and it should be nontoxic toward healthy cells. Therefore, to study its biocompatibility, the synthesized Gr@PIn nanocomposite, red blood cell (RBC) lysis test was conducted. The exposure of our nanocomposite with healthy cells revealed little or no toxicity and led to minimal lysis of healthy cells. As demonstrated in Figure 12, the Gr3@PIn NCs around 27.2% and Gr1@PIn showed lysis of 27% (P ≤ 0.001) additionally at a higher concentration of 512 μg/mL. Although, similar to the concentration increases, the cell viability declined proportionally.

**Antibacterial Potential of Gr@PIn Nanocomposite against S. aureus Skin Infection in Balb/C Mice.** Keeping into consideration the strong antibacterial potential of Gr3@PIn combating against both MRSA ATCC 43300 in addition to ATCC BAA-1708 isolates, we evaluated its probability to cure acute cutaneous dermatitis contagion in the animal model. As shown in Figure 13A, reddening of the skin in Balb/ C mice because of being exposed against MRSA triggered cutaneous bacterial infections and resulted in localized skin disruption. The remaining bacterial load in the skin was analyzed through the details of bacteria in the given specimen by culturing in the solid agar medium. The outcomes revealed that the Gr3@PIn treatment efficiently eliminated skin infection (Table 3, Figure 13 B). Supplementing with Gr3@PIn causes immense reduction in the bacterial burden (∼45% reduction) when compared to untreated control (P < 0.005).

**Histopathological Analysis.** With the various Gr3@PIn formulation, we studied histopathological analysis of the infected Balb/C mice through the bacterial burden, skin architecture, and sustained inflammatory changes. On day 11 postinfection, the treated skin samples were taken out aseptically and stained using hematoxylin and eosin (HE) staining. The healthy group exhibiting normal skin histology has a regular intact epidermal layer, unlike MRSA-infected skin experiencing thin epidermal layer with dysfunction through the existence of a significant number of inflammatory cells. Figure 13C reveals that treatment with Gr3@PIn leads to healing of epidermal layer with negligible skin damage. In conclusion, right after the Gr3@PIn NCs formulation treatment, the skin attained a typical architecture.

**CONCLUSIONS**

In summary, we have successfully synthesized an active antibacterial Gr@PIn nanocomposite via eco-friendly and facile strategy, and RGO was achieved through the reduction of exfoliated GO using the glucose as a reducing agent. The Gr@PIn was effective in inhibiting MRSA and was seen to be resistant to a vast area of antibiotics. The mechanism of

![Figure 11](image1.png)

**Figure 11.** Electron microscopic observation of MRSA when coincubated with different Gr@PIn. (A) SEM micrograph depicting the interaction of various Gr3@PIn with MRSA ATCC BAA-1708 strain. B. TEM showing the interaction of various Gr3@PIn with MRSA ATCC BAA-1708 strain.

![Figure 12](image2.png)

**Figure 12.** Effect of varying concentrations of Gr@PIn nanocomposite on RBC leakage.
The antibacterial action of Gr@PIn nanocomposite could adhere toward the bacterial surface, irreversibly interrupting the membrane layer structure of the bacteria, eventually penetrating cells, and efficiently impeding protein activity, which inherently turns into bacteria apoptosis in vitro. Electron microscopy revealed the bacterial surface deterioration through the Gr@PIn nanocomposite. Additionally, Gr@PIn nanocomposite has the potential to inhibit the S. aureus-mediated lysis of RBCs. In conclusion, our study suggests that the synthesized Gr@PIn nanocomposites have great antibacterial characteristics against MRSA with minimal toxicity and biocompatibility as found in vitro studies. Remarkably, Gr@PIn was additionally efficient in suppressing MRSA and found to become resistant up to a range; this is undoubtedly large for antibiotics.

**EXPERIMENTAL SECTION**

**Synthesis of RGO.** To synthesis GO, we used modified Hummer method with 20 μm mesh and Sigma-Aldrich graphite powder as a precursor, in continuation of our earlier work.16−19 RGO sheets were synthesized through the followed method: 50 mL of homogeneous GO dispersion (3 and 5 mg mL⁻¹ in two distinct beakers) was ultrasonicated for 2 h in deionized water; a colloidal solution obtained in the clear beaker. Following that, 150 mg of glucose was added, followed by the stirring for 24 h. Then, 50 μL of liquor ammonia solution (25 w/w %) was added to the colloidal solution of GO in each beaker and vigorously agitated during 30 min and after that retained at 90 °C for 1 h. The solution was obtained at room temperature, centrifuged, and cleaned out with ethanol and water and then was dehydrated at 40 °C in a vacuum oven for 24 h. RGO powder was obtained.

**Synthesis of Gr@PIn (Gr₁@PIn and Gr₃@PIn) Nanocomposite.** For the synthesis of Gr@PIn (Gr₁@PIn and Gr₃@PIn) by varying the RGO concentration for the 3 mg mL⁻¹ (Gr₁@PIn) and 5 mg mL⁻¹ (Gr₃@PIn), 1 M sodium dodecyl sulfate surfactant was dissolved in ethanolic solution (1:1 ethanol and water) and stirred continuously for 1 h at room temperature. Then, 1 M indole was introduced under constant stirring, followed by RGO. After stirring for 3 h, 1.5 M anhydrous FeCl₃ was added slowly into the stirred solution. The solution rapidly turned into light green as anhydrous FeCl₃ was dropped into the solution. The commixture was placed inside a conventional microwave oven (Samsung Electronics, 750 W) at an irradiation power of 180 W for 3 min. The dark green precipitate was filtered and rinsed by dissolving in ethanol, dichloromethane, and deionized water repeatedly to remove monomer/oligomer and unreacted oxidant.34 Eventually, the synthesized sample was dried out into a vacuum oven and stored for analysis. The same process was used for indole polymerization through the conventional strategy, utilizing anhydrous FeCl₃ as the oxidant. Also, PIn samples were synthesized through the microwave irradiation according to a similar procedure stated above. Nevertheless, synthesized PIn was washed repetitively through ethanol and after that by dichloromethane to eliminate the unreacted monomer and dehydrated in a vacuum desiccator.

**Table 3. Values of log_{10} CFU/mL in Tabular Form**

| pathogen     | groups          | log_{10} CFU/mL |
|--------------|-----------------|-----------------|
| MRSA 43300   | positive control| 5.969           |
|              | PIn             | 5.393           |
|              | Gr₁@PIn        | 4.872           |
|              | Gr₃@PIn        | 4.300           |
|              | vancomycin      | 4.170           |
|              | PIn             | 5.393           |

**Figure 13.** Effect of Gr@PIn formulations on experimental skin infection (A); efficacy of Gr₃@PIn against the examination of mice skin. (B) Mice were infected topically with MRSA ATCC BAA-1708 and treated with various Gr@PIn groups concurrently after infection. Mice inoculated with phosphate-buffered saline alone were used as a control. After treatment with various Gr@PIn, skin lesions were cut; the homogenized and bacterial count was determined by CFU assay on the 11th day; (C) on day 11, biopsy specimens were taken instantly after the conclusion of the experiment, fixed in 4% neutral-buffered formalin and embedded in paraffin. The biopsy specimens were stained with HE. Experiments were performed in triplicates; results are shown as mean ± SD; ***P ≤ 0.001.
Assessment of Antibacterial Activity. All of the antibacterial in vitro and in vivo assay are embedded in the Supporting Information file.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b00326.

TEM image of RGO; FTIR spectrum of RGO; RAMAN spectrum of RGO; and assessment of antibacterial activity (DOCX)

AUTHOR INFORMATION

Corresponding Author

*E-mail: drmMobin@hotmail.com (M.M.).

ORCID

Mohammad Mobin: 0000-0003-4829-7491

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

M.S. gratefully acknowledged the financial assistance in terms of “SRF” by UGC-MANF, New Delhi. The authors are extremely thankful to Sumairah Kareem and Sharique Ahmad for providing help in the pictorial representation of the data obtained and drawing graph structures for the manuscript, respectively. We are also grateful for the TEM and SEM facilities at USIF-AMU, Aligarh and also to Abul Maaz and Shamim for the technical support.

REFERENCES

(1) Lam, S. J.; O’Brien-Simpson, N. M.; Pantarat, N.; Sulistio, A.; Wong, E. H. H.; Chen, Y.-Y.; Lenzo, J. C.; Holden, J. A.; Blencowe, A.; Reynolds, E. C.; Qiao, G. G. Combating multidrug-resistant Gram-negative bacteria with structurally nanoengineered antimicrobial peptide polymers. Nat. Microbiol. 2016, 1, 16162.
(2) Boyle-Vavra, S.; Daum, R. S. Community-acquired methillin-resistant Staphylococcus aureus: the role of Panton-Valentine leukocidin. Lab. Invest. 2006, 87, 3–9.
(3) Yamamoto, T.; Hung, W.-C.; Takano, T.; Nishiyama, A. Genetic nature and virulence of community-associated methillin-resistant Staphylococcus aureus. Biomedicine 2013, 3, 2–18.
(4) Kenawy, E.-R.; Worley, S. D.; Broughton, R. The Chemistry and Applications of Antimicrobial Polymers: A State-of-the-Art Review. Biomacromolecules 2007, 8, 1359–1384.
(5) Hassaniem, R.; Al-Hinali, M.; Farha Al-Said, S. A.; Little, R.; Şiller, L.; Wright, N. G.; Houlton, A.; Horrocks, B. R. Preparation and Characterization of Conductive and Photoluminescent DNA-Templated Polytopic Nanowires. ACS Nano 2010, 4, 2149–2159.
(6) Dögli, S. T.; Mert, B. D.; Yazıcı, B. Polyindole top coat on TiO2 sol–gel films for corrosion protection of steel. Corros. Sci. 2013, 66, 51–58.
(7) Bober, P.; Liu, J.; Mikkonen, K. S.; Ihalainen, P.; Pesonen, M.; Plumed-Ferrer, C.; von Wright, A.; Lindfors, T.; Xu, C.; Latonen, R.-M. Biocomposites of Nanofibrillated Cellulose, Polypyrrole, and Silver Nanoparticles with Electroconductive and Antimicrobial Properties. Biomacromolecules 2014, 15, 3655–3663.
(8) Ma, G.; Chen, Y.; Li, L.; Jiang, D.; Qiao, R.; Zhu, Y. An attractive photocatalytic inorganic antibacterial agent: Preparation and property of graphene/zinc ferrite/polyaniline composites. Mater. Lett. 2014, 131, 38–41.
(9) Poyraz, S.; Cerkiz, I.; Huang, T. S.; Liu, Z.; Kang, L.; Luo, J.; Zhang, X. One-Step Synthesis and Characterization of Polyaniline Nanofiber/Silver Nanoparticle Composite Networks as Antibacterial Agents. ACS Appl. Mater. Interfaces 2014, 6, 20025–20034.
(10) Ebrahimial, S.; Zakaria, A.; Kassim, A.; Basri, S. N. Novel conductive polypyrrole/zinc oxide/chitosan bionanocomposite: synthesis, characterization, antioxidant, and antibacterial activities. Int. J. Nanomed. 2015, 10, 217–227.
(11) Youssel, A. M.; Moustafa, H. A.; Barhoum, A.; Hakim, A. E.-F. A.; Dufresne, A. Evaluation of the Morphological, Electrical and Antibacterial Properties of Polyaniline Nanocomposite Based on Zn/Al-Layered Double Hydroxides. ChemistrySelect 2017, 2, 8553–8566.
(12) Hinds, L.; Kenny, O.; Hossain, M. B.; Walsh, D.; Sheehy, E.; Evans, P.; Gaffney, M.; Rai, D. K. Evaluating the Antibacterial Properties of Polycrylactene and Glucosinolate Compounds with Further Identification of Their Presence within Various Carrot (Daucus carota) and Broccoli (Brassica oleracea) Cultivars Using High-Performance Liquid Chromatography with a Diode Array Detector and Ultra Performance Liquid Chromatography–Tandem Mass Spectrometry Analyses. J. Agric. Food Chem. 2017, 65, 7186–7191.
(13) Zhu, J.; Wang, J.; Hou, J.; Zhang, Y.; Liu, J.; Van der Bruggen, B. Graphene-based antimicrobial polymeric membranes: a review. J. Mater. Chem. A 2017, 5, 6777–6793.
(14) Ma, Y.; Bai, D.; Hu, X.; Ren, N.; Gao, W.; Chen, S.; Chen, H.; Lu, Y.; Li, J.; Bai, Y. Robust and Antibacterial Polymer/Mechanically Exfoliated Graphene Nanocomposite Fibers for Biomedical Applications. ACS Appl. Mater. Interfaces 2018, 10, 3002–3010.
(15) Zou, X.; Zhang, L.; Wang, Z.; Luo, Y. Mechanisms of the Antibacterial Activities of Graphene Materials. J. Am. Chem. Soc. 2016, 138, 2064–2077.
(16) Singh, B. R.; Shoeb, M.; Khan, W.; Naqvi, A. H. Synthesis of graphene/zirconium oxide nanocomposite photocatalyst for the removal of rhodamineB dye from aqueous environment. J. Alloys Compd. 2015, 651, 598–607.
(17) Shoeb, M.; Singh, B. R.; Mobin, M.; Afreen, G.; Khan, W.; Naqvi, A. H. Kinetic Study on Mutagenic Chemical Degradation through Three Pot Synthesized Graphene/ZnO Nanocomposite. PLoS One 2015, 10, No. e0135055.
(18) Shoeb, M.; Mobin, M.; Ali, A.; Zaman, S.; Naqvi, A. H. Graphene-mesoporous anatase TiO2 nanocomposite: A highly efficient and recyclable heterogeneous catalyst for one-pot multi-component synthesis of benzodiazepine derivatives. Appl. Organomet. Chem. 2018, 32, No. e3961.
(19) Ansari, M. Z.; Shoeb, M.; Nayab, P. S.; Mobin, M.; Rahisuddin; Khan, I.; Siddiqi, W. A. Honey mediated green synthesis of graphene based NiO2/Cu2O nanocomposite (Gr@NiO2/Cu2O NCs): Catalyst for the synthesis of functionalized Schiff-base derivatives. J. Alloys Compd. 2018, 738, 56–71.
(20) Wu, X.; Tan, S.; Xing, Y.; Pu, Q.; Wu, M.; Zhao, J. X. Graphene oxide as an efficient antimicrobial nanomaterial for eradicating multidrug resistant bacteria in vitro and in vivo. Colloids Surf., B 2017, 157, 1–9.
(21) Lam, S. J.; Wong, E. H. H.; Boyer, C.; Qiao, G. G. Antimicrobial polymeric nanoparticles. Prog. Polym. Sci. 2018, 76, 40–64.
(22) Liu, S.; Zeng, T. H.; Hofmann, M.; Burcombe, E.; Wei, J.; Jiang, R.; Kong, J.; Chen, Y. Antibacterial Activity of Graphite, Graphite Oxide, Graphene Oxide, and Reduced Graphene Oxide: Membrane and oxidative Stress. ACS Nano 2011, 5, 6971–6980.
(23) Wang, G.; Yang, J.; Park, J.; Gou, X.; Wang, B.; Liu, H.; Yao, J. Facile Synthesis and Characterization of Graphene Nanosheets. J. Phys. Chem. C 2008, 112, 8192–8195.
(24) Zhou, Q.; Zhu, D.; Ma, X.; Xu, J.; Zhou, W.; Zhao, F. High-performance capacitive behavior of layered reduced graphene oxide and polypyrrole nanocomposite materials. RSC Adv. 2016, 6, 29840–29847.
(25) Chatterjee, S.; Layek, R. K.; Nandi, A. K. Changing the morphology of polyaniline from a nanotube to a flat rectangular nanopipe by polymerizing in the presence of amino-functionalized
reduced graphene oxide and its resulting increase in photocurrent. *Carbon* 2013, 52, 509−519.

(26) Meng, Y.; Wang, K.; Zhang, Y.; Wei, Z. Hierarchical Porous Graphene/Polyaniline Composite Film with Superior Rate Performance for Flexible Supercapacitors. *Adv. Mater.* 2013, 25, 6985−6990.

(27) Ma, X.; Zhou, W.; Mo, D.; Lu, B.; Jiang, F.; Xu, J. One-step template-free electrodeposition of novel poly(indole-7-carboxylic acid) nanowires and their high capacitance properties. *RSC Adv.* 2015, 5, 3215−3223.

(28) Wang, R.-X.; Fan, Y.-J.; Wang, L.; Wu, L.-N.; Sun, S.-N.; Sun, S.-G. Pt nanocatalysts on a polyindole-functionalized carbon nanotube composite with high performance for methanol electrooxidation. *J. Power Sources* 2015, 287, 341−348.

(29) Ferrari, A. C.; Basko, D. M. Raman spectroscopy as a versatile tool for studying the properties of graphene. *Nat. Nanotechnol.* 2013, 8, 235−246.

(30) Wadatkar, N. S.; Waghuley, S. A. Complex optical studies on conducting polyindole as-synthesized through chemical route. *Egypt. J. Basic Appl. Sci.* 2015, 2, 19−24.

(31) Pandey, R. K.; Singh, A. K.; Prakash, R. Enhancement in performance of polycarbazole-graphene nanocomposite Schottky diode. *AIP Adv.* 2013, 3, 122120.

(32) Mudila, H.; Rana, S.; Zaidi, M. G. H.; Alam, S. Polyindole/Graphene Oxide Nanocomposites: The Novel Material for Electrochemical Energy Storage. *Fullerenes, Nanotubes, Carbon Nanostruct.* 2015, 23, 20−26.

(33) Kugelberg, E.; Norstrom, T.; Petersen, T. K.; Duvold, T.; Andersson, D. I.; Hughes, D. Establishment of a Superficial Skin Infection Model in Mice by Using *Staphylococcus aureus* and *Streptococcus pyogenes*. *Antimicrob. Agents Chemother.* 2005, 49, 3435−3441.

(34) Tiwari, M.; Kumar, A.; Umre, H. S.; Prakash, R. Microwave-assisted chemical synthesis of conducting polyindole: Study of electrical property using Schottky junction. *J. Appl. Polym. Sci.* 2015, 132, 42192.