Highly Efficient Nuclear Delivery of Anti-cancer Drugs using a Bio-functionalized Reduced Graphene Oxide

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Abstract

Targeted drug delivery has become important, attractive and challenging in biomedical science and applications. Anti-HER2 antibody-conjugated poly-L-lysine functionalized reduced graphene oxide (anti-HER2-rGO-PLL) nanocarriers were prepared to efficiently deliver doxorubicin targeting at the nucleus of HER2 over-expressing cancer cells. The polycationic PLL was first covalently grafted to graphene oxide (GO) nanosheets followed by reduction to obtain rGO-PLL with high drug loading and good colloidal stability. The anti-HER2 antibodies were subsequently conjugated to the amino groups of PLL to achieve excellent cell uptake capability. Cellular uptake of anti-HER2-rGO-PLL into MCF7/HER2 cells is significantly higher than that of rGO-PLL due to the specific targeting of anti-HER2 to HER2 overexpressing breast cancer cells. Additionally the anti-HER2-rGO-PLL enables a fast accumulation of DOX inside the nucleus, its subcellular site of action. In vitro cytotoxicity measurements clearly reveal a seven fold improvement in the anticancer efficacy for anti-HER2-rGO-PLL/DOX in comparison to rGO-PLL/DOX. The enhanced anticancer efficacy could be ascribed to the different intracellular DOX distributions resulted from the different internalization routes that are energy-dependent macropinocytosis and energy-independent direct penetration by anti-HER2-rGO-PLL and rGO-PLL, respectively. The results demonstrate that anti-HER2 conjugated rGO-PLL developed is a promising vehicle for efficient nuclear delivery of chemotherapeutic agents to HER2 over-expressing tumours.

Key words: drug delivery, reduced graphene oxide, anti-cancer drugs, breast cancer, poly-L-lysine, endocytosis

Introduction

Recently, graphene and its derivatives have attracted an overwhelming interest for potential medical or biological applications such as biosensors, cellular imaging, and drug delivery ^[1-9]. Graphene based nanomaterials possess large specific surface areas (theoretical value $\sim 2630 \text{ m}^2\text{g}^{-1}$) ^[2, 10] and are able to complex with aromatic drugs via π - π stacking and/or hydrophobic interaction ^[11,12] for excellent drug storage property ^[13]. Moreover, drugs loaded on graphene are highly stable to avoid any premature release outside the target cell. One challenge is that graphene-based materials are easy to agglomerate in buffer or cell medium to significantly decrease the drug loading surface areas and thus reducing the cellular uptakes. It has been reported that graphene and its derivative nanomaterials can be functionalized with polyethylene glycol (PEG) ^[6,14,15], polyethylenimine (PEI) ^[16], gelatin ^[17], chitosan ^[18] or sulfonic acid groups ^[13] via covalent or noncovalent strategies for good dispersion and stability in physiological solutions. Further conjugation with targeting ligands such as folic acid ^[13,19,20] or Rituxan (anti-CD20) ^[14] can enhance the internalization into specific cell types for an improved therapeutic efficacy. However, as many drug targets are localized to subcellular compartments, the cell specific targeting with this approach is still not sufficient and leads to either drug sequestration in endocytotic pathways or pervasive drug distribution throughout a cell. Thus, an efficient subcellular targeting strategy is very challenging but is highly demanded to achieve high therapeutic efficacy in clinical applications ^[21-23].

Many anticancer drugs such as doxorubicin (DOX), camptothecin (CPT) and cisplatin kill cancer cells by intercalating nuclear DNA for direct damage or inhibiting topoisomerase involved in DNA replication^[24-27]. The therapeutic potentials can only be fully realized if the drug can be specifically delivered into the subcellular site of action, the nucleus. To efficiently deliver doxorubicin to the nucleus of HER2 over-expressing cancer cells, poly-L-lysine (PLL) is covalently conjugated to graphene oxide (GO) nanosheets, followed by reduction of the GOs to form PLL-functionalized reduced GOs (rGOs), and subsequently labelling with anti-HER2 antibodies (Scheme 1). PLL, a cationic polymer that easily penetrates cell and organelle membranes^[24], is conjugated to graphene oxide (GO) nanosheets for stabilizing the nanocarriers in physiological solutions by electrostatic repulsions, while its plentiful active amino groups allow functionalization with targeting ligands^[28]. PLL renders the nanocarriers with positive charges to enhance the interactions between the nanocarrier and the negatively charged cell membrane for efficient nanocarrier uptake. Results indicate that rGO-PLL has high drug loading capacity and good colloidal stability. For subcellular targeting, rGO-PLL is further labelled with anti-HER2 to bind with HER2, a transmembrane tyrosine kinase receptor over-expressed in 25-30% of human breast cancer^[29]. The internalization ability of HER2 can have an efficient uptake of the anti-HER2 antibodies or their conjugates^[30].

It is discovered that anti-HER2 conjugated rGO-PLL nanocarriers allow a rapid nuclear accumulation of DOX within 4 h whereas DOX molecules are still trapped in the cytoplasm for the rGO-PLL nanocarriers. The fast nuclear transport of DOX leads

to a significant improvement of anti-cancer efficacy. Investigation of the cellular uptake routes reveals that the anti-HER2 conjugated rGO-PLL nanocarrier is internalized via energy-dependent macropinocytosis, while the uptake of rGO-PLL is energy-independent. It is believed that the greatly improved anticancer efficacy could be ascribed to the synergistic effect of the high drug loading from the reduced graphene oxide, effective colloidal stability provided by polycationic PLL and specific targeting of anti-HER2.

Materials and Methods

2.1 Chemicals

Chlorpromazine hydrochloride, Wortmannin from *Penicillium funiculosum*, Amiloride hydrochloride hydrate, Methyl- β -cyclodextrin (M β CD), Genistein and Poly-L-lysine hydrochloride (PLL), mol wt 15,000-30,000 was purchased from Sigma (St.Louis, MO). Doxorubicin hydrochloride (DOX), Cytochalasin D and Monoclonal anti-HER2 antibodies (anti-HER2) produced in mouse was obtained from Merck. The deionized water (18.2 M Ω •cm) was obtained from a Millipore Milli-Q water purification system. All other reagents were of analytical grade.

2.2 Synthesis of poly-L-lysine functionalized reduced graphene oxides (rGO-PLL)

Graphene oxide (GO) was prepared by a modified Hummer's method^[31,32]. The rGO-PLL was prepared by vigorously stirring a solution of 5 mg GO, 20 mg PLL, and 25 mg KOH in 25 mL of H₂O at 70°C for 24 h. PLL is covalently grafted onto GO

through the reaction of epoxy groups on GO and amino groups on PLL in alkaline conditions. Then 2 mL of 1 M NaBH₄ solution was added and reacted at 70°C for another 2 h to reduce GO. After that, the rGO-PLL obtained was centrifuged and washed with H₂O for 3 times to remove impurities and physically adsorbed PLL^[28].

2.3 Immobilization of anti-HER2 to PLL-rGO

Anti-HER2 antibodies were coupled to the amino groups of PLL via glutaldehyde bifunctional linkages. Briefly, 2 mL of rGO-PLL solution was mixed with 40 µL of glutaldehyde solution (50% v/v) and stirred at room temperature for 15 min. 5 µL of anti-HER2 (1 mg/mL) was then added to the mixture to react for 2 h. The reaction mixture was centrifuged and washed with PBS to obtain the anti-HER2 labelled rGO-PLL.

2.4 Characterization

The product morphology was examined by transmission electron microscope (TEM; JEOL, JEM-2100F, 200 kV) and field-emission scanning electron microscope (FESEM; JEOL, JSM-6700F, 5 kV). Ultraviolet-visible (UV-vis) absorption spectra of samples were collected using a spectrophotometer (Nanodrop 2200). Zeta potentials and hydrodynamic sizes were measured using a Zetasizer 3000 (Malvern Instruments). Fourier transform infrared spectroscopy (FTIR) spectra were obtained with a Perkin Elmer FT-IR Spectrum GX.

2.5 Determination of drug loading capacity

Aqueous anti-HER2 labelled or non-labelled rGO-PLL suspensions (2 mL, 0.7 mg

mL⁻¹) were mixed with DOX solution (0.2 mL, 2 mg mL⁻¹) and stirred at room temperature for 24 h. At specified time points, the DOX loaded sample was centrifuged and the characteristic DOX absorbance at 490 nm in the supernatant was measured using an UV-vis spectrophotometer. Loading capacity was calculated according to Equation 1.

$$\text{Drug loading capacity} = \frac{\text{Mass of drug loaded}}{\text{Mass of drug carrier}} \times 100\% \quad (1)$$

2.6 In vitro release study

The release of DOX was tested in phosphate-buffered saline (PBS) at 37°C. The mixture was centrifuged every 24h to measure the absorbance of the supernatant to estimate the released amount. The percentage of drug released was given by Equation 2.

$$\text{Percentage of drug released} = \frac{\text{Mass of drug released}}{\text{Mass of drug loaded}} \times 100\% \quad (2)$$

2.7 Cell culture

Human breast cancer MCF7 cells were grown in Minimum Eagle's Medium (MEM) (PAA Laboratories, Pasching, Austria) supplemented with 10% heat inactivated fetal bovine serum (FBS) (PAA Laboratories), 1 mM sodium pyruvate (PAA Laboratories) and 50 U/mL penicillin/streptomycin (Gibco, Grand Island, NY). HER2 over-expressing MCF7 (MCF7/HER2) cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM) (PAA Laboratories) supplemented with 10% heat inactivated FBS, 0.5 mg/mL G418 (PAA Laboratories) and 50 U/mL penicillin/streptomycin (Gibco). The cells were grown in a humidified incubator at 37°C with 5.0% CO₂. Cells

were seeded at a density of 1×10^4 cells cm^{-2} onto poly-L-lysine (0.1 mg mL^{-1}) coated coverslips for cell attachment.

2.8 Cell viability assay

The viability and proliferation of cells were evaluated using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay in triplicate. MCF7/HER2 cells were seeded into 96-well plates at a density of 1×10^4 per well in 200 μL of media and grown overnight. The cells were then exposed to various concentrations of DOX, DOX free samples or DOX loaded samples for 24 h. Following this incubation, cells were incubated in DMEM containing 0.5 mg/mL of MTT for 4 h. Thereafter, the MTT solution was removed and the precipitated violet crystals were dissolved in 200 μL of DMSO. The absorbance was measured at 570 nm using a BioTek microplate reader.

2.9 Cellular uptake

To investigate cellular uptake, the non-labelled or anti-HER2 labelled rGO-PLL samples were mixed with DOX overnight at room temperature to form complexes. Unbound DOX was removed by repeated washing. The cells were incubated with 50 $\mu\text{g/mL}$ DOX-rGO- PLL or anti-HER2-rGO-PLL. After specified durations, the cells were washed three times with PBS buffer and the fluorescence images were acquired by confocal laser scanning microscopy (CLSM) (LSM 510 META, Carl Zeiss, Germany).

2.10 Endocytotic inhibition study

The effect of temperature block was studied by pre-incubating the cells at 4°C for

1h and then treated with the DOX complexed samples (50 $\mu\text{g/mL}$) for 2 h at 4 $^{\circ}\text{C}$. To study the effect of various inhibitors on the uptake of samples, cells were pre-incubated with the following inhibitors individually at concentrations as listed in Table 1. In the study, the group without any treatment represents the negative control, while the groups in the presence of samples but without inhibitor treatment were used as positive controls and their fluorescence intensities were expressed as 100%. Following the preincubation for 1 h, the inhibitor solutions were removed, and the freshly prepared samples in media containing inhibitors at the same concentrations were added and further incubated for 2h. Subsequently, the cells were washed three times with PBS, collected according to the methods described above and analyzed.

Results and discussion

Fig. 1a shows that the UV-vis spectra of PLL, GO and rGO have absorption peaks at 200 nm, 230 nm and 263 nm, respectively. After rGO-PLL formation, the absorption peaks appear at 196 nm and 263 nm, indicating the successful grafting of PLL and the restoration of electronic conjugation within the graphene basal planes. These two characteristic peaks also exist in the anti-HER2 labeled rGO-PLL. The reduction of GO is evident in the inset of Fig. 1a. The rGO-PLL solution (III) is black, similar to that of the rGO solution (II), whereas GO solution (I) exhibits a distinct brown colour. FTIR spectra (Fig. 1b) confirm the existence of C=O (1723 cm^{-1}), C=C (1590 cm^{-1}), and C-O (1042 cm^{-1}) functional groups in GO, whereas for rGO and rGO-PLL, the peak at 1723 cm^{-1} diminishes, signifying the reduction of GO to rGO. The FTIR spectrum of rGO-PLL displays typical PLL absorption peaks including N-H (in free

NH₃⁺) at 3267 cm⁻¹, NH-CO stretching vibration at 1649 cm⁻¹ and N-H II band at 1539 cm⁻¹.^[33]

FESEM images show that the rGO-PLL nanosheets (Fig. 1c) are much rougher covering with condensed PLL particles as compared to the plain rGO (Fig. 1d). The average hydrodynamic sizes of rGO, rGO-PLL and anti-HER2-rGO-PLL determined from Fig. 1d are 130 nm, 340 nm and 270 nm, respectively. The size of nanocarriers increases significantly after PLL functionalization increases but decreases upon conjugation with anti-HER2. The decrease in size may be due to the crosslinking effect of glutaldehyde.

The measured zeta potentials of rGO, rGO-PLL and anti-HER2-rGO-PLL are -45.3±13.8 mV, 55.1±8.0 mV and 40.7±9.3 mV, respectively (Fig. 1e). After functionalization with polycationic PLL, the rGO-PLL possessed plentiful active amino groups thus exhibiting very positive zeta potential. When anti-HER2 antibodies were conjugated onto rGO-PLLs, a substantial amount of the amino groups participated in the amide bond formation thus leading to a less positive zeta potential of anti-HER2-rGO-PLL as compared to rGO-PLL. It is observed that rGO, rGO-PLL and anti-HER2-rGO-PLL disperse well in water. rGO quickly aggregates whereas both rGO-PLL and anti-HER2-rGO-PLL remain well dispersed in phosphate buffered saline (PBS) (Fig. S1).

It is postulated that DOX can be efficiently loaded on the rGO based nanocarriers via π - π stacking and hydrophobic interactions, and we expect these interactions to be

stronger than the charge repulsions. The DOX loading capacities of rGO-PLL and anti-HER2-rGO-PLL are measured to be 41.2% and 37.2%, respectively (Fig.S2a). The slightly lower loading capacity for anti-HER2-rGO-PLL as compared to rGO-PLL might be due to the slightly increased steric hindrance for DOX molecules to interact with the rGO surfaces after the conjugation with antibodies. Overall, these two nanocarriers exhibit loading capacities lower than unmodified GO/rGO,^[7] but much higher than conventional drug carriers such as polymer and silica nanoparticles.^[34] Negligible DOX releases were observed from both samples after three day incubation in PBS (Fig.S2b). This confirms the stability of DOX on rGO-PLL and anti-HER2-rGO-PLL, and further suggests that DOX can be protected in the bloodstream before reaching the target tumour site.

As shown in Fig. 2a and b, the anti-cancer efficacy of DOX loaded anti-HER2-rGO-PLL to MCF7/HER2 cells is much higher than that of DOX loaded rGO-PLL. At 24 h, the 50% growth inhibition concentration (IC₅₀) for anti-HER2-rGO-PLL/DOX is about 0.8 $\mu\text{g mL}^{-1}$, more than 7 times lower than rGO-PLL/DOX (6 $\mu\text{g mL}^{-1}$). Although the IC₅₀ value for anti-HER2-rGO-PLL/DOX is comparable to using DOX alone, drug delivery using this nanocarrier offers several critical advantages over applying the drug alone. Firstly, DOX loaded on anti-HER2-rGO-PLL are highly stable so as to avoid any premature release before reaching the target tumor site. Secondly, anti-HER2-rGO-PLL nanocarriers enable specific targeting to HER2 over-expression cancer cells, so as to avoid the severe side effects of DOX to off-target cells such as cardiac cells. Further increase of incubation

time to 48 h leads to a 4-fold reduction of the IC₅₀ value for anti-HER2-rGO-PLL/DOX to 0.2 $\mu\text{g mL}^{-1}$. The effective dosage of anti-HER2-rGO-PLL/DOX needed to kill 80% of cancer cells is only $\sim 5 \mu\text{g mL}^{-1}$, while the anti-HER2-rGO-PLL nanocarrier itself has negligible cytotoxicity against MCF7/HER2 cells even at a high concentration of 80 $\mu\text{g mL}^{-1}$ (Fig.S3). These results show that anti-HER2 labelled nanocarrier greatly enhances anti-cancer efficacy and improves targeting specificity to minimize side effects, thus offering an ideal nanocarrier for drug delivery.

Highly efficient internalization of nanocarriers into cancer cells and the specific accumulation of anti-cancer drugs to its subcellular site of action play a critical role in achieving high anti-cancer efficacy. In order to understand the effect of different nanocarriers on anti-cancer efficacy, DOX fluorescence was monitored to investigate cellular uptake, DOX release from the nanocarriers and DOX intracellular distribution. We have confirmed that adsorption of DOX on rGO-PLL or anti-HER2-rGO-PLL could completely quench fluorescence via fluorescence resonance energy transfer (FRET),^[35] while releasing DOX from the nanocarriers upon isopropanol addition can recover the fluorescence (Fig.S4).

As shown in Fig. 3a & b, much stronger DOX fluorescence was observed in MCF7/HER2 cells (HER2 positive) than in MCF7 cells (low HER2 expression), clearly indicating higher uptake of anti-HER2-rGO-PLL/DOX by MCF7/HER2 cells and specific targeting of anti-HER2-rGO-PLL/DOX at the cells with HER2 receptors. The HER2 expression in MCF7/HER2 cells has been confirmed using Western blot

(Fig.S5) and immunofluorescence (Fig.S6). Notably, the DOX fluorescence is more intense in the nucleus as compared to the cytoplasm proving the effective nuclear accumulation of DOX molecules after the internalization of anti-HER2-rGO-PLL/DOX into MCF7/HER2 cells (Fig. 3a). On the contrary, for the non-labelled rGO-PLL, the DOX fluorescence only appears in the cytoplasm surrounding the nucleus after incubation with both MCF7 and MCF7/HER2 cells for the same incubation duration (Fig. 3c & d). Many bright fluorescence spots are found in the cytoplasm of MCF7/HER2 cells, suggesting that the internalized DOX is released from rGO-PLL but trapped in endocytic compartments such as endosomes or lysosomes, thus compromising its anti-cancer efficacy (Fig. 3c). These results indicate that anti-HER2 labelling not only enables specific targeting to HER2 over-expressing breast cancer cells but also promotes the nuclear accumulation of DOX.

We further examined the intracellular localization of FITC labelled anti-HER2-rGO-PLL, rGO-PLL and IgG-rGO-PLL. As shown in Fig. 4a, intense green fluorescence is observed in MCF7/HER2 cells after the addition of FITC labeled anti-HER2-rGO-PLL, whereas only very weak fluorescence is found in cells treated with FITC labeled rGO-PLL (Fig. 4b). In addition, negligible fluorescence is observed in non-specific IgG labelled rGO-PLL, reconfirming the specific binding role of anti-HER2 to its receptors is necessary for efficient uptake. The untreated MCF7/HER2 cells show no fluorescence as expected ruling out the influence of autofluorescence (Fig.S7). These results clearly demonstrate that the highest uptake is achieved by anti-HER2-rGO-PLL, which helps to explain the significant nuclear DOX

accumulation observed previously in MCF7/HER2 cells treated with DOX loaded anti-HER2-rGO-PLL.

The time-dependent uptake of anti-HER2-rGO-PLL/DOX into MCF7/HER2 cells was also monitored (Fig. 5a & b). After 2 h incubation, DOX fluorescence was observed throughout the entire cell with a significant accumulation in the perinuclear region. This observation agrees with the report that endocytosed HER2 conjugates are rapidly transported towards the perinuclear region along microtubules.^[36] As the incubation time increases to 4 h, the DOX fluorescence in the nucleus became more intense proving the nuclear accumulation of DOX. The cross-section analysis of Z-stacked fluorescence images confirms the localization of DOX in the nuclear compartment and it is clear that the nuclear DOX concentration increases quickly with the incubation time. Since DOX is an anticancer drug that targets the DNA, effective nuclear accumulation of DOX leads to a better anti-cancer effect for anti-HER2-rGO-PLL/DOX.

The cell uptake mechanisms of the nanocarriers were subsequently studied to identify the internalization pathways utilized and explain the efficient nuclear drug accumulation. Endocytic uptake is an energy-dependent mechanism that can be strongly inhibited by lowering the temperature.^[37] We first investigated if the cellular uptakes of anti-HER2 labelled and non-labelled rGO-PLL nanocarriers were energy-dependent. Upon reducing the incubation temperature from 37°C to 4°C, the cellular uptake of anti-HER2 labelled rGO-PLL nanocarriers was also significantly reduced (Fig. 6a & b), indicating that anti-HER2-rGO-PLL is internalized *via*

energy-dependent pathways. On the contrary, the cellular uptake of non-labelled rGO-PLL nanocarriers was not significantly affected by temperature reduction (Fig. 6c & d), suggesting an energy-independent uptake route.

To further elucidate the cellular uptake pathways, we studied the nanocarrier uptake in the presence of a series of chemical inhibitors for clathrin-mediated endocytosis, macropinocytosis and caveolae-mediated endocytosis. The uptake of rGO-PLL is not affected by any of these inhibitors, which agree with its energy-independent nature of uptake (Fig. 7a). In contrast, anti-HER2-rGO-PLL is only significantly affected by cytochalasin D (Fig. 7b), displaying a ~ 45% reduction in the cellular uptake. Cytochalasin D is known to inhibit actin polymerization and membrane ruffling, which is involved in macropinocytosis. Thus, we believe that the main uptake route of anti-HER2-rGO-PLL is via macropinocytosis. The endocytosis inhibition experiments reveal that rGO-PLL enters cells by passive penetration due to the positive charges in an energy-independent manner while the anti-HER2-rGO-PLL is uptaken into cells via an active macropinocytosis route. The different cellular uptake routes utilized by the anti-HER2 labelled vs. unlabelled nanocarriers may explain the large differences in their anti-cancer efficacies. The energy-independent uptake of rGO-PLL is limited due to its passive and uncontrolled nature. In contrast, the energy-dependent internalization of anti-HER2-rGO-PLL is much higher because of an active receptor-mediated process. The specific cell targeting and higher cellular uptake of anti-HER2-rGO-PLL leads to improved nuclear accumulation of DOX and consequently higher anti-cancer efficacy.

325

326 **Conclusions**

327 A highly stable anti-HER2 antibody-conjugated poly-L-lysine functionalized reduced
328 graphene oxide (anti-HER2-rGO-PLL) drug delivery system was successfully
329 developed to facilitate the targeted uptake in HER2 overexpressing breast cancer cells
330 and subsequently enhance the accumulation of an anti-cancer drug to the nucleus. The
331 seven-fold improvement of anticancer efficacy for anti-HER2-rGO-PLL as compared to
332 rGO-PLL nanocarrier can be attributed to its energy-dependent macropinocytosis
333 internalization route revealed by the endocytosis inhibition study. This work not only
334 provides an efficient vehicle for targeted delivery of anticancer therapeutics, but also
335 holds a great promise for broad applications in nanomedicine.

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337 **Acknowledgements**

338 This work is financially supported by National Science Foundation of China (No.
339 21205097), National Key Basic Research Program of China (973 Program) under
340 contract No.2013CB127804, Institute for Clean Energy & Advanced Materials
341 (Southwest University, Chongqing, China), Chongqing Key Laboratory for Advanced
342 Materials and Technologies of Clean Energies (Chongqing, China), Start-up grant
343 under SWU111071 from Southwest University (Chongqing, China), Chongqing
344 Engineering Research Center for Rapid diagnosis of Dread Disease (Chongqing, China)
345 and Chongqing development and reform commission (Chongqing, China).

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347 **References**

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Figures and Captions

Scheme 1 Synthesis of anti-HER2-rGO-PLL is performed in two steps: (I) GO is functionalized with PLL under alkaline conditions followed by NaBH_4 reduction to form rGO-PLL and (II) rGO-PLL is subsequently conjugated with anti-HER2 antibodies via glutaraldehyde bifunctional linkers.

Fig. 1 (a) UV-vis spectra of poly-L-lysine (PLL), graphene oxide (GO), reduced graphene oxide (rGO), PLL functionalized rGO nanocomposite (rGO-PLL) and anti-HER2 conjugated rGO-PLL (anti-HER2-rGO-PLL). Inset shows the photo of (I) GO, (II) rGO and (III) rGO-PLL solutions. (b) FTIR spectra of (I) PLL, (II) GO, (III) rGO-PLL and (IV) Anti-HER2- rGO-PLL. FESEM images of (c) rGO-PLL and (d) rGO and; (e) Hydrodynamic sizes and (f) Zeta potential distributions of rGO, rGO-PLL and anti-HER2-rGO-PLL.

Fig. 2 In vitro cytotoxicity of free DOX, DOX loaded rGO-PLL and DOX loaded anti-HER2-rGO-PLL against MCF7/HER2 cells after (a) 24 h and (b) 48 h incubation.

Fig. 3 Fluorescence (a-d) and merged bright-field and fluorescence images (e-h) of MCF7 (b, f, d & h) and MCF7/HER2 (a, e, c & g) incubated with DOX loaded rGO-PLL nanocarriers (a, b, e & f) or anti-HER2-rGO-PLL nanocarriers (c, d, g & h) for 4 h.

Fig. 4 The uptake of FITC labeled (a, d) Anti-HER2- rGO-PLL, (b, e) rGO-PLL and (c, f) IgG-rGO-PLL in MCF7/HER2 cells.

Fig. 5 Section analysis of MCF7/HER2 cells incubated with DOX loaded anti-HER2-rGO-PLL nanocarriers for (a) 2 h and (b) 4 h.

Fig. 6 The fluorescence images showing the cellular uptake of (a & b) anti-HER2 conjugated rGO-PLL nanocarriers vs. (c & d) non-labeled rGO-PLL nanocarriers after 2 h incubation at (a & c) 37°C and (b & d) 4 °C, respectively.

Fig. 7 Effects of inhibitors on the cellular uptake of (a) rGO-PLL nanocarriers vs. (b) anti-HER2-rGO-PLL nanocarriers

Table 1 The application of endocytotic inhibitors.

Inhibitor	Concentrations ($\mu\text{g/mL}$)	Process inhibited
Chlorpromazine	6	Clathrin-mediated endocytosis
Methyl- β -cyclodextrin	15	Non-clathrin-mediated endocytosis
Amiloride	1.5	Macropinocytosis
Wortmannin	0.21	Macropinocytosis
Genistein	50	Caveolae-mediated endocytosis
Cytochalasin D	10	Macropinocytosis, Caveolae uptake
Incubation at 4°C		All active processes