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Highly Efficient and Excitation Tunable Two-Photon Luminescence Platform For Targeted Multi-Color MDRB Imaging Using Graphene Oxide

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Multiple drug-resistance bacteria (MDRB) infection is one of the top three threats to human health according to the World Health Organization (WHO). Due to the large penetration depth and reduced photodamage, two-photon imaging is an highly promising technique for clinical MDRB diagnostics. Since most commercially available water-soluble organic dyes have low two-photon absorption cross-section and rapid photobleaching tendency, their applications in two-photon imaging is highly limited. Driven by the need, in this article we report extremely high two-photon absorption from aptamer conjugated graphene oxide ($\sigma_{2PA} = 50800 \text{ GM}$) which can be used for highly efficient two-photon fluorescent probe for MDRB imaging. Reported experimental data show that two-photon photoluminescence imaging color, as well as luminescence peak position can be tuned from deep blue to red, just by varying the excitation wavelength without changing its chemical composition and size. We have demonstrated that graphene oxide (GO) based two-photon fluorescence probe is capable of imaging of multiple antibiotics resistance MRSA in the first and second biological transparency windows using 760–1120 nm wavelength range.

In the last thirty years, the evolution of multiple drug resistance bacteria (MDRB) has increased day by day and are spread all over the world^{1–9}. The emergence of MDR methicillin-resistant *Staphylococcus aureus* (MRSA), and extensively drug-resistant (XDR) *Mycobacterium tuberculosis* have presented a significant challenge for the treatment of infectious diseases^{1–9}. Without proper treatment, early diagnosis is the key for survival from MDRB infection disease. For biological imaging applications, near-infrared (NIR) light in two biological transparency windows located in 650–950 nm for the first NIR window or 1000–1350 nm for the second NIR window must be used to avoid the absorption of light from physiological fluids, tissues and biological water^{10–15}. Since hemoglobin has substantially less absorption between 650 to 1350 nm and the blood is sufficiently transparent from 1000 to 1350 nm, the second window thus provides a maximum radiation penetration through tissue^{10–15}. But unfortunately, despite huge advances in organic fluorescence probes in the last 50 years, fluorescence imaging using NIR light remains a big challenge till today^{10–15}. As an alternative, two-photon (TPF) imaging has been introduced for NIR imaging, which exhibits several advantages such as a larger penetration depth, minimized tissue autofluorescence background and reduced photodamage^{10–15}. The efficiency of two-photon fluorescence imaging is highly dependent on the two-photon absorbing materials, which should have large two-photon absorption cross-sections in water and also they should have very good photo-stability. However, due to rapid photobleaching and low two-photon absorption cross-section (50 Goeppert-Mayer (GM)), use of organic dyes for TPF has been hampered for real life imaging^{10–15}. In the last decade, quantum dots (QDs) have been shown to be one of the valuable fluorescent probes which can be used as NIR probe. Since QDs are made from highly toxic metal ions, the toxicity of semiconductor QDs is a huge concern for in vivo bio-imaging^{10–15}. Recently, several articles^{29–35} have reported that graphene oxide nanoparticles with size below 40 nm can be used as graphene quantum dots for bio-labeling. In their report, color of the fluorescence imaging has been varied by changing the size of graphene dots. In this manuscript we have reported graphene oxide sheet based two-photon multi-color bio-imaging of MDRB, where multicolor imaging is based on the fact that two-photon fluorescence wavelength of graphene oxide sheet can be tuned just by varying the excitation energy without changing its chemical composition and size. As per our knowledge, current manuscript reports for the first time multicolor imaging of MRSA using aptamer attached 2D graphene oxide sheet.

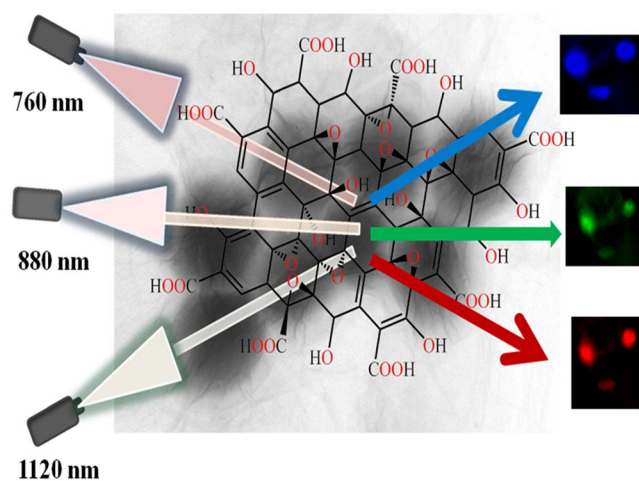


Figure 1 | Schematic representation of two-photon image of MRSA bacteria, shows graphene oxide based tunable two-photon fluorescent platform for selective biological sensing in the first and the second biological transparency windows.

After the discovery of isolated graphene flakes about ten years ago, due to its remarkable electronic and structural properties, the two dimensional (2D) graphene has revolutionized the scientific community^{16–20}. Unfortunately, graphene sheet cannot exhibit luminescent properties due to the presence of zero optical band gap^{21–24}. As a result, pure graphene cannot be used as fluorescence imaging material^{20–24}. Last few years, several articles have demonstrated that photoluminescence of graphene can be observed by modifying with various oxygen-containing groups or by reducing its size to the nanometer scale^{24–32}. Though the fluorescence of graphene oxide (GO) can be tuned from the visible to NIR region, it is not suitable for biological imaging using NIR light at biological transparency windows^{24–30}. In this communication, we have demonstrated that two-photon photoluminescence imaging color, as well as luminescence peak position can be tuned from deep blue to red, just by varying the excitation wavelength without changing its chemical composition and size, as shown in Figure 1. Since annually two million patients suffer from hospital-acquired *Staphylococcus aureus* strains infections and 99,000 deaths come from MRSA infections every year in the United States^{1–4}, in the current manuscript we report that aptamer APT^{SEB1}-modified graphene oxide based two-photon luminescence platform can be used for selective and multi-color imaging of MRSA in the first and the second biological transparency windows.

Results and Discussions

Graphene oxide from graphite was synthesized using strong oxidizing agents using modified Hummers reported method^{17,18}. As shown in Figure 2A–2B, the fluorescence of graphene oxide can be tuned from the visible to NIR in water at room temperature. It is very interesting to observe that fluorescence wavelength can be tuned just by varying the excitation energy from the UV to the visible range without changing its chemical composition and size. According to Michael Kasha's rule³³, the photoluminescence peak is independent of the wavelength of the excitation, as it is always observed for organic dyes and inorganic quantum dots fluorophores. Usually, the fluorophore is excited to the excited states in femto-second (10^{-15} s) time scale via the absorption of light and after that it undergoes nonradiative relaxation to the band edge in pico-second (10^{-12} s) time scale^{24–32}. At the end, radiative recombination of the electron and hole to emit a photon as photoluminescence occurs at nano-second (10^{-9} s) time scale. As a result, all the excited electrons, independent of initial energy, have to relax to the band edge before it

comes back to the ground state by releasing luminescence^{24–32}. Due to the above fact, the fluorescence from organic chromophores or quantum dots does not depend on the excitation energy. The excitation energy dependent photoluminescence spectral shift for graphene oxide can be due to the several factors^{36–39} and these are as follows: 1) possible excited-state protonation of the $-\text{COOH}$ group. In the excited state, intramolecular proton transfer occurs from hydroxy to carboxylate anions; 2) excitation wavelength dependent fluorescence from the $-\text{OH}$ moiety in the graphene oxide sheets; 3) Due to the local reorganization of photoexcited GO sheets in a polar solvent, the solvent relaxation time becomes comparable to the fluorescence lifetime. As reported in the Figure 2, though the GO photoluminescence can be tuned from the visible to the near NIR region just by varying the excitation energy from 440–600 nm, we have not observed any clear fluorescence above 600 nm excitation, which clearly indicates that GO is not suitable for biological imaging applications using near-infrared (NIR) light within the biological transparency windows. As a result, TPF imaging at different excitation wavelengths with near-infrared light in two biological transparency windows has been used for MDRB imaging. For this purpose, we have used Nikon multiphoton microscope (FV1000MPE). 80 MHz Ti:sapphire laser with 100 femto second (fs) pulse width and 80 MHz repetition rate was used as an excitation source.

Tunable wavelengths in two biological transparency windows were generated using optical parametric amplifier. Experimental details for two-photon imaging has been reported before⁴⁰. Figure 2C shows the excitation wavelength dependent two-photon photoluminescence from freshly prepared graphene oxide, which clearly indicates that two-photon photoluminescence from GO can be tuned just by varying the NIR excitation energy from 760 to 1120 nm, without changing its chemical composition and size. To verify whether the observed luminescence was induced by two-photon excitation, we have performed excitation wavelength power dependent two-photon intensity measurement. Figure 2D shows how 680 nm two-photon emission intensities from GO vary for 1120 nm fs laser excitation with various average powers. Our experimental data clearly show that the emission intensity is proportional to the square of the fs excitation intensity, which really confirms the presence of the two-photon process. Next, to find out the photostability of aptamer APT^{SEB1}-modified graphene oxide as a TPF material, we have performed continuous 1120 nm fs laser illumination experiment for 40 minutes. As shown in Figure 3E, two-photon luminescence signals remain about unchanged till 40 minutes of illuminations, which indicates very good photo-stability of aptamer APT^{SEB1}-modified graphene oxide as a TPF material.

As it is well documented^{42,43} that the two-photon luminescence intensity depends on the two-photon absorption cross section (σ_{2p}) and the emission quantum yield (Φ_f), we have determined the two-photon action cross-sections from two-photon luminescence data. Xu et. al⁴² and Albota et. al.⁴³ reported the absolute values of TPA cross sections of fluorescein as a function of wavelength from 690–960 nm, and therefore, we have used fluorescein as the reference. We have also used Albota et. al.⁴³ reported method for the measurement of two-photon fluorescence quantum yield of graphene oxide using fluorescein as the reference. By using fluorescence quantum yield as 0.9 for fluorescein^{42,43}, two-photon emission quantum yield of synthesized aqueous graphene oxide is determined to be 0.34. Next, we have measured two-photon absorption cross-section values using the following equation^{42,43}.

$$\sigma_{\text{GO}} = \sigma_{\text{FL}} \cdot (F_{\text{GO}}/F_{\text{FL}}) \cdot (\Phi_{\text{FL}}/\Phi_{\text{GO}}) \cdot (C_{\text{FL}}/C_{\text{GO}}) \quad (1)$$

where the observed fluorescence intensity has been represented by F , the quantum yield is represented by Φ and C is the concentration. Using $\sigma_{\text{FL}} = 36 \text{ GM}$ [$1 \text{ GM} = 10^{-50} (\text{cm}^4 \text{ s/photon})$] at 760 nm excitation, as reported by Albota et. al.⁴³, we found that the two-photon absorption cross-section is 50840 Goeppert-

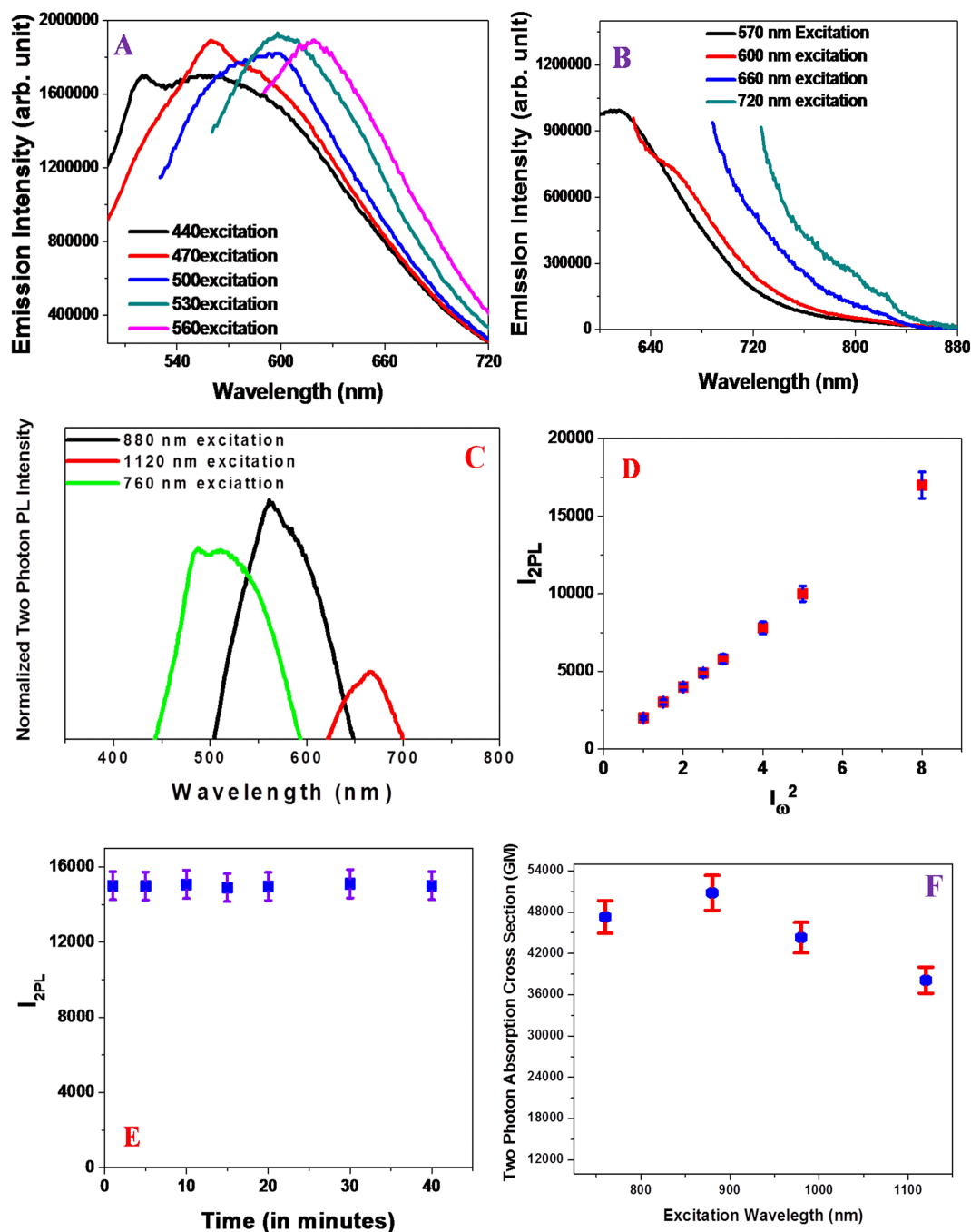


Figure 2 | (A), (B) Excitation wavelength dependent single photon photoluminescence from freshly prepared graphene oxide. Plot clearly shows that water soluble GO photoluminescence can be tuned from the visible to near NIR region just by varying the excitation energy, without changing its chemical composition and size. (C) Excitation wavelength dependent two-photon photoluminescence from freshly prepared graphene oxide. Plot clearly shows that two-photon photoluminescence from GO can be tuned just by varying the NIR excitation energy from 760 to 1120 nm, without changing its chemical composition and size. (D) Plot shows how the photoluminescence at 680 nm varies with the square of intensity of 1140 nm excitation. Our result shows that the observed photoluminescence is indeed a two-photon process. (E) Plot demonstrates the photostability of aptamer APT^{SEB1}-modified graphene oxide. Our result clearly shows that even after an hour of exposure, the two-photon luminescence intensity remains constant. (F) Plot demonstrates the wavelength dependent two-photon absorption cross-section of aptamer APT^{SEB1}-modified graphene oxide.

Mayer units (GM), at 760 nm excitation for -NH modified aptamer APT^{SEB1} attached graphene oxide, which is the highest reported value in literature. Our measured σ_{2PA} for APT^{SEB1} attached graphene oxide is few orders of magnitude larger than that of organic molecules and even higher than that of the highest reported two-photon absorption cross-section for QDs^{14,41}. Figure 2F shows the wavelength dependent two-photon absorption cross-section. Very high σ_{2PA} for APT^{SEB1} attached graphene

oxide can be due to several highly efficient intramolecular charge transfer between large π -conjugated systems of water soluble graphene oxide and the strong electron denoting carboxy, hydroxyl and amine group. Very high intramolecular charge transfer enhanced the two-photon absorption cross-section and as a result, strong two-photon-induced fluorescence has been observed. Due to the very high two-photon absorption cross-section and good quantum yield, graphene oxide synthesized by our group is an

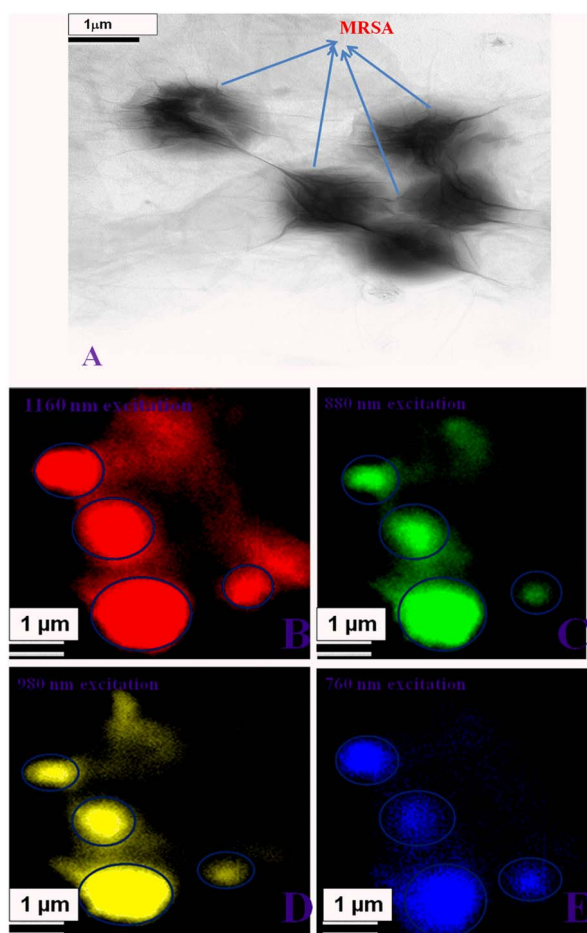


Figure 3 | (A) TEM image shows aggregation of MRSA bacteria inside the aptamer APT^{SEB1}-modified graphene oxide sheet after the addition of 4.4×10^2 cfu/mL MRSA. (B–E) Multicolor two-photon luminescence Imaging of MRSA using aptamer APT^{SEB1}-modified graphene oxide sheet. MRSA positions have been indicated by “circle”. (B) TPL image performed at 1160 nm excitation. (C) TPL image performed at 880 nm excitation. (D) TPL image performed at 980 nm excitation. (E) TPL image performed at 760 nm excitation.

highly promising two-photon luminescence microscopy imaging contrast agent for bio-imaging.

Since it is known that aptamer APT^{SEB1} can be used for very selective detection of *Staphylococcus aureus*³⁵, for the selective detection of MRSA, we have used an aptamer APT^{SEB1}-modified graphene oxide. After that, graphene oxide sheets were modified with polyethylene glycol (PEG) to avoid the aggregation in media. Next, we have incubated aptamer APT^{SEB1}-modified graphene oxide with different concentrations of MRSA for 30 minutes. After that, unconjugated MRSA were separated using centrifugation followed by washing with buffer. We have performed this process three times to make sure that unconjugated MRSA are separated nicely from graphene oxide sheets. Figure 3A clearly shows that in the presence of aptamer-modified hybrid GO, MRSA is conjugated inside of the graphene oxide sheet. Next, to understand whether the two-photon luminescence of graphene oxide can be tuned just by varying the excitation energy from the first to the second biological transparency windows, we have performed two-photon photoluminescence experiments at 760, 880, 980 and 1120 nm excitation wavelength. Figures 3B–3E clearly show that the two-photon luminescence of graphene oxide can be used for multi-color bio-imaging and it is due to the fact that two-photon photoluminescence from GO can be tuned just by varying the NIR excitation energy without changing its chemical composi-

tion and size, as we have discussed before. Due to the interaction of aptamer attached graphene oxide sheets and bacterial surface, GO density is much higher on the top of MRSA than in other places, as shown in Figure 3A. As a result, we have observed much brighter two-photon fluorescence from graphene oxide on top of MRSA, as shown as “circle” in Figure 3B–3E. Our experimental data show that the fluorescence image becomes brighter for bigger “circle” due to the strong interaction between hybrid GO and aggregated MRSA. The above interaction helps us to locate MRSA in the fluorescence image in the presence GO two-photon fluorescence background. To find out the biocompatibility of our aptamer APT^{SEB1}-modified graphene oxide, biocompatibility of graphene oxide sheet was found out using MRSA. For this purpose, 2.4×10^2 cfu/mL MRSA was incubated with aptamer APT^{SEB1}-modified graphene oxide for different time intervals till 24 hours. As shown in Figure 4B, even after 24 hours of incubation with MRSA, we have observed roughly 98% cell viability, which clearly indicates very good biocompatibility of water soluble GO synthesized in our lab.

Next, to find out that whether aptamer APT^{SEB1}-modified graphene oxide based two-photon luminescence is selective for MRSA or not, we have incubated aptamer APT^{SEB1}-modified graphene oxide with 4.4×10^3 cfu/mL of Salmonella DT104 for 30 minutes. After that, unconjugated Salmonella DT104 were separated using centrifugation followed by washing with buffer. We have performed this process three times to make sure that unconjugated Salmonella DT104 are separated nicely from graphene oxide sheets. Two-photon fluorescence image as shown in Figure 4C and TEM image as shown in Figure 4A clearly show that Salmonella DT104 does not bind with aptamer APT^{SEB1}-modified graphene oxide sheet. The above experimental data clearly indicate that aptamer APT^{SEB1}-modified graphene oxide based two-photon luminescence is highly selective for MRSA imaging.

Conclusion

In conclusion, in this manuscript, we have reported the development of aptamer conjugated graphene oxide as an excitation tunable two-photon luminescence platform for targeted bio-imaging. Our experimental results show extremely high two-photon absorption cross-section (50800 GM) from aptamer conjugated graphene oxide. Excitation wavelength dependent two-photon photoluminescence data indicate that two-photon photoluminescence from GO can be tuned from blue to red, without changing its chemical composition and size. One can tune the color by just varying the NIR excitation energy from 760 to 1120 nm. This very high two-photon absorption cross-section and wavelength tunable good photoluminescence efficiency indicate that APT^{SEB1}-modified graphene oxide can be a highly efficient two-photon fluorescent probe for multi-color MDRB imaging. Our results show that aptamer APT^{SEB1}-modified graphene oxide based two-photon luminescence platform can be used for selective and multi-color imaging of MRSA, in the first and second biological transparency windows. Our reported data demonstrate that aptamer modified graphene oxide possesses very bright two-photon fluorescence, high photostability, as well as excellent biocompatibility, which is essential for two-photon microscopy. After proper engineering, we believe that graphene oxide based two-photon photoluminescence can be a good choice for multicolor bio-imaging applications.

Methods

Materials and experiments. Graphite, KMNO₄, HNO₃, were purchased from Sigma-Aldrich and used without further purification. Aptamers were purchased from Midland Certified Reagent. MRSA bacteria were purchased from the American Type Culture Collection (ATCC, Rockville, MD).

Synthesis of graphene oxide and aptamer APT^{SEB1}-modified graphene oxide.

Graphene oxide from graphite was synthesized using strong oxidizing agents as we have reported recently^{17,18}. In brief, we have used graphite exfoliation by strong oxidizing agents to yield graphene oxide using modified Hummers reported the

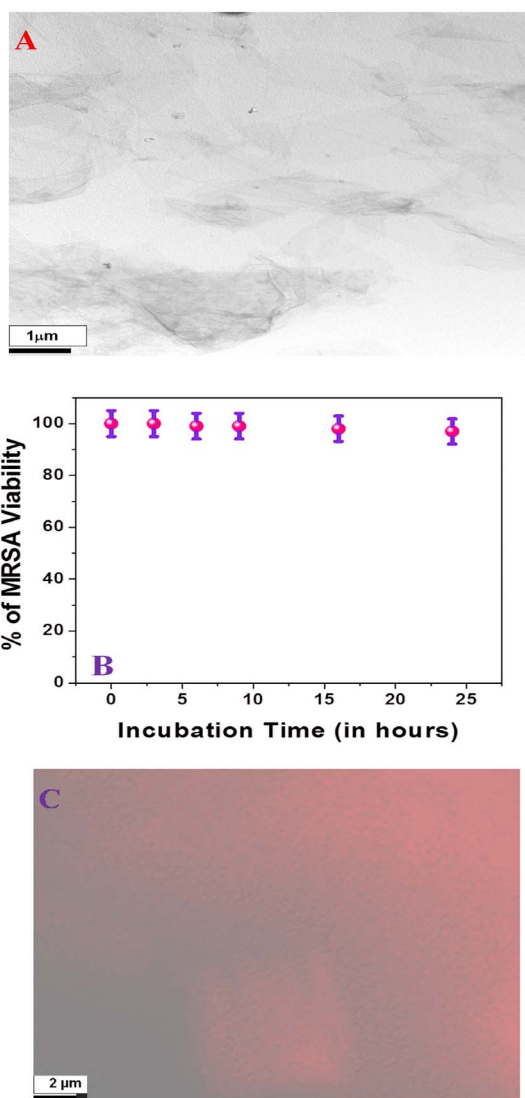


Figure 4 | (A) TEM image also shows that *Salmonella* DT104 does not bind with aptamer APT^{SEB1}-modified graphene oxide sheet. (B) Plot demonstrates the biocompatibility of aptamer APT^{SEB1}-modified graphene oxide sheet. Even after 24 hours of incubation with MRSA, we have observed about 98% of cell viability. (C) TPL imaging of *Salmonella* DT104 using aptamer APT^{SEB1}-modified graphene oxide sheet. Our data show that *Salmonella* DT104 does not bind with aptamer APT^{SEB1}-modified graphene oxide sheet.

method. After that, we have used FTIR, Raman and high-resolution TEM to characterize graphene oxide, as we have reported before. Next, to attach -NH modified aptamer APT^{SEB1} to graphene oxide, acid chloride functionalized graphene oxide was prepared by treating -COOH functionalized graphene oxide with thionyl chloride.

Bacteria sample preparation. For culturing MRSA bacteria, we followed ATCC protocol. After culturing MRSA bacteria, from the stock solution, we have different concentrations of MRSA from 10–10⁷ CFU/mL. Next, we have incubated different amounts of bacteria with APT^{SEB1} aptamer modified graphene oxide solution at room temperature. After that, unconjugated bacteria were separated using centrifugation followed by washing with buffer. We have performed this process three times to make sure that unconjugated MRSA were separated nicely from graphene oxide sheets.

Two-photon photoluminescence measurement. For two-photon imaging at different excitation wavelengths with near-infrared light, we have used Nikon multiphoton microscope (FV1000MPE) as we have reported before⁴⁰. We have used 80 MHz Ti:sapphire laser as an excitation source with 100 femto second (fs) pulse width and 80 MHz repetition rate. Tunable wavelengths in two biological transparency windows were generated using optical parametric amplifier.

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Author contributions

P.C.R. designed the experiment. A.P., Z.F., S.R. and S.S.S. conducted all experiments. All the authors contributed on the discussion part of all the results. P.C.R. wrote the manuscript. A.P., Z.F., S.R. and S.S.S. commented on the manuscript at all stages.

Additional information

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