An EGF-modified PLGA-lanthanide nanoplatform for combined NIR-II cancer imaging and targeted drug delivery

Yuan Yuan He¹, Zhenfeng Yu¹, Timo Schomann³, Hong Zhang², Christina Eich¹* and Luis J. Cruz¹*

¹Translational Nanobiomaterials and Imaging (TNI) Group, Department of Radiology, Leiden University Medical Center, 2333 ZA Leiden, the Netherlands; ²Van ’t Hoff Institute for Molecular Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, the Netherlands
*Correspondence: L.J.Cruz_Ricondo@lumc.nl (L. J. C.), Tel.: +31 71 5265764; *C.Eich@lumc.nl (C.E.), Tel.: +31 71 5261848

Abstract: The use of multifunctional nanopreformats for synergistic therapy and imaging is a promising approach in cancer treatment. In this study, we exploited the imaging properties of lanthanides by encapsulating CaF₂:Y, Nd along with the chemotherapeutic drug doxorubicin (DOX) into poly (D,L-lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) to prepare a nanoplaform suitable for imaging in the second near-infrared (NIR-II) window and simultaneous anti-cancer therapy. To facilitate the accumulation of CaF₂:Y, Nd+DOX@PLGA NPs in breast cancer cells, we modified the NPs with EGF. The diameter of the obtained CaF₂:Y, Nd+DOX@PLGA/PEG/NPs was approximately 150 nm, with a nearly round shape and homogeneous size distribution. In addition, analysis of the drug release behaviour showed that DOX was released more readily and had a longer release time in acidic environments. Accordingly, MTS results indicated that DOX-loaded NPs were significantly cytotoxic. Furthermore, fluorescence microscopy and flow cytometry studies revealed that CaF₂:Y, Nd+DOX@PLGA/PEG and CaF₂:Y, Nd+DOX@PLGA/PEG/EGF NPs were gradually taken up by 4T1 breast cancer cells over time, and EGF-coated Nd+DOX@PLGA NPs exhibited increased uptake rates after 72 h. Moreover, we found that EGF increased the solubility of Nd+DOX@PLGA NPs in water by comparing the aqueous solutions of the different NPs formulations. Finally, NIR imaging demonstrated strong fluorescence of PLGA NPs carrying CaF₂:Y, Nd NPs at 900-1200 nm under 808 nm laser excitation. In conclusion, the developed CaF₂:Y, Nd+DOX@PLGA/PEG/EGF NPs could be monitored for an extended period of time, and co-encapsulated DOX could be efficiently released to kill breast cancer cells.

Keywords: Drug delivery; Real-time monitoring; Nanomedicine; Tumor targeting
Introduction

Cancer is currently a leading cause of human mortality, mainly due to its high metastasis and recurrence as well as low cure rates[1]. In addition to surgery, most patients require further treatment, such as chemotherapy, radiation, immunotherapy, hormone therapy and targeted therapy[2]. Although drug-assisted therapy is a common cancer treatment, the uncertainty of drug distribution in the body poses potential hazards to the patient. Nanocarriers have emerged as a promising solution to accurately track the drug distribution in vivo. However, deep tissue tracking remains a major challenge in current research. Rare-earth-doped nanoparticles (RENPs) exhibit high tissue penetration power and low auto-fluorescence properties in the NIR-II region, making them an effective tool for deep tissue drug tracking[3,4]. Consequently, there is increasing attention and exploration of multifunctional nanoplatforms with tracking, targeting, and therapeutic capabilities.

In recent years, metallic elements have become increasingly prevalent in the preparation of multifunctional nanoplatforms for simultaneous cancer diagnostic and therapy[5]. Lanthanide elements play an important role in this field due to their rich electric, optical, magnetic and nuclear properties[6]. To date, a large number of lanthanide-based fluorescent materials, such as upconversion nanoparticles (NPs), have been applied for tracking and drug delivery[7]. For example, Kuang et al. combined photothermal and radiotherapy nanoplatforms with MRI guidance using the luminescent properties of Gd and the radiosensitizing function of hafnium[8]. Shapoval and his colleagues improved the sensitivity of in vivo imaging by doping Nd3+ into fluoride contrast agents[9]. A study by Yu et al. demonstrated that Nd3+-doped LuOF nanophosphors (LuOF: Nd3+ NPs) displayed excellent NIR-II fluorescence and computed tomography imaging capabilities[10]. Fan et al. found that modification of RENPs using ligands enhanced their imaging capabilities and showed good tumor-killing capacity when combined with chemotherapeutic agents[11]. However, some limitations, such as the tendency of lanthanides to aggregate and exhibit burst fluorescence, greatly reduce their sensitivity[12]. Fluorides possess unique optical properties (spectral window 190-1100 nm), highly stable structural properties as well as low phonon energies, and are often used as fluorescent host substrates to reduce the bursting of rare earth ions[13]. Recently, we demonstrated that co-doping of calcium fluoride (CaF2) with ytterbium (Y2+), gadolinium (Gd3+), and neodymium (Nd3+) broke the quenching threshold of Nd3+ and enhanced the NIR-II fluorescence and paramagnetic properties of these multifunctional CaF2:Y,Gd,Nd NPs with trimal imaging capabilities[14].

Doxorubicin (DOX) is a widely used chemotherapeutic agent for the treatment of various types of cancer[15]. As a hydrophilic compound, DOX can inhibit or slow down tumour cell growth in vivo[16]. However, its clinical use is limited by severe cardiotoxicity, drug resistance and damage to healthy tissues, which offsets its survival benefits in cancer patients[17]. To address this issue, PEG-PLGA copolymers have been extensively used for drug delivery due to their biocompatibility, low toxicity, biodegradability and solubility[18-21]. These copolymers can effectively control drug release, improve drug aggregation at the tumor site and reduce side effects[22].

With advances in scientific research, the concept of active targeting has gained significant attention from researchers. NPs can be actively targeted to specific receptors through peripheral ligands[23]. A common ligand-receptor interaction is the binding of epidermal growth factor (EGF) to epidermal growth factor receptor (EGFR), which is overexpressed on cancer cell membranes[24]. EGFR has been shown to promote cancer cell growth, invasion and angiogenesis[25] in numerous cancer cell lines, including lung, colorectal, breast, ovarian, head and neck, prostate, kidney, pancreatic, bladder cancer and glioblastoma[26,27].

To deliver chemotherapeutic drugs while tracking NP distribution in vivo, we prepared multifunctional NPs by combining CaF2:Y,Nd NPs and DOX with PLGA/PEG NPs modified with EGF through a water-in-oil approach. In this platform, Nd3+ acts as the main NIR-II imaging centre and Y2+ enhances the imaging signal for real-time tracking. The selection of PLGA NPs can locally concentrate CaF2:Y,Nd NPs within PLGA NPs, and surface modification with PEG effectively reduces non-specific binding of NPs to blood components, and thus prolongs circulation time in vivo, and serves as a chemical linker to attach targeting motifs to the NP surface. Active targeting and enhanced uptake of CaF2:Y, Nd+DOX@PLGA/PEG/EGF NPs were achieved by binding to overexpressed EGF receptors on tumor cell membranes. Here, we analyzed the physicochemical properties of CaF2:Y, Nd+DOX@PLGA/PEG/EGF NPs, such as particle size, morphology, and zeta potential, as well as NPs stability, cytotoxicity, in vitro drug release behavior, cellular uptake and NIR II imaging properties.
Figure 1: Schematic illustration of the preparation of CaF$_2$:Y, Nd+DOX@PLGA/PEG/EGF NPs.

2 Materials and methods

2.1. Materials

Neodymium(III) chloride hexahydrate (NdCl$_3$ 6H$_2$O, 99.9%), polyvinyl alcohol (PVA, Mw 13,000-23,000, CAS number is 9002-89-5, 87-89% hydrolyzed), chloroform (CHCl$_3$, CAS 67-66-3), yttrium (III) chloride heptahydrate (YCl$_3$6H$_2$O, 99.99%), Calcium chloride dihydrate (CaCl$_2$ 2H$_2$O, 99.5%), ammonium fluoride (NH$_4$F, ≥98.0%), bovine serum albumin and potassium citrate tribasic monohydrate (HOC(COOC)(CH$_2$COOK)$_2$ H$_2$O, ≥99.0%) were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The PLGA polymer (DL-lactide/glycolide molar ratio of 50:50, MW=17,000) was purchased from Caribon PURAC (Amsterdam, the Netherlands). Doxorubicin hydrochloride (DOX, powder, 98.0-102.0 % (HPLC) was acquired from Euroasia Co., Ltd. (Delhi, India). Trypsin, Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin (P/S, 10,000 U/mL) were provided by Gibco (Darmstadt, Germany). Alexa Fluor™ 488 Phalloidin (Cat# A12379), 4',6-Diamidino-2-phenylindole (DAPI, Dilactate Cat#D3571), Pierce™ Coomassie (Bradford) Protein Assay Kit (Cat# 23200), and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Agarose was purchased from Bioline (Bioline AgroSciences Ltd., Essex, United Kingdom). CellTiter 96(R) Aqueous MTS Reagent Powder were provided by Promega (Madison, Wisconsin, USA). NdCl$_3$ (>99%) and YCl$_3$ (>99%) were provided by Carlo Erba (Milano, Italia).

2.2. Synthesis of CaF$_2$:Y, Nd+DOX@PLGA/PEG/EGF NPs

Based on previous studies[28-30], CaF$_2$:Y, Nd NPs were prepared by a hydrothermal synthesis process. Briefly, 3.5 mmol stoichiometric CaCl$_2$ (>99%, J.T. Baker, Avantor Performance Materials, NJ, United States), YCl$_3$ and NdCl$_3$ were first added to a 50 mL beaker and dissolved in 7 mL of deionized water. Under vigorous stirring conditions, 20 mL of aqueous potassium citrate (1 M) was added dropwise and after a few minutes, 8.75 mmol of NH$_4$F was added and mixed thoroughly. Finally, the clarified mixture was placed in a teflon-lined autoclave, screwed down and baked for 10 h at 180 °C in an oven. After the temperature of the autoclave had been brought to room temperature (RT), the NPs were collected by centrifugation at 2.4 x g for 30 minutes. The samples were washed 4 times with deionized ethanol and water (1:2) to remove any raw materials that did not participate in the reaction or were incompletely reacted, and then freeze-dried for 3 days.

For the preparation of CaF$_2$:Y, Nd+DOX@PLGA/PEG/EGF NPs, a double emulsion evaporation technique was used[31,32]. Briefly, 30 mg of DOX and 7.5 mg of CaF$_2$:Y, Nd NPs were added to 1 mL of ultrapure deionized water and set aside. Next, 50 mg of PLGA and 10 mg PLGA/PEG-NHS were dissolved in 5 mL of chloroform. After sufficient dissolution, the aqueous solutions of DOX and CaF$_2$:Y, Nd NPs were added dropwise to the PLGA chloroform solution. This resulted in the formation of a water-in-oil (W1/O) emulsion, which was emulsified in an ice bath for 65 s using an ultrasonograph (Branson, Danbury, USA). Subsequently, 20 mL of the W1/O emulsion containing 2 % polyvinyl
Alcohol (PVA, w/v) was added and emulsified by the ultrasonograph to obtain an aqueous W1/O/W2 emulsion. The emulsion was stirred at 4 °C on a magnetic stirrer for 12 h to evaporate the chloroform. The NPs were then collected by high-speed centrifugation at 14,000 x g for 25 min and washed 3 times with deionized water to remove impurities. Next, the NPs were resuspended in 50 µL of EGF solution (1 mg/mL ultrapure deionized water) and stirred at 4 °C overnight. The NPs were collected by high-speed centrifugation (14,000 x g, 25 min) and washed three times to remove the excess EGF. Finally, the obtained NPs were freeze-dried (Martin Christ, Osterode, Germany).

2.3. Dynamic light scattering measurements.

Zeta potential, particle size distribution and size of the NPs were determined using a Zetasizer Nano ZS90 instrument (Malvern Panalytical, UK). First, 0.5 mg of NPs were dissolved in 1 mL of ultrapure deionized water. The aqueous solution of NPs was then treated in an ultrasonicator for 2 min, and subsequently analyzed using Zetasizer Software (Version 7.13).

2.4 Characterization of CaF$_2$:Y, Nd NPs

2.4.1 Fourier transform infrared spectroscopy

To analyze the binding properties of CaF$_2$:Y, Nd NPs, an iRSpirit FTIR spectrophotometer (Shimadzu, Kyoto, Japan) was used. Briefly, CaF$_2$:Y, Nd NP powder was placed into the machine and spectra were recorded in the range 400-4000 cm$^{-1}$, with a resolution of 4 cm$^{-1}$ and 15 scans per spectrum. The data were analyzed after subtraction of the blank control (KBr).

2.4.2 X-ray diffraction

The crystal structure of CaF$_2$:Y, Nd NP powder was characterized by X-ray Diffractogram (XRD) analysis. Briefly, the NPs were analyzed using a Panalytical X’pert PRO powder diffractometer (Malvern Panalytical, UK) operating at 40 kV and 40 mA. The analysis was performed at RT with a scanning speed of 6.0 degrees/min over a 2θ range of 20-80 degrees.

2.4.3 Infrared thermal imaging

To examine the effect of photothermal conversion of CaF$_2$:Y, Nd NPs under laser irradiation, real-time thermograms and temperature changes after irradiation were recorded using an infrared thermographer (Fluke Ti32, Fluke Corporation, USA)[33-36]. Specifically, 100 µg/mL of CaF$_2$:Y, Nd NPs and saline (control) were stored in a 12-well plate. These wells were then irradiated by an 808 nm laser (1.5 W/cm²) for 4.5 min and temperature changes were recorded by infrared (IR) thermography during irradiation.

2.5. Infrared spectra analysis

The fluorescence of NPs was measured on the Fluorolog®-3 with FluorEssence™ spectrometer (Horiba, Kyoto, Japan). The intrinsic fluorescence was measured by exciting the NP solution at 808 nm (Ex) and obtaining emission (Em) spectra at 900-1200 nm in increments of 0.5 nm.

2.6. Drug loading measurements

To measure the drug loading capacity of NPs for the potential antitumor drug DOX, an enzymatic calibrator was used. As described previously[37], 1 mg of NPs was completely dissolved in 0.3 mL of dimethylformamide (DMF) and treated in an ultrasonicator for 30 s to disrupt the structure of the NPs, allowing for complete release of DOX. Next, 0.7 mL of ultrapure water was then added to dilute the solution, and the resulting NP solution was transferred to a 96-well plate. The amount of DOX was analyzed by measuring the absorbance at 480 nm using a microplate reader. The actual concentration of DOX in the NPs was calculated using the following formula:

\[
\text{Drug loading (\%)} = \left( \frac{\text{amount of drug found in lyophilized NPs}}{\text{amount of lyophilized NPs}} \right) \times 100\%
\]

Three independent measurements were performed for this experiment, and data are expressed as mean ± standard deviation (SD).

2.7. In vitro drug release study
The release behavior of the NPs was measured using a dynamic dialysis method under different conditions (pH=5.0, 6.5 and 7.4) for 7 days[38]. To accomplish this, an aqueous solution containing 3 mg NPs (with a DOX amount equal to 20 μg) was placed in a dialysis tube (MWCO 3500) and stirred continually in a shaker incubator at 100 rpm and 37 °C. At specific time intervals, 500 μL of the sample was collected and an equal volume of fresh release medium was added. The release of DOX was analyzed at 480 nm using a microplate reader.

2.8. Transmission Electron Microscope (TEM)

The morphology and size of DOX-loaded CaF$_2$:Y, Nd+DOX@PLGA/PEG/EGF NPs were visualized using transmission electron microscopy (TEM). An aqueous suspension of 1 mg/mL NPs was dropped onto a glow-discharged copper grid surface and the NPs were allowed to settle. The TEM was equipped with a Gatan OneView Camera (Model 1095, Pleasanton, USA) and operated at an accelerating voltage of 120 kV for inspection.

2.9. Cell culture

The 4T1 (ATCC No. CRL-2539™) cell line was cultured in DMEM supplemented with 10 % FBS and 1 % P/S and maintained at 37 °C in a 5 % CO$_2$ incubator. When the 4T1 cells reached 85-90 % confluence, they were passaged.

2.10. In vitro cytotoxicity

The MTS assay was used to assess the cytotoxicity of PLGA/PEG/NPs, PLGA/PEG/EGF NPs, DOX@PLGA/PEG NPs, DOX@PLGA/PEG/EGF NPs, CaF$_2$:Y, Nd@PLGA/PEG NPs and CaF$_2$:Y, Nd+DOX@PLGA/PEG/EGF NPs. Briefly, 4T1 cells (1 × 10$^3$ cells/well) were inoculated into 96-well plates and incubated at 37 °C for 24 h. Subsequently, the cells were treated with different concentrations (31.25, 62.5, 125, 250, 500, 1000 and 2000 μg/mL) of PLGA/PEG NPs, PLGA/PEG/EGF NPs, CaF$_2$:Y, Nd @PLGA/PEG NPs, CaF$_2$:Y, Nd @PLGA/PEG/EGF NPs, DOX@PLGA/PEG/EGF NPs, CaF$_2$:Y, Nd+DOX@PLGA/PEG/EGF NPs and CaF$_2$:Y, Nd+DOX@PLGA/PEG/EGF NPs. After 24 and 72 h of incubation, the culture medium was removed and replaced with 100 μL of fresh medium and 20 μL of MTS reagent. The cells were then incubated at 37 °C for 1.5 h. The absorbance of samples was measured at 490 nm using a Molecular Devices SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices, USA) to assess the cytotoxicity of the NPs.

2.11. Bradford Protein Assay

The concentration of EGF protein on the NP surface was measured using the Bradford method[39]. Protein standards with a concentration range of 0.5 to 1000 μg/mL were prepared by diluting bovine serum albumin (BSA) in ultrapure water in triplicate according to the manufacturer’s instructions. A 96-well plate was used to mix 50 μL of the solution with 150 μL of Bradford’s reagent, and the mixture was incubated at 37 °C for 30 min. The absorbance of the samples was then measured at 562 nm using a Molecular Devices SpectraMax M3 Multi-Mode Microplate Reader.

2.12 NIR-II Fluorescence Imaging

For NIR-II Fluorescence Imaging, 3 mg of PLGA/PEG NPs, PLGA/PEG/EGF NPs, DOX@PLGA/PEG NPs, DOX@PLGA/PEG/EGF NPs, CaF$_2$:Y, Nd@PLGA/PEG NPs, CaF$_2$:Y, Nd@PLGA/PEG/EGF NPs, CaF$_2$:Y, Nd+DOX@PLGA/PEG/EGF NPs, and CaF$_2$:Y, Nd+DOX@PLGA/PEG/EGF NPs were dissolved in 1.5 mL of ultrapure deionized water. The fluorescence intensity of the aqueous NPs was measured using a laser power density of 100 mW/cm$^2$, an excitation wavelength of 808 nm and an exposure time of 5 ms.

2.13. Statistical analysis

All data are expressed as mean ± standard deviation (SD) unless stated otherwise. Variance and t-tests were used for statistical analysis. A p-value of $p < 0.05$ was considered statistically significant. The symbols *, **, *** and **** denote p-values of $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively. All data analyses were performed using GraphPad Prism 8 software.

3. Results and discussion

3.1 Characterization of CaF$_2$:Y$^{3+}$,Nd$^{3+}$ NPs
Figure 2 depicts representative TEM images of CaF₂ NPs doped with Y and Nd at different magnifications (Figure 2A). As previously reported[40], our CaF₂:Y,Nd NPs had uniform morphology and size (Figure 2A), with an average size of 11.8 ± 1.1 nm and a zeta potential of -28.2 ± 0.751, as measured by Malvern Zetasizer Nano (Figure 2B). XRD was used to analyze the crystal structure and dimensions of the NPs, as shown in Figure 2C. An significant peak was observed near the 2θ value at a specific angle of 28 degrees, which corresponds to the (111) face of the CaF₂ phase. In addition, several small peaks were observed near the 2θ values of 32 degrees, 67.5 degrees and 76.5 degrees, corresponding to the facets of the (200), (400) and (331) phase, respectively, in line with previous reports[41-43]. The TEM results showed that the crystals ranged between 10 and 15 nm in diameter. Thus, CaF₂:Y,Nd NPs were highly crystalline, had small grain sizes and exhibited a single-phase cubic fluorite structure.

Next, the CaF₂:Y,Nd NPs were excited by a NIR diode laser at an excitation wavelength of 808 nm, and strong NIR emission was detected at 1058 nm, 1064 nm and 1330 nm, corresponding to the 4F_{3/2} - 4I_{11/2} and 4F_{3/2} - 4I_{13/2} intra-4f electronic transitions of Nd³⁺, respectively. Since the laser wavelengths fall within the biological first (650-1000 nm) window and the emission wavelengths fall within the biological second (1000-1400 nm) window[28], the NPs can be used for in vivo imaging of deep tissues. FT-IR techniques were employed to detect the NPs’ functional groups. As shown in Figure 1E, the adsorption peaks at 1410 and 1590 cm⁻¹ corresponded to the stretching vibrations of the carboxyl group in the NPs in the wave number range of 400 to 4000 cm⁻¹[44-46]. The absorption peak at 3450 cm⁻¹ corresponded to the hydroxyl group stretching vibration in the NPs[47]. In agreement with previous studies, Sisubalan et al. and Muhammad et al. reported a C-O stretching band at 1410 and 1590 cm⁻¹ and an O-H stretching vibration at 3450 cm⁻¹, respectively[47,48]. In summary, the properties of our CaF₂:Y,Nd NPs were consistent with previous results[10,28], demonstrating successful preparation of CaF₂:Y,Nd NPs, which provided a strong basis for constructing a RENP/PLGA hybrid nanoparticle.
Figure 2. Morphology of CaF$_2$:Y,Nd NPs. (A) TEM images of CaF$_2$:Y,Nd NPs; (B) Hydrate particle size and zeta potential of CaF$_2$:Y,Nd NPs. (C) X-ray diffraction pattern of CaF$_2$:Y,Nd NPs. (D) Emission spectra of CaF$_2$:Y,Nd NPs (excited by an 808 nm xenon lamp); (E) FT-IR spectra of CaF$_2$:Y,Nd NPs.

3.2 Morphology of CaF$_2$:Y, Nd+DOX@PLGA/PEG/EGF NPs

To facilitate the delivery of chemotherapeutic drugs while monitoring the distribution of NPs in vivo, we prepared CaF$_2$:Y, Nd+DOX@PLGA/PEG/EGF NPs using non-toxic degradable PLGA via the compound emulsion method (W1/O/W2). In order to delay drug release, we modified the resulting PLGA-NPs with PEG by a chemical reaction between the carboxyl group (-COOH) of PLGA and the hydroxyl group (-OH) of PEG. To improve the targeting of chemotherapeutic agents and reduce side effects, we further modified the PEG layer of the NPs with EGF via a reaction between the amine-reactive sulfo-NHS ester of PEG and the carboxyl group (-NH$_2$) of EGF. To verify the successful encapsulation of CaF$_2$:Y,Nd NPs into PLGA NPs, we examined the morphological characteristics of the NPs by TEM. As shown in Figure 3A, the NPs were relatively uniform in size and mostly round. The images of CaF$_2$:Y,
Nd+DOX@PLGA/PEG/EGF NPs clearly showed the crystal structure of encapsulated CaF$_2$:Y,Nd NPs, in line with previous studies that demonstrate the appearance of crystal structures in TEM images as a clear indication of inorganic nanocrystals encapsulated into NPs [49]. The particle size and zeta potential of the NPs were measured using the Zetasizer Nano, which showed that the NPs had a narrow polydispersity index (0.18 ± 0.02) and that their size ranged from 300-600 nm (Figure 3B), with the DLS measurements being similar to the size demonstrated by TEM imaging. In addition, the zeta potential values for PLGA/PEG/EGF NPs, CaF$_2$:Y, Nd@PLGA/PEG/EGF NPs, DOX@PLGA/PEG/EGF NPs and CaF$_2$:Y, Nd+DOX@PLGA/PEG/EGF NPs were -15.4 ± 0.06 mV, -7.94 ± 0.12 mV, -14.5 ± 0.17 mV and -6.67 ± 0.45 mV, respectively (Figure 3C). Our experimental results were consistent with previous research. For example, Lei et al. reported a zeta potential of -13.2 ± 2.3 mV for DOX@PLGA NPs [50]. Notably, both NP formulations encapsulating CaF$_2$:Y,Nd were less negative than the NPs without lanthanides.

![Figure 3. The TEM (A) images and the size distribution (B and C) of NPs in control and experimental groups.](image)

To determine the amount of EGF attached to our CaF$_2$:Y Nd+DOX@PLGA/PEG/EGF NPs, we utilized the Bradford Protein Assay [51]. As shown in Supplementary Figure 1, the amount of EGF detected in 1 mg of CaF$_2$:Y Nd+DOX@PLGA/PEG/EGF NPs was 0.93 ± 0.1 μg/mg. In comparison, background levels of 0.086 ± 0.006 μg/mg and 0.048 ± 0.01 μg/mg were detected in CaF$_2$:Y Nd+DOX@PLGA/PEG NPs and ultrapure water, respectively. These results validate that EGF was successfully conjugated to the surface of CaF$_2$:Y Nd+DOX@PLGA/PEG/EGF NPs.

![Supplementary Figure 1. The concentration of EGF protein in NPs was measured using the Bradford protein assay, ***p < 0.001 compared with control group by t-test.](image)

3.3. NIR-II spectra Analysis
The NIR fluorescence spectrum of CaF$_2$:Y,Nd+DOX@PLGA/PEG/EGF NPs was characterized by the Fluorolog®-3 with FluorEssence™ spectrometer. As shown in Figure 4, PLGA NPs containing CaF$_2$:Y,Nd NPs exhibited enhanced fluorescence under 808 nm excitation. In contrast, the fluorescence spectra of PLGA NPs without CaF$_2$:Y,Nd NPs showed no peaks. This indicates that the fluorescence performance of CaF$_2$:Y,Nd+DOX@PLGA/PEG/EGF NPs was attributed to the doped CaF$_2$:Y,Nd NPs. Our previous study confirmed that Y$^{3+}$ and Nd$^{3+}$ could be doped in CaF$_2$, forming NPs that emit light stably under 808 nm laser excitation[28]. Several studies have shown that lanthanides can be efficiently loaded into other nanoplatforms while retaining their original fluorescence properties[52-56]. For example, Kang et al. created multifunctional nanocomposites by loading polyacrylic acid hydrogels with gadolinium vanadate, which acted not only as cellular imaging bioprobes but also facilitated the efficient release of co-loaded DOX in cancer cells[52]. Our data demonstrated that after the addition of DOX, the NPs retained their fluorescence properties, but the fluorescence intensity was slightly reduced (Figure 4). We believe that this reduction was likely due to the co-encapsulation of DOX, which reduced the available space for CaF$_2$:Y,Nd, causing a reduction in fluorescence signal in CaF$_2$:Y,Nd+DOX@PLGA/PEG/EGF NPs.

Figure 4. The fluorescence spectra of CaF$_2$:Y,Nd+DOX@PLGA/PEG/EGF NPs under 808 nm excitation. Emission spectra of PLGA/PEG/EGF NPs (orange), CaF$_2$:Y,Nd@PLGA/PEG/EGF NPs (green), DOX@PLGA/PEG/EGF NPs (purple), and CaF$_2$:Y,Nd+DOX@PLGA/PEG/EGF NPs (blue).

3.4 Drug release

The therapeutic efficiency of NPs is directly influenced by the efficiency of drug loading. Figure S2 displays the standard curve of DOX at RT at a concentration range of 1.95-500 μg/mL. The loading efficiency of DOX in our NPs was calculated by determining the absorption spectrum at 490 nm of solutions of NPs dissolved in DMF[57-60]. The DOX loading rates were analyzed after adding different amounts of DOX during the NP preparation, as shown in Figure S3. By analyzing the data, we found that the DOX loading rate was proportional to the amount of added DOX. The loading of DOX into CaF$_2$:Y,Nd+DOX@PLGA/PEG NPs and CaF$_2$:Y,Nd+DOX@PLGA/PEG/EGF NPs was calculated to be 8.4% and 8.9%, respectively. This indicates that DOX was efficiently co-encapsulated into PLGA NPs.

Next, the cumulative release of DOX from CaF$_2$:Y,Nd+DOX@PLGA/PEG NPs and CaF$_2$:Y,Nd+DOX@PLGA/PEG/EGF NPs was investigated over time at different pH values (pH=5.0, 6.5 and 7.4). The pH 5.0 was used to mimic the microenvironment of endosomes and lysosomes[61], pH 6.5 mimicked the weakly acidic microenvironment in tumor cells[62], and pH 7.4 mimicked the physiological environment that NPs encounter in the bloodstream[63]. After 24 h, 63.0%, 47.5%, and 35.0% of drugs were released from CaF$_2$:Y,Nd+DOX@PLGA/PEG NPs in PBS at pH 5.0, 6.5, and 7.4, respectively. The drug release was monitored for up to 30 days (720 h) reaching a cumulative DOX release of 65.0% at pH 5.0, 50% at pH 6.5, and 36% at pH 7.4. Under more acidic conditions, the release of DOX was more rapid and the cumulative release was greater, indicating that the pH significantly affected the release of DOX from
rapidly in an acidic environment.

Additionally, we observed that the release of DOX from CaF$_2$:Y, Nd+DOX@PLGA/PEG NPs was more complete than that from NPs surface-modified with EGF NPs (Figure 5A-D), suggesting that the presence of EGF limited the release of DOX. This finding is in line with other studies showing that modification with EGF, for example, reduced the curcumin release rate from EGF-modified curcumin/chitosan NPs[65]. Similarly, Wang et al. also found that cisplatin was slowly released from cisplatin-loaded EGF-modified mPEG-PLGA-PLL NPs[66].

![Figure 5. Cumulative releases of DOX from CaF$_2$:Y, Nd+DOX@PLGA/PEG NPs and CaF$_2$:Y, Nd+DOX@PLGA/PEG/EGF NPs in PBS at pH 5.0, pH 6.5, and pH 7.4 with 37°C (n=3).](image)

**Supplementary Figure 2. Standard curve of DOX.**
Supplementary Figure 3. Drug loading efficiency of the drug-carriers with quantity change of DOX.

3.5 Cellular NP uptake

NPs are transported into the cell via the endocytic pathway, which is a time dependent process that also limits cellular uptake[67]. Effective internalization of NPs enhances their intracellular delivery and increases therapeutic effects[68]. In this study, we used the fluorescent signal of DOX to quantify the NP uptake and intracellular location by fluorescence microscopy (Figure 6A). The 4T1 cells exhibited strong punctate fluorescence, which indicated that NPs were taken up by endocytosis. As the incubation time increased, NP uptake and DOX release proportionally increased. After 48 h of incubation, we observed a numerous red fluorescent dots around the nuclei (the fluorescent dots are NPs) and a significant amount of red fluorescence overlapping with the DAPI-stained (blue) nuclei. DOX is known to promote cell death by targeting the nucleus and inhibiting topoisomerase activity[51,69]. Next, we measured the NP uptake by flow cytometry and obtained similar to the results obtained by fluorescence microscopy, quantification of the fluorescence intensity of CaF₂:Y Nd+DOX @ PLGA/PEG/EGF NPs in 4T1 cells by FACS analysis (Figure 6C) indicated higher cellular uptake rates of NPs with increasing incubation time.

EGF receptors are known to be present in large numbers on the surface of tumor cells. To determine the effect of EGF-containing NPs on cellular uptake, we treated cells with both CaF₂:Y Nd+DOX @ PLGA/PEG/EGF NPs and CaF₂:Y Nd+DOX @ PLGA/PEG NPs. Flow cytometric analysis of the DOX fluorescent signal showed that after 4 h of treatment, uptake of CaF₂:Y Nd+DOX @ PLGA/PEG/EGF NPs was significantly higher in 4T1 cancer cells, compared to CaF₂:Y Nd+DOX @ PLGA/PEG NPs (Figure 6C). To determine the contribution of EGF to the uptake of CaF₂:Y Nd+DOX@PLGA/PEG/EGF NPs in 4T1 cells, we performed a competition experiment. As shown in Figure 6C, the uptake of CaF₂:Y Nd+DOX@PLGA/PEG/EGF NPs by cells cultured in medium supplemented with soluble EGF was significantly lower than of cells cultured with control medium. However, we did not observe any difference in uptake at later time points, suggesting that the presence of EGF improves the initial NP uptake.
First, we investigated whether CaF$_2$, Nd NPs and CaF$_2$, Nd@PLGA NPs in the absence of DOX (with and without EGF modification) had an effect on the viability of breast cancer cells. Treatment with different concentrations (31.25-2000 µg/mL) of PLGA/PEG NPs, PLGA/PEG/EGF NPs, CaF$_2$, Y, Nd@PLGA/PEG NPs and CaF$_2$, Y, Nd@PLGA/PEG/EGF NPs did not affect the viability of 4T1 cells after 24 h (Supplementary Figure 4A-B). After 72 h, CaF$_2$, Y, Nd@PLGA/PEG/EGF NPs significantly induced cytotoxicity at concentrations greater than or equal to 500 µg/mL (Supplementary Figure 4 B), suggesting that RENPs were slightly cytotoxic compared to control PLGA NPs (Supplementary Figure 4A). Similar results were obtained when 4T1 cells were treated with DOX-containing NPs (Figure 7A). CaF$_2$, Y, Nd+DOX@PLGA/PEG/EGF NPs were more efficient in inducing cell death in 50 % of 4T1 cells (IC50=8.032 µg/mL, equivalent to 90 µg/mL of NPs), compared to DOX@PLGA/PEG/EGF NPs (IC50=12.93 µg/mL, equivalent to 145 µg/mL of NPs).

Furthermore, CaF$_2$, Y, Nd@PLGA/PEG/EGF NPs induced greater cytotoxicity than CaF$_2$, Y, Nd@PLGA/PEG NPs, indicating that EGF increased the uptake of RENPs and the cytotoxicity at high concentrations (Supplementary Figure 4B).

To assess the impact of EGF-modification on cellular cytotoxicity, we treated 4T1 cells with NPs containing 8.032 µg/mL DOX (equivalent of 90 µg/mL of NPs). After 24 h, DOX@PLGA/PEG/EGF NPs reduced the survival rate of 4T1 cells by 45.9 % compared to the control group, and CaF$_2$, Y, Nd+DOX@PLGA/PEG/EGF NPs significantly reduced the survival rate of 4T1 cells by 59.7 % (Figure 7 C). As the amount of NP was significantly lower than the ≥500 µg/mL of RENPs that can cause cytotoxicity in the absence of DOX (Supplementary Figure 4B), these results suggest that DOX was the major component causing cell death in DOX@PLGA/PEG/EGF NPs and CaF$_2$, Y, Nd+DOX@PLGA/PEG/EGF NPs, and that CaF$_2$, Y, Nd+DOX@PLGA/PEG/EGF NPs had a more significant effect on reducing cell activity.

Then, 4T1 cells were treated with NPs containing different concentrations (DOX concentrations equal to 1, 2 , 4, 8, 16, and 32 µg/mL) of DOX@PLGA/PEG NPs, DOX@PLGA/PEG/EGF NPs, CaF$_2$, Y, Nd+DOX@PLGA/PEG NPs and CaF$_2$, Y, Nd+DOX@PLGA/PEG/EGF NPs. As shown in Figure 7D-E, after 24 h of incubation, DOX@PLGA/PEG/EGF NPs and CaF$_2$, Y, Nd+DOX@PLGA/PEG/EGF NPs were less toxic than their non-EGF-modified counterparts in 4T1 cells. However, when the incubation time was extended to 72 h, EGF-modified NPs significantly reduced the cellular activity of 4T1 cells (Figure 7 D-E). In fact, Figure 5 showed that more DOX was released more rapidly from CaF$_2$, Y, Nd+DOX@PLGA/PEG NPs within the first 24 h compared to CaF$_2$, Y, Nd+DOX@PLGA/PEG/EGF NPs. Thus, CaF$_2$, Y, Nd+DOX@PLGA/PEG NPs were likely more effective in inducing cytotoxicity in the first 24 h due to the faster DOX release. With increasing treatment time, an increased initial cellular NP uptake due to EGF counteracted the faster release of DOX from CaF$_2$, Y, Nd+DOX@PLGA/PEG NPs.

3.6 In vitro cell viability assay

To assess the cytotoxicity of the different NP formulations in vitro, we treated 4T1 cells with increasing concentrations of NPs and measured cell viability using an MTS assay. The cell viability of each group was compared to the untreated control (100 % viability; Figure 7 and Supplementary Figure 4).
Figure 7. Effect of nanoparticles on cell viability. The effect on cell viability was determined by MTS after incubation with different concentrations of DOX@PLGA/PEG/EGF NPs (A) and CaF$_2$:Y Nd+DOX@PLGA/PEG/EGF NPs (B). The *in vitro* cytotoxicity of experimental and control NPs on 4T1 cells (C). Relative viability of 4T1 cells incubated with different concentrations of NPs modified with EGF or not for 24 h and 72 h incubation via MTS assay (D-E; n=3). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.001 compared with each groups by t-test and Bonferroni’s multiple comparisons test.

Supplementary Figure 4. Effect of nanoparticles on cell viability. The effect on cell viability was determined by MTS after incubation with different concentrations of PLGA/PEG NPs and PLGA/PEG/EGF NPs (A), CaF$_2$:Y Nd@PLGA/PEG NPs and CaF$_2$:Y Nd@PLGA/PEG/EGF NPs (B) for 24 h and 72 h, respectively. Bonferroni’s multiple comparisons test (n=3; **p < 0.01, ***p < 0.001.

3.7 NIR-II Fluorescence Imaging of NP
NIR-II fluorescence bioimaging has attracted a significant amount of attention due to its advantages over conventional imaging, such as high biosafety, high spatial resolution, strong penetration and high sensitivity. However, the effectiveness of current nanoprobe for NIR-II fluorescence imaging is poor. In this study, we explored the effectiveness of CaF$_2$:Y, Nd NPs as NIR-II imaging probes. Firstly, the aqueous solution of each sample was imaged under white light. As shown in Figure 8A, CaF$_2$:Y, Nd NPs appeared as white powders, which were dissolved in water and appeared as white emulsions. The powdered DOX-loaded NPs were of orange-red color, and after dissolving in water, they formed an orange-red emulsion. These results demonstrated that the NPs were water-soluble. However, we observed that CaF$_2$:Y, Nd+DOX @PLGA/PEG NPs had better water solubility than CaF$_2$:Y, Nd+DOX @PLGA/PEG/EGF NPs.

To verify the validity of CaF$_2$:Y, Nd NPs as NIR-II imaging probes, we performed NIR-II fluorescence imaging of aqueous solutions of NPs at a concentration of 2 mg/mL using the KIS NIR-II optical imaging system (Kaer Labs, Nantes, France). As shown in Figure 8, we did not observe fluorescence for water, PLGA/PEG NPs, PLGA/PEG/EGF NPs, DOX@PLGA/PEG NPs, and DOX@PLGA/PEG/EGF NPs under excitation at 808 nm (Figure 8B). However, CaF$_2$:Y, Nd@PLGA/PEG NPs, CaF$_2$:Y, Nd@PLGA/PEG/EGF NPs, CaF$_2$:Y, Nd+DOX @PLGA/PEG NPs, and CaF$_2$:Y, Nd+DOX @PLGA/PEG/EGF NPs exhibited NIR-II fluorescence under an excitation wavelength of 808 nm (Figure 8B). This demonstrates the strong absorption of CaF$_2$:Y, Nd NPs in the NIR-II window. Comparing the fluorescence intensities of CaF$_2$:Y, Nd@PLGA/PEG NPs, CaF$_2$:Y, Nd@PLGA/PEG/EGF NPs, CaF$_2$:Y, Nd+DOX @PLGA/PEG NPs, and CaF$_2$:Y, Nd+DOX @PLGA/PEG/EGF NPs, we found that CaF$_2$:Y, Nd+DOX @PLGA/PEG NPs were less water-soluble, and precipitation was observed. Our data suggest that NPs carrying EGF may be more soluble in water.

4. Conclusion

In summary, we developed a multifunctional nanoplatform that combines chemotherapeutic drugs (DOX) with lanthanide NPs. Characterization of the NPs using TEM, DLS and UV spectrophotometry showed consistent size, shape and spectra, indicating successful NP synthesis. Cytotoxicity experiments demonstrated that encapsulation of DOX in the NPs was responsible for the cytotoxic effects in cancer cells at a NP concentration below 500 μg/mL. In addition, CaF$_2$:Y Nd+DOX@PLGA/PEG/EGF NPs exhibited distinct emission peaks and a strong fluorescence response under 808 nm laser excitation, demonstrating their potential for NIR-II fluorescence imaging. Our versatile nanoplatform effectively combines the benefits of PLGA and CaF$_2$:Y, Nd NPs, which not only reduces the side effects...
caused by DOX but also allows real-time tracking of the NP location. This also provides evidence for exploring the value of NIR-II fluorescence imaging as a probe to track the distribution of NPs in cancer therapy. In conclusion, multifunctional CaF$_2$:Y Nd+DOX@PLGA/PEG/EGF NPs represent an efficient and targeted anti-cancer drug delivery system. Our drug delivery system can deliver chemotherapeutic agents and target tumor cells, as well as possesses NIR-II fluorescence imaging capabilities, which holds great promise for future applications in optical imaging detection and therapy in the medical field.

**Supplementary Information**
The online version contains supplementary material available at

**Author Contributions:**
Contributed equally. Z.Y. and Y.H. conceived and designed the experiments. Y.H. and Z.Yu performed the experiments and responsible for the statistical analysis. Y.H. wrote the paper. T.S. performed the TEM analysis. Z.Y., T.S., C.E. and L.J.C. revised the text. All authors have read and agreed to the published version of the manuscript.

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