Loading harmine on nanographene changes the inhibitory effects of free harmine against MCF-7 and fibroblast cells

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Abstract
Today cancer is one of the main causes of death all over the world. Chemotherapy, which is one of the main therapies in the treatment of cancer, causes several side effects by damaging healthy cells. Therefore, carbon nanomaterial systems have been developed to optimize therapeutic procedures with the least negative consequences. Targeting nanographene oxide (NGO) with folic acid (FA) molecules allows the recognition of MCF-7 cells, which are folic acid receptor (FR) positive. Harmine is a pharmacologically active secondary metabolite that is produced by Peganum harmala. It is found that this metabolite induces apoptosis to human breast cancer cell lines by intercalating DNA molecules. In this study, harmine was loaded on FA-NGO (FA-NGO/harmine) via π–π stacking and hydrophobic interactions and the cytotoxicity against MCF-7, as FR positive cancerous cell, and fibroblast cells, as normal FR negative cell, were investigated. The in vitro studies illustrated that FA-NGO/harmine have remarkably higher cytotoxicity against MCF-7 cells, about 60% cell loss, in comparison with free harmine with 40% cell loss (in the concentration of 40 μg mL⁻¹). However, the released amount of harmine into normal fibroblast cells was considerably low, only 28% cell loss in dose of 40 μg mL⁻¹. Our results suggest that the controlled release of harmine into FR positive cancerous cells might have a substantially high cytotoxicity effect.

Keywords Nanographene · Drug delivery · Graphene oxide · Cancer cells · Cancer therapy · Harmine

Introduction
Cell membranes, as cellular barriers, lessen drugs’ therapeutic efficacy, so small molecules need to be passed from these membranes and delivered into cells. Various targeted cell delivery systems have been developed in the last decade to tackle this issue. These delivery systems are helpful in different therapeutic areas, such as cancer therapy [1–3].

Cancer is one of the most life-threatening diseases, and cancer therapy has received a significant attention due to its serious health threats. Physicians use chemotherapy to cure cancer; however, chemotherapy itself can cause various undesired and toxic side effects. Medicines with natural sources may decrease these toxic and noxious side effects [4, 5]. Harmine, which is derived from Peganum harmala, has been used in folk medicine for a long time [6, 7]. According to the studies, this natural β-carboline alkaloid has DNA intercalating and topoisomerases I and II inhibiting functions [8]. It causes DNA frameshift mutation and cytotoxic effects that is occurred by a significant inhibition of telomerase activity [9, 10]. Also, harmine was found to induce apoptosis in human breast cancer cell line by downregulation of TAZ gene, which encodes tafazzin protein [11].

Researchers examine different cancer-targeted delivery strategies in the last decade in order to establish a promising targeted delivery system and control the specified release of drugs. Graphene oxide (GO) is a two-dimensional nanomaterial [12] constructed from single-layer sheets of sp², in which each carbon is bonded to three carbon atoms with the bond angle of 120° and length of 1.42 Å [13]. Its intrinsic physical–chemical and structural properties have attracted much attention in various fields [14]. GO has been widely used in drug delivery due to its extensive hydrophobic surface area, high biocompatibility, and various surface functionalization [15]. A wide range of aromatic biomolecules can be loaded on this nanomaterial owing to its large

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specific surface area, and it could be efficient for gene transfection as well [5].

One way of achieving specific and targeted delivery is to modify the surface of nanomaterials with determined ligands, including small molecules such as folic acid (FA) [1]. Studies have shown that employing FA as a targeting agent would allow for the intercellular uptake of nanoparticles [2, 16]. Surface functionalization leads nanocarriers to identify the target tumor cells. This feature reduces the adverse effects of therapy while enhancing its therapeutic potential. A high selective tendency exists between folate receptors (FR) (which are overexpressed on some specific cancerous cells) and FA molecules. Therefore, cancer therapy could become more efficient after targeting nanocarriers with FA [17].

Deb and Vimala (2018) loaded anticancer drug, camptothecin (CPT) on GO-polyethylene glycol-GA and studied the enhanced cytotoxicity of CPT against the MCF-7 cell line by MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] [18]. Kun Ma et al. [1] developed a nanocarrier for the first time and targeted it with FA. In this study, the doxorubicin (DOX) loaded on GO was delivered to cells by soy phosphatidylcholine (SPC) nanocarriers. They represented GO/DOX@SPC-FA as a novel targeted nanohybrids that could improve delivery and cellular uptake [1]. Zhang et al. [17] investigated the controlled loading of two anticancer drugs, DOX and CPT, on GO-FA nanocarriers for the first time. They found that the ratio loading of nanoscale graphene oxide (NGO) for DOX could reach about 400%, which is much higher than other nanocarriers. They applied a targeted delivery system with FA conjugated GO to observe the effective delivery of DPX/CPT into cells through receptor-mediated endocytosis [17]. Ma et al. (2017) constructed FA-bovine serum albumin decorated GO as nanohybrids to enhance anticancer activity. Their work proved that FA-BSA/GO is a safe drug carrier. Also, cellular uptake analysis showed that this construct could have more efficiency than DOX, individually [19].

Along with the vast applications of graphene and other carbon-based nanomaterials, some investigations have evaluated the cytotoxicity of them against different types of cell lines [20, 21]. These recent experiments have demonstrated that graphene could have toxicity effect depend on the type of cell line, the injected dosage and the time duration of treating. Gurunathan et al. [22] found that only doses up to 60 μg mL⁻¹ of GO and bacterially reduced GO decrease MCF-7 cell viability. Meanwhile, Wang et al. [23] demonstrated that dose-dependent toxicity of GO against fibroblast cells is only for doses higher than 50 μg mL⁻¹. A noticeable study [24] showed obvious evidence about the reduced toxicity effect of GO or pristine graphene, when some molecules were tied to their surfaces. Lactobionic acid-polyethylene glycol-GO, PEG-GO, and polyethylenimine-GO could not damage human lung fibroblast cells as much as GO alone [23].

In this work, nanocarriers were synthesized, and harmine was loaded on nanocarriers as an anticancer drug. In order to enhance nanomaterials stability under physiological conditions, sulfonate groups were attached to NGOs, and also FA molecules were introduced to NGOs to target particular cells with FA receptors. Here, two groups of cells were used; MCF-7 cells, human breast cancer cells with overexpressed FA receptors, and fibroblast cells, as normal cells with minor FA receptors. Breast cancer was chosen as the disease model because it is one of the most prevalent diseases among women in the world [25, 26]. Harmine, a β-carboline alkaloid that has a water-insoluble aromatic structure, was loaded on NGO via π–π stacking and hydrophobic interactions. The loading capacity and releasing ratio of harmine were measured, and its in vitro cytotoxic effects against MCF-7 and fibroblast cells were determined. Furthermore, the effect of FA and targeted drug delivery against FR positive cells (MCF-7) was observed by a fluorescent microscope.

Results and discussion

Study the changes in surface functionalities

Figure 1a shows β-carboline structure of harmine, as well as the schematic diagram of nanocarriers’ preparation (Fig. 1b). The chemical structure of these nanocarriers was characterized using Fourier-transform infrared (FTIR) spectroscopy. FTIR is a fast, accessible, and simple instrumental technique, which has helped efficiently to determine the chemical structure of several biomasses.

As revealed in Fig. 2, the FTIR spectra of NGO-SO₃H and FA-NGO demonstrated the same bands at 1630–1660, 1100, 1046, 1186 cm⁻¹ and several bands at 660–800 cm⁻¹. The range of 1630–1660 cm⁻¹ corresponds to aromatic cycles. The peak at 1640 cm⁻¹ is assigned to C = O stretching vibration of COOH groups, and the considerable band at 1100 cm⁻¹ is allocated to C–O stretching vibration. The sulfonate groups that exist in both structures produce a number of significant absorption spectra. The range of 660–800 cm⁻¹ is assigned to S = O bending vibration, while 1046 and 1186 cm⁻¹ peaks correspond to symmetrical stretching and asymmetrical stretching of S = O, respectively [27].

In FA-NGO, the reaction between NH₂ groups in FA and COOH in NGO-SO₃H formed an amide bond, which creates new peaks in the FTIR spectrum. Absorption peaks at 1293 and 1700 represent C–N stretching band and C = O stretching in amide I vibration, respectively. Also, a broad peak in 3100–3500 cm⁻¹ refers to amide stretch (NH)
vibration. A distinct further absorption peak in 1593 cm$^{-1}$ is observed, which indicates C=N in FA structure [27].

In order to confirm the FA-NGO and FA-NGO/harmine formation, UV–Vis spectroscopy was carried out. The signature absorption peak of the NGO is at 235 nm, which shifted to 280 nm through the formation of an amide bond in FA-NGO (Fig. 3). After loading harmine on the nanocarrier sheets, in Fig. 4, the loading of the drug is recognizable.

**Loading and releasing harmine, as an anticancer alkaloid**

According to the wide two-dimensional surface of the NGO, and its large capacity of loading, we utilized targeted NGO to carry harmine into the cells. Harmine, which was investigated in our previous studies in terms of cytotoxicity features [7], was chosen as an anticancer drug, and its loading and releasing ratio on the graphene surface was evaluated.

**Loading drug**

After mixing harmine with FA-NGO aqueous suspension and removing the unbound excess harmine by repeated centrifugation, the resulted product was measured by UV–Vis spectroscopy. Figure 4 compares the absorbance plot of FA-NGO/harmine with harmine. It shows that harmine has been loaded on the nanographene surface. The characteristic absorbance peaks of harmine in 250, 300, and 370 nm can be observed in the mixed product at 255, 285, and 365 nm, respectively. The loading of harmine on the nanosheets resulted in this slight fluctuate. The main interaction between harmine and FA-NGO is $\pi-\pi$ stacking and
hydrophobic interactions, and the aromatic groups in both of these structures are the main cause of this linkage. Different concentrations of harmine, which were dissolved in dimethyl sulfoxide (DMSO), were mixed with FA-NGO to measure the loading ratio of harmine on the graphene sheets. After removing the unbound drugs, the best-loaded concentration of harmine on NGOs was chosen for the next steps. The amount of harmine loaded on FA-NGO was assessed by measuring harmine absorbance at 250 nm, after deducting FA-NGO absorbance.

As shown in Fig. 5, the loading ratio of 4000 μg mL⁻¹ harmine on FA-NGO is 180%. It is a remarkable percentage for loading drugs on nanographene sheets. This efficient loading has occurred due to the high number of π–π stacking interactions between harmine and nanographene. The surface of the sheets saturates gradually when harmine concentration increases. Therefore, after sheet saturation, the interactions among harmine molecules reduce the loading ratio, and the loaded molecules release from the sheets' surface.

Releasing ratio

The release of drug at pH 5 and 7 was investigated during 24 and 48 h. A direct correlation between drug-releasing and acidic pH was observed. In acidic pH, the hydrophilicity of harmine (mainly because of nitrogen atoms in the harmine structure) increases. Its hydration causes the release from the nanocarriers. It was found that after 48 h, the cumulative release of harmine at pH 5 is 65%. However, it is only 35% at neutral conditions (Fig. 6). Most of the malignant tumors’ environments are acidic with pH 6.5–6.9, while normal cells pH changes between 7.2 and 7.4 [28]. The acidic environment of tumor cells may lead to an increased risk in metastases occurrence [27, 29]. Therefore, due to this acidic environment of MCF-7 cells, pH-dependent releasing of drugs is beneficial.
FA receptors mediated pathway

The entrance mechanism of FA-NGO/harmine into MCF-7 cells through folate receptors mediated pathway is shown in Fig. 7, as a scheme. After endocytosis, endosomes turn into lysosomes in an acidic environment. Then their release ratio enhances, and the free drugs could pass through the lysosome membrane and spread out into the cytosol. In order to intercalate DNA and inhibit DNA topoisomerase enzymes, free harmine can enter the cell nucleus.

Rhodamine 6G uptake

The effect of FA molecules on targeted uptake of MCF-7 cells was analyzed by labeling FA-NGO and NGO with a fluorescent dye, rhodamine 6G. Rho 6G can be loaded on FA-NGO and GO by π-π stacking, hydrogen bonding, and hydrophobic interactions. After treating the cells with FA-NGO/Rho6G and NGO/Rho6G, the targeted uptake of MCF-7 by FA receptors was observed through the fluorescence microscope. Figure 8 demonstrates that FA receptors on MCF-7 surface cells could determine the FA molecules and improve the absorption of drugs. The results also represent that in extracellular environment, when Rho 6G is tied to FA-NGO or NGO, the fluorescent quenching occurs. However, in intercellular environment, in which pH condition is different, Rho releases from nanocarrier surface and therefore, the fluorescence emission of Rho 6G occurs.

MTT assays against MCF-7 cell lines and primary fibroblast cells were also carried out to investigate the targeted delivery of harmine. MCF-7 was used as cancerous FR positive cell, and primary fibroblast cell, as the normal FR negative cell.

Targeted drug delivery against MCF-7

The viability of MCF-7 cells, which were treated by 15, 30, and 40 μg mL⁻¹ of FA-NGO/harmine, NGO-SO₃H/harmine, and free harmine, respectively, and FA-NGO is shown in Fig. 9. 40 μg mL⁻¹ of FA-NGO/harmine and NGO-SO₃H could reduce cell viability to 42% and 47%, respectively, while 40 μg mL⁻¹ of harmine as a free drug could decline cell viability to 60%. These assays were done in 24 h. The loaded harmine on nanocarriers at higher concentrations, whether targeted via FA or not, was more capable of killing cancerous cells in comparison with free harmine. The controlled release of harmine from nanographene surface in acidic environment of MCF-7 cells has effectively raised its cytotoxicity. Each nanosheet contains a large number of harmine molecules (according to loading ratio of 180%), which are being released in the cell, cumulatively. The cytotoxicity of harmine when it is bonded to FA-NGO is higher than harmine bonded to untargeted NGO due to the delivery of harmine into cells by receptor-mediated endocytosis. The toxicity of FA-NGO without harmine was also evaluated using MTT assay for MCF-7, and no apparent toxicity was found.

Targeted drug delivery against fibroblast cells

The cytotoxic effects of FA-NGO/harmine were investigated against normal cells, and lower harmful effects of FA-NGO/harmine were observed, in comparison with free harmine. The primary fibroblast cells could not appropriately uptake the drug via the endocytosis-mediated pathway due to having a few FA receptors. Therefore, the released harmine and its cytotoxic effect on cells were much lower, especially at the lower concentrations. As it is evident in Fig. 10, 15 μg mL⁻¹ of loaded FA-NGO/harmine had no significant effect against fibroblast cells. However, 15 μg mL⁻¹ of free harmine reduced fibroblast cell viability to 80%. 30 and 50 μg mL⁻¹ concentrations of FA-NGO/harmine decrease the fibroblast cell growth to only 94% and 72%, respectively. In contrast, the same concentrations (30 and 50 μg mL⁻¹) of free harmine demonstrated more cytotoxic effect against normal cells and lessen cell viability to 73% and 48%, respectively. Moreover, loading drugs on targeted nanosheets at 15 and 30 μg mL⁻¹ doses significantly protected normal cells compared with untargeted nanocarriers. However, as drug dosage increased (at 50 μg mL⁻¹), no remarkable difference was observed between
targeted and untargeted drugs. But still due to slow controlled release of harmine in neutral condition of fibroblast cell environment, the loaded harmine caused less cytotoxic effect. Furthermore, it should be mentioned that NGO and FA-NGO have no cytotoxic effect on the concentrations we used [30]. Overall, fibroblast cells by expressing less FA receptors absorbed lower concentrations of loaded harmine and accordingly, illustrated a higher resistance to it. Furthermore, pH environment of normal cells is almost neutral, hence the release ratio of the drug would be lower in normal cells, and the cytotoxic effect of FA-NGO/harmine after 24 h was much less.

Other similar investigations have been carried out recently, which contained the same procedures and results. Singh et al.

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**Fig. 7** FA-NGO/harmine endocytosis into MCF-7 cells via the folic acid receptor-mediated pathway. The blue spheres represent harmine molecules, and the pink ones represent folic acid molecules. The FA existing on the surface of nanographene sheets can bind to folate receptor, and therefore the absorbance of harmine in MCF-7 cells would be promoted by folate motifs (color figure online)

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**Fig. 8** Fluorescent images of MCF-7 cells treated by a FA-NGO/Rho 6G and b NGO/Rho 6G. The intracellular delivery of Rho 6G is observed under a fluorescent microscope. a Rho 6G is released into MCF-7 cells and emits fluorescent. The remarkable role of FA as the target motif is comprehensible. b The uptake of Rho 6G by MCF-7 cells is less efficient due to lack of FA. No fluorescent emission is observed in the ×40 figure of MCF-7 cells
activity of DOX loaded on double-targeted GO (transferrin/FA-NGO). Their high stability and non-toxicity nanocarrier construction depict desirable drug-loading and drug-releasing functions. They also observed a higher cytotoxicity of loaded DOX at high concentrations rather than free DOX [31].

Conclusion

Overall, the aim of this research was to enhance the cytotoxicity efficacy of cancer drugs against breast cancer cells by loading it on nanoparticle, and in particular to decrease this effect in normal cells by means of assembling a targeted nanocarrier. In this study, the loading of harmine on targeted nanographene oxide increased not only the drug stability but also led harmine to be released cumulatively from the FA-NGO surface in acidic environments of tumoral cells. Furthermore, we used the high loading capacity of GO to load the highest percentage of harmine. Therefore, harmine could be delivered into MCF-7 cells with improved lethal efficacy. To conclude, this investigation, which demonstrated applying functionalized NGO for loading cancer drugs on its surface, seems potentially helpful to target several tumoral cells and eliminate them with less side effects. This promising system can be utilized in future clinical studies.

Materials and methods

Graphite flake was provided from Santa Cruz; sulfuric acid, potassium persulfate, hydrochloric acid, phosphorus pentoxide, potassium permanganate, rhodamine G, sodium monochloroacetate, and FA were purchased from Sigma; and harmine freebase was purchased from Sigma. Dulbecco’s Modified Eagle’s medium (DMEM), the culture medium, pen/strep antibiotics, and fetal bovine serum (FBS) were obtained from Gibco.

FTIR spectra were measured by a Bruker optics IFS 66v/S Vacuum FTIR spectrum. UV/Vis spectral measurement was carried out by SPECORD® 50 plus Analytik Jena. Fluorescence imaging was captured by Canon EOS 350D. Targeted cells were observed by Mshot MF31 LED fluorescence microscopy. Absorbance in the MTT assays was read by the Elisa spectrum. Cells were incubated with a water-jacketed CO2 Thermo Fisher 3010 incubators. Ultrasonication of nanocarriers was fulfilled by Jac-2010 ultrasonic.

**Nanocarrier synthesis**

GO was synthesized based on the literature [17], by hummer’s method with minor modification [32, 33]. The obtained GO was cracked by an ultrasonic bath at 500 W for 100 min to generate nanoscale graphene oxide dispersion.

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Fig. 9 Relative cell viability of MCF-7 treated with FA-NGO/harmine, NGO-SO3H/harmine, and free harmine, at different concentrations of 15, 30, and 40 μg/mL for 24 h. At all different concentrations, the loaded harmine on nanosheets could significantly reduce cell viability compared to free harmine. Nanosheets containing folic acid demonstrated even more significant fatal outcomes in comparison with NGO-SO3H. Data are presented as mean ± SD throughout three independent experiments. Cell viability evaluation for NGO constructions was also carried out and no cytotoxicity for these concentrations was observed. *P < 0.05, **P < 0.01, ***P < 0.001 compared with cells treated via free harmine.

Fig. 10 Cell viability of fibroblast cells treated by 15, 30, and 50 μg mL⁻¹ of harmine, FA-NGO/harmine, and NGO-SO3H/harmine for 24 h. Data are arranged in terms of a types of treatment and b different doses. Data are presented as mean ± SD throughout three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with cells treated by free harmine.
NGO-COOH preparation

First, COOH groups were substituted for hydroxyl, ester, and epoxide groups in GO to increase its water solubility as well as promoting the interaction of NGOs with FA. Therefore, as first step, 10 mL of NGO dispersion with 1 mg mL\(^{-1}\) concentration was prepared, then 0.5 g NaOH and 0.5 g CICH2COONa were added to NGO dispersion, and sonicated for 2 h in order to convert the hydroxyl groups into the carboxyl groups. The resulting product was neutralized by a diluted HCl, followed by repeated centrifugation and washing the suspension since the pellet was well dispersed in deionized water (DW). Then the resulted suspension, NGO-COOH, was dialyzed against DW for 48 h to remove the ions in solution [34].

NGO-SO\(_3\)H preparation

For enhancing the stability of nanocarriers in physiological solutions and preventing their precipitation, sulfonate groups were introduced to the NGO. Moreover, sulfonate groups boost the stability of these carriers in the presence of FBS, which exists in the flask cultures. For this purpose, the diazonium salt solution was prepared. Sulfanilic acid (20 mg) and sodium nitrite (8 mg) were dissolved in 2 mL of 0.25% NaOH, and then in an ice bath, this solution was added to 2.5 mL of 0.1 N HCl. The diazonium salt solution was added to NGO-COOH dispersion, stirred and kept in an ice bath for 2 h, then dialyzed for 48 h against DW. The NGO-SO\(_3\)H dispersion was stored at 4 °C.

FA-NGO preparation

A protocol introduced in 2005 was used to conjugate FA molecules with the NGO-SO3H [35]. 182.5 mg of N-hydroxysuccinimide and 125 mg of 1-Ethyl-3-(3-dimethyl aminopropyl)-carbodiimide were added to NGO-SO3H (200 mg) and ultra-sonicated for 2 h. Eventually, 5 mL of 2% FA was regulated to pH 8.0 by using sodium bicarbonate solution and added to the mixture; then, the result was stirred overnight in cold room. The free FA were separated by dialysis against sodium bicarbonate solution with pH 8.0 for 48 h followed by dialysis against DW for 24 h. Structural study of the result product was evaluated by FTIR and ultraviolet–visible spectroscopy (UV/Vis spectroscopy).

Study of controlled loading and release of harmine

In order to load drugs on nanocarriers, different concentrations of harmine (1–6, 12 mg mL\(^{-1}\) dissolved in DMSO) were added to FA-NGO aqueous suspension (50:50 V/V) and stirred for 24 h. The product was repeatedly centrifuged at 6000 rpm, to remove undissolved drug molecules, until no pellet was observed. The loading ratio was calculated by UV/Vis spectra at 250 nm absorbance (\(\lambda_{max}\) of harmine), while the FA-NGO absorbance was subtracted. According to the beer-lambert equation, harmine loading ration was drawn, and the concentration with the highest loading ratio was measured.

The mixture of loaded harmine on FA-NGO was added to phosphate-buffered saline at different pH values of 5 and 7 to measure the release ratio of the drug from FA-NGO. The resulted mixture incubated in 37 °C for 24 and 48 h. Then the mixtures were repeatedly centrifuged at 6000 rpm to remove released drugs, and after subtracting FA-NGO absorbance, the remaining drugs absorbed at 250 nm were calculated by UV/Vis spectra.

MTT assay

MCF-7 human breast cancer cells (FA receptor positive) were purchased from Iranian Biological Resource Center, and fibroblast cells (FA receptor negative) were extracted from primary human foreskin in national institute of genetic engineering and biotechnology. Cells were maintained in DMEM medium supplemented with 10% FBS and 1% pen/strep. For the colorimetric MTT assay, cells were seeded in 96-well plates with a density of 10,000 cells per well for MCF-7 and 6000 cells per well for fibroblast cells. After treating by different concentrations of harmine, FA-NGO/harmine, and NGO-SO\(_3\)H/harmine, cells were incubated at 37 °C containing 5% CO2 for 24 h. After the treatment period, cells were incubated with 0.5 mg mL\(^{-1}\) MTT reagent for 6 h. When the medium was removed, 100 μL of DMSO was added to each well to dissolve the formazan crystals formed by the cells.

Targeted uptake

MCF-7 cells were applied to investigate the cellular uptake of FA-NGO and NGO, and also the effect of FA receptors on targeted drug delivery. In this experiment, 10 μL Rho G (10 μmol L\(^{-1}\)) was loaded on 2 mL FA-NGO and NGO, separately, and stirred for 2 h; then, the excess Rho G was washed by repeated centrifuge. The MCF-7 cells were seeded in 96-well plates and treated by the FA-NGO/Rho G and NGO/Rho G. After incubating for 2 h, and cellular uptake was observed, using a fluorescent microscope.

Statistical analysis

Statistical analysis of the data in this article was carried out by GraphPad Prism 8.0.2 software and significant differences among mean values were evaluated by two-way ANOVA (Tukey’s multiple comparisons test). All the data are presented as mean result ± SD, and the differences between the experimental data groups were considered statistically significant (\(P\) value < 0.05).
Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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