Responsive nanoprobes play an important role in bioassay and bioimaging, early diagnosis of diseases and treatment monitoring. Herein, a upconversion nanoparticle (UCNP)-based nanoprobe, Ru@UCNPs, for specific sensing and imaging of hypochlorous acid (HOCl) is reported. This Ru@UCNP nanoprobe consists of two functional components, i.e., NaYF₄:Yb, Tm UCNPs that can convert near infrared light-to-visible light as the energy donor, and a HOCl-responsive ruthenium(II) complex [Ru(bpy)₂(DNCH-bpy)][PF₆]₂ (Ru-DNPH) as the energy acceptor and also the upconversion luminescence (UCL) quencher. Within this luminescence resonance energy transfer nanoprobe system, the UCL OFF–ON emission is triggered specifically by HOCl. This triggering reaction enables the detection of HOCl in aqueous solution and biological systems. As an example of applications, the Ru@UCNPs nanoprobe is loaded onto test papers for semiquantitative HOCl detection without any interference from the background fluorescence. The application of Ru@UCNPs for background-free detection and visualization of HOCl in cells and mice is successfully demonstrated. This research has thus shown that Ru@UCNPs is a selective HOCl-responsive nanoprobe, providing a new way to detect HOCl and a new strategy to develop novel nanoprobes for in situ detection of various biomarkers in cells and early diagnosis of animal diseases.

1. Introduction

The rapid development in material science and analytical chemistry has advanced the design and fabrication of responsive bio-probes, including molecular chemosensors and nanoprobes for various applications in sensing and quantitative analysis of biological and environmental samples. Notably, nanoprobes are fabricated by integrating highly specific recognition units with organic/inorganic nanomaterials of unique electronic and optical characteristics, appearing as a new stratigical tool for bioanalysis and imaging. However, the real application of this novel tool is still a challenge due to the limited number of nanoprobes available and the lack of nanomaterials with suitable optical properties.

Upconversion nanoparticles (UCNPs) are capable of converting near-infrared (NIR) light to short wavelength emission ranging from deep-UV to NIR and emerge as a promising optical material in fabricating of nanoprobes for the detection of biomolecules and chemicals. Abundant photophysical properties of UCNPs allow for a broad range of applications in luminescence sensing and imaging. Since the luminescence emission of UCNPs is inert toward surroundings, the luminescence resonance energy transfer (LRET) mechanism is normally associated with the development of responsive UCNP-based nanoprobes, where upconversion luminescence (UCL) is used as the energy donor to activate the responsive chemosensor integrated on the surface. Following this principle, a number of UCL nanoprobes have been constructed for the detection of various analytes. Nevertheless, only fewer UCNP-based nanoprobes that can be used both for detecting highly reactive oxygen species (hROS) by paper-based analysis and visualizing hROS in live organisms are reported. Herein, we developed a new responsive UCNP-based nanoprobe for the detection of hypochlorous acid (HOCl) and demonstrated its utilities in paper-based assay and bioimaging in live organisms.

HOCl is an important biomarker relevant to the diseased states. In live organisms, HOCl is generated from chloride ion (Cl⁻) and hydrogen peroxide (H₂O₂) in an oxidation reaction catalyzed by myeloperoxidase. As well documented,
endogenous HOCl plays important roles in the immune system, while elevated HOCl level is associated with a wide range of inflammatory diseases, such as those in lung, liver, and kidney, atherosclerosis, neurodegenerative disorders, myocardial infarction, and cancers.[10] Recently, a few metal complex-based luminescence probes have been developed for the detection of HOCl in our previous research.[11] The complexes are normally excited by UV or visible light, while their luminescence in the practical application is severely affected by the background autofluorescence. To eliminate the background interference, a new strategy has been proposed (Scheme 1) to develop specific HOCl-responsive nanoprobe through LRET by elegantly combining UCNP and ruthenium (Ru(II)) complex molecules.

In this contribution, a unique responsive nanoprobe, Ru@UCNP, was developed by integrating UCNP s (NaYF 4 with 20 mol% Yb, 0.5 mol% Tm) with a Ru(II) complex-based chemosensor, [Ru(bpy) 2(DNCH-bpy)](PF 6 ) 2 (Ru-DNPH). UCNP s were employed as the antenna nanophosphor to covert the NIR light to blue emissions, which subsequently activate Ru-DNPH through the LRET process. The prepared nanoprobe was successfully demonstrated for ratiometric luminescence detection and quantification of HOCl in aqueous solution. The semiquantitative detection of HOCl in aqueous solution was also achieved using a test paper in a background-free approach. Using Ru@UCNP s as the nanoprobe, visualization of HOCl in live cells and mice was further realized.

2. Results and Discussion
2.1. Rationality of the Ru@UCNP s for the Detection of HOCl

As illustrated in Scheme 1A, the Ru@UCNP s nanoprobe was designed based on the LRET process. In this energy transfer (ET) system, NaYF 4:Yb (20%), Tm (0.5%) UCNP s with blue emissions centered at 450 and 475 nm (originated from 1D 2 →3F 4 and 1G 4 →3H 6, respectively) is used as the energy donor,[12] and Ru-DNPH (maximum absorbance located at λ abs = 478 nm) is employed as the energy acceptor. Thus, the energy transfer occurs from the blue emissions of UCNP s to the surface-adsorbed Ru-DNPH, leading to the quenching of blue UCLs. The ET efficiency from NaYF 4:Yb, Tm UCNP blue emissions to Ru-DNPH absorption is modulated by the degree of the reaction with HOCl, i.e., the HOCl concentration. In the presence of HOCl, a specific reaction occurs with Ru-DNPH to yield [Ru(bpy) 2(COOH-bpy)](PF 6 ) 2 (Ru-COOH) (Scheme 1B), accompanied by suppressing and blue-shifting the absorption of this mixed solution (Scheme 1C). The spectral overlap between ruthenium(II) complex and UCNP s is thus decreased and the UCL emissions at 450 and 475 nm are switched on. As a result, inert NaYF 4:Yb, Tm UCNP s can be functionalized as a specific nanoprobe to sense the HOCl level in aqueous solution. More interestingly, the NIR emission of UCNP s at 800 nm can be employed as an internal reference since this emission is essentially not influenced by the ET process. Hence, the ratiometric upconversion luminescences at 450 and 475 nm to that at 800 nm (I 450/I 800 and I 475/I 800) can be used to measure the HOCl level with minimal measurement errors. Together with the NIR excitation, the interference of the background fluorescence can be then effectively eliminated.

2.2. Characteristics of Ru@UCNP Nanoprobe

The hybrid nanoprobe system was thus prepared by loading of Ru-DNPH on the surface of UCNP s through a α-cyclodextrin (α-CD) assisted self-assembly method. Specifically, the OA-UCNP s
with the oleic acid (OA) as the surface ligand were synthesized by the thermal decomposition method. As-prepared UCNPs were well dispersed in cyclohexane with the average particle diameter of 38.5 nm (Figure 1E), as measured in transmission electron microscope (TEM) images (Figure 1A). The typical high-resolution TEM (HRTEM) image of as-prepared NaYF$_4$:Yb, Tm UCNPs shows a legible lattice space of 0.52 nm (Figure 1D), corresponding to the (100) plane of the hexagonal NaYF$_4$ structure. All X-ray diffraction (XRD) peaks of prepared OA-UCNPs confirm the hexagonal phase of NaYF$_4$ (Figure S1, Supporting Information). OA on the UCNP surface was also confirmed by the Fourier transform infrared (FTIR) characteristic vibrations. The IR peaks at $\approx$2853 and 2923 cm$^{-1}$ can be assigned to the symmetric ($v_s$) and asymmetric ($v_{as}$) stretching vibrations of methylene (–CH$_2$–) in the fatty acid chain, respectively (Figure S2, Supporting Information). Further the weak peak at around 3001 cm$^{-1}$ is due to the –C–H stretching vibration of OA ligand.

Hydrophobic UCNPs were then made hydrophilic by coating α-CD (Scheme 1) via ultrasonic treatment for 30 min. As shown in Figure 1B, as-obtained α-CD-UCNPs were monodispersed in aqueous solution without any obvious changes in the size and shape. The successful ligand self-assembly with α-CD was further confirmed by additional antisymmetric glycosidic vibration $v_{\text{a}}$(C–O–C) at 1154 cm$^{-1}$ and the coupled stretching vibrations $\nu$(C–C/O–C) at 1032 and 1080 cm$^{-1}$ in the FTIR spectrum (Figure S2, Supporting Information). The hydrodynamic diameter was 60.3 nm (PDI 0.150), corresponding to the addition of the core size of UCNPs, the ligand α-CD length and hydration layer. The ζ-potential of α-CD-UCNPs was $\approx$-23.3 mV.

The proposed nanoprobe Ru@UCNP was then prepared by adsorbing Ru-DNPH onto the hydrophobic oleic acid layer of α-CD-UCNPs via the electrostatic and hydrophobic interactions. As shown in Figure S3 (Supporting Information), the loading amount of Ru-DNPH was concentration-dependent, and the saturated loading was achieved at the Ru-DNPH concentration of $>500 \times 10^{-6}$ M. The saturate loading amount of Ru-DNPH was 8.5 wt% of Ru@UCNPs by measuring the absorbance at 478 nm ($\varepsilon_{478}$ mm$^{-1}$ = 2.88 $\times$ 10$^4$ cm$^{-1}$ M$^{-1}$). The morphology of Ru@UCNPs was not changed after Ru-DNPH loading (Figure 1C). The hydrodynamic diameter was 73.6 nm (Figure 1F), and the ζ-potential was changed to $\approx$-12.6 mV due to the loading of positively charged Ru-DNPH.

Upconversion luminescence spectra of OA-UCNPs in cyclohexane and α-CD-UCNPs in water are shown in Figure S4 of the Supporting Information. Under 980 nm excitation, both OA-UCNPs and α-CD-UCNPs displayed strong blue emissions at 450 and 475 nm, and red emission at 800 nm, which can
be assigned to $^1\text{D}_2 \rightarrow ^3\text{F}_4$, $^1\text{G}_4 \rightarrow ^3\text{H}_6$, and $^3\text{H}_4 \rightarrow ^3\text{H}_6$ transitions of Tm$^{3+}$, respectively. Due to the luminescence quenching by the surrounding water, $\sim 10\%$ decrease in UCL intensity was observed for the emission of $\alpha$-CD-UCNPs in water.

Under the excitation at 980 nm, the effect of UCL ratiometric value against the Ru-DNPH loading was investigated in water. In the presence of increasing concentrations of Ru-DNPH, the UCL intensity at 450 and 475 nm was decreased. The UCL quenching efficiency gradually increased with the increase amount of Ru-DNPH loading to UCNPs, and the maximum quenching efficiency was 89.3%, as estimated from the upconversion luminescence intensity with the saturate Ru-DNPH loading (Figure S5, Supporting Information). To further confirm the energy transfer between UCNPs and Ru-DNPH, the emission lifetime of UCNPs at 475 nm was investigated before and after loading of Ru-DNPH. The lifetime of $\alpha$-CD-UCNPs was decreased from 419.1 to 189.8 $\mu$s after Ru-DNPH loading (Figure S6, Supporting Information). The corresponding energy transfer efficiency was calculated to be 54.7$\%$ according to formula $E_{\text{ET}} = 1 - \tau_{\text{DA}}/\tau_{\text{D}}$. The ET efficiency calculated by the lifetime is smaller than that of the quenching efficiency calculated by the changes of UCL intensity, suggesting that the quenching of UCL is attributed to both ET and reabsorption.

The association stability of Ru@UCNPs was examined by measuring the changes of absorbance at 478 nm in water. As shown in Figure S7 of the Supporting Information, 91$\%$ of Ru-DNPH was still associated with suspended Ru@UCNPs after 30 h incubation in water at room temperature, indicating the good association stability of Ru@UCNPs in aqueous solution.

The UCL emissions at 450 and 475 nm are stable in aqueous solutions with the pH value from 5.0 to 9.0 and in cell culture medium. Very small changes of Ru@UCNPs emission spectra were noticed in different pH solutions and culture medium (Figure S8A, Supporting Information). In addition, no obvious diameter changes of Ru@UCNPs in different pH solutions (Figure S8B, Supporting Information), suggesting high pH stability of Ru@UCNPs nanoprobe. The size increase of Ru@UCNPs was observed in cell culture medium, which might be ascribed to the formation of protein corona where fetal bovine serum existed in this solution.

The Ru@UCNPs are also photostable in aqueous solution upon continuous laser irradiation at 980 nm. As shown in Figure S9 of the Supporting Information, negligible changes of $I_{450}/I_{800}$ and $I_{475}/I_{800}$ ratiometric values of Ru@UCNPs were noticed, suggesting that the Ru@UCNPs are resistant to photobleach.

### 2.3. Detection of HOCl in Aqueous Solution by Ru@UCNPs

As shown in Figure 2A, a strong absorption band with a maximum absorption wavelength at 478 nm was observed for the Ru@UCNPs in water. Following the addition of HOCl, a significant decrease of this absorption was observed and blue-shifted for 22 nm. As shown in Figure 2B, the UCL intensity at 450 and 475 nm was increased with the HOCl concentration increasing from 0 to $20 \times 10^{-6}$ m. The ratiometric values ($I_{450}/I_{800}$ and $I_{475}/I_{800}$) were also increased and showed a good linearity with the HOCl concentration (Figure 2C), which can be employed as the standard curves for HOCl semiquantification in aqueous solution. The detection limits (LOD), calculated according to the concentration corresponding to three standard deviations of the
background signal (LOD = \(3\sigma/k\)), were \(1.66 \times 10^{-7}\) M and \(1.36 \times 10^{-7}\) M by employing the \(I_{800}/I_{564}\) and \(I_{450}/I_{564}\) signals, respectively.

Similarly to the rapid response of Ru-DNPH to HOCl\(^{[11e]}\), Ru@UCNPs exhibited a fast UCL response toward HOCl in a few seconds. As shown in Figure S10 of the Supporting Information, upon the addition of HOCl, the UCL intensity at 450 and 475 nm was significantly increased and reached to a steady level within 60 s. These data suggest that Ru@UCNPs can serve as a nanoprobe for rapid HOCl detection, one of the key requirements for a useful nanoprobe in practical detection of a target analyte in biological systems.

The UCL response of Ru@UCNPs also exhibited high selectivity toward HOCl over other ROS, RNS, metal ions, anions, and biomolecules. As shown in Figure 3A, luminescence spectra in the presence of various species in Ru@UCNPs solution were little changed, in sharp contrast with significant luminescence enhancement at 450 and 475 nm when HOCl was added. The ratiometric luminescence values of Ru@UCNPs toward other ROS, RNS metal ions, anions, and biomolecules were much smaller (mostly around 0.1) than that for HOCl (0.6–0.65) (Figure 3B; Figure S11, Supporting Information). Thus spectrometric response was specifically triggered by HOCl, indicating that Ru@UCNPs is a selective UCL nanoprobe for HOCl detection. Furthermore, the detection of HOCl by Ru@UCNPs is not effected in the presence of various competitive species (Figure 3B; Figure S11, Supporting Information), demonstrating high selectivity of Ru@UCNPs toward HOCl. In comparison with other HOCl probes (Table S1, Supporting Information), our Ru@UCNPs probe has a lower detection limit, and quick response toward HOCl.

2.4. Paper-Based Analysis of HOCl

Considering the important roles of HOCl in both clinical practice and disease progression in live organisms, it is important to engineer effective methods to quantify the HOCl concentration both in aqueous solution and in vivo. Recently, lots of efforts have been made to develop molecular/nanoprobes for HOCl detection in aqueous solutions.\(^{[2c,9,10]}\) As we know, it would be more convenient to use a probe in practical detection cases if the probe could work in the solid state, such as on test papers. In this “dip-stick” method, on-site semiquantification information of HOCl levels is possibly obtained without using any instrumental analysis. Toward this end, the colorimetric, luminescence, and UCL responses of Ru@UCNPs to HOCl on test papers were investigated, as depicted in Figure 4. The test paper was easily prepared by dropping Ru@UCNPs solution onto a circular Whatman filter paper disk (6 mm diameter) and dried at room temperature (Figure S12, Supporting Information). As shown in Figure 4A(a), Ru@UCNPs test papers showed brown color under natural light before adding HOCl, while the color was gradually changed to pale yellow with the HOCl concentration increasing from \(5 \times 10^{-9}\) to \(1 \times 10^{-8}\) M \((1 \times 10^{-9}\) M). Under UV lamp (365 nm), Ru@UCNP test paper showed no luminescence, but then red-emitting luminescence enhanced with the HOCl concentration (Figure 4A(b),B). The luminescence enhancement is attributed to the production of emissive Ru-COOH after Ru-DNPH reacts with HOCl. Under 980 nm illumination, blue UCL luminescence was observed to significantly correlate with the HOCl concentration (Figure 4A(c),C). Thus detection of HOCl can be achieved using a user-friendly test paper by combined changes in color, luminescence, and UCL.

Very interestingly, the detection of HOCl by the UCL channel is not interfered with Rhodamine b when it is used as the artificial background fluorescence. After reaction with HOCl, the test papers were treated with 20 µL of Rhodamine b \((30 \times 10^{-6}\) M in EtOH) and dried in the fume cupboard and then the color change examined. As shown in Figure 4A(d),(e), under natural light and UV-illumination, the colors were kept the same, i.e., bright yellow fluorescence \((\lambda_{em} = 542\) nm, \(\lambda_{ex} = 564\) nm) of Rhodamine b in all cases (Figure 4D). Thus detection of HOCl seemed impossible. In sharp contrast, under excitation of 980 nm laser, enhancement in blue UCL was not affected by Rhodamine b (Figure 4A(f)), having a very similar trend in color change with the HOCl concentration (Figure 4E). Therefore, the Ru@UCNPs test papers prepared in this work offer a simply yet robust approach for the background-free detection of HOCl. As well documented, the UCL is capable of eliminating the background fluorescence from samples, attributed to the unique NIR light excitation. Although conventional methods for HOCl analysis remain important, this kind of test paper is simpler and more straightforward for the detection of HOCl without any spectroscopic instrumentation.

Figure 3. Selectivity of Ru@UCNPs nanoprobe toward HOCl. A) UCL spectrum of Ru@UCNPs in aqueous solution in the presence of various ROS \((2 \times 10^{-4}\) M), metal ions, and biomolecules. B) Changes in ratiometric values of \(I_{450}/I_{564}\) (pink and blue) and \(I_{475}/I_{564}\) (cyan and green) of Ru@UCNPs in the presence of various ROS and RNS.
2.5. Sensing and Imaging of HOCl In Vitro

Encouraged by the results of Ru@UCNPs for the detection of HOCl in aqueous solution, we further demonstrated the application of Ru@UCNPs in biological systems through visualization of HOCl under the excitation of NIR light (980 nm). Prior to the luminescence imaging, cytotoxicity of Ru@UCNPs was evaluated by a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on MDA-MB-231 and J774A.1 macrophage cells with the increasing concentration of Ru@UCNPs. As shown in Figure S13 of the Supporting Information, no significant changes in the proliferation of both MDA-MB-231 and J774A.1 macrophage cells were observed after incubation with Ru@UCNPs for 24 h at 37 °C. Even after incubation with Ru@UCNPs at the concentration of 400 µg mL⁻¹, the MDA-MB-231 cell viability was still greater than 80%, suggesting the Ru@UCNPs had low cytotoxicity.

Figure 4. Semi-quantitative determination of the HOCl level using paper-based nanoprobe. A) Ru@UCNPs prestained Whatman filter paper responding toward HOCl at the concentration from 0 to $10^6 \times 10^{-9}$ M. The test papers were then stained with Rhodamine b (d, e, f, $30 \times 10^{-6}$ M in EtOH) to investigate the interference from background fluorescence. a, d) under natural light, b, e) under UV light, and c, f) under 980 nm light. Curve of luminescence intensity changes against the concentration of HOCl of the test papers B) A(b), C) A(c), D) A(e), and E) A(f), respectively.
Luminescence imaging of exogenous HOCl in MDA-MB-231 cells was performed after incubating with Ru@UCNPs (50 µg mL⁻¹) for 4 h and then treating with 5 × 10⁻⁶ M HOCl for another 0.5 h. As shown in Figure 5A, cells in the control group showed no luminescence under the excitation of 980 and 458 nm, respectively. After MDA-MB-231 cells were incubated with Ru@UCNPs for 4 h, very weak blue and red luminescences were observed under the excitation of 980 and 458 nm, respectively, indicating that there was a limited amount of HOCl produced in MDA-MB-231 cells. In sharp contrast, when the Ru@UCNPs loaded cells were further treated with HOCl for 30 min, significant enhancement of intracellular blue UCL was observed under 980 nm laser excitation. Under 458 nm laser irradiation, red-emitting luminescence of ruthenium(II) complexes was also observed. A higher relative mean UCL intensity was noticed when the Ru@UCNPs-internalized MDA-MB-231 cells were treated with 10 × 10⁻⁶ M of HOCl (Figure S14, Supporting Information). The red emission of ruthenium(II) complexes was well overlapped with the blue emission of UCNPs in the cells, indicating that the Ru@UCNPs is stably associated with the subcellular structures in cells.

**Figure 5.** Confocal luminescence (λex = 458 nm) and UCL (λex = 980 nm) imaging of exogenous HOCl in A) MDA-MB-231 cells and B) endogenous HOCl generation in J774A.1 macrophage cells. A) Control (top row); the MDA-MB-231 cells were incubated with Ru@UCNPs (50 µg mL⁻¹) for 4 h (middle row), followed by the treatment with HOCl (5 × 10⁻⁶ M) for another 30 min (bottom row). B) Control (top row); the J774A.1 macrophage cells were incubated with Ru@UCNPs (50 µg mL⁻¹) for 4 h (middle row), the J774A.1 macrophage cells were treated with LPS (100 ng mL⁻¹) for 2 h and then incubated with Ru@UCNPs (50 µg mL⁻¹) for 4 h (bottom row). Scale bar: 20 µm.
Luminescence imaging of endogenous HOCl in live J774A.1 macrophage cells was conducted. Lipopolysaccharide (LPS) stimulation (100 ng mL\(^{-1}\)) was performed on macrophage cells for 2 h, followed by incubation with Ru@UCNPs (50 µg mL\(^{-1}\)) for another 4 h. As shown in Figure 5B, control cells showed dark luminescence under both 980 and 458 nm laser excitation. The Ru@UCNPs-internalized cells exhibited weak luminescence, which could be ascribed to the reaction of internalized Ru@UCNPs with a small amount of HOCl production in macrophage.[2c] By contrast, bright luminescence was clearly observed when J774A.1 macrophage cells were pretreated with LPS. It is well documented that hROS, including HOCl are increased in macrophage cells under LPS stimulation.[2c,d] The luminescence images show the feasibility of Ru@UCNPs in visualization of endogenous HOCl in LPS-stimulated macrophage. In agreement with the image in MDA-MB-231 cells, good overlap of the red emission of ruthenium(II) complexes with blue emission of UCNPs was noticed in inflamed J774A.1 macrophage cells, corroborating the high stability of Ru@UCNPs in live cells. Therefore, this reliable nanoprobe can be used for visualization of intracellular HOCl production under NIR and UV light activation.

2.6. Sensing and Imaging of HOCl In Vivo

The availability of Ru@UCNPs for in vivo imaging was further confirmed by administering exogenous HOCl after the subcutaneous injection of Ru@UCNPs to a live mouse, and then recording the images at 0.5, 5, 10, 15, and 20 min post HOCl injection. No emission from both Ru(II) channel and UCL channel was noticed from the control group (area “1”) of the exogenous HOCl (middle and bottom row, Figure 6A). The luminescence intensity of both Ru(II) channel (Figure 6B) and UCL channel (Figure 6C) was gradually increased, indicating Ru@UCNPs can be used as the nanoprobe for imaging of HOCl in living animals.

Visualization of endogenous HOCl at the inflammation site of a live mouse was then demonstrated. The mouse was subcutaneously injected with LPS (area pointed by the red arrow, Figure 7A), and after 4 h with Ru@UCNPs in the same area for imaging of endogenous HOCl. As shown in Figure 7, background fluorescence emission was noticed in all luminescence images recorded before and after Ru@UCNPs injection (area pointed by the white arrow, middle row, Figure 7A) while there was no signal in UCL images (bottom row, Figure 7A). Before injection of Ru@UCNPs, no luminescence signal from Ru(II) complex channel was noticed in inflamed J774A.1 macrophage cells, corroborating the high stability of Ru@UCNPs in live cells. Therefore, this reliable nanoprobe can be used for visualization of intracellular HOCl production under NIR and UV light activation.
3. Conclusions

In summary, a new NIR-light excitable UCL nanoprobe for HOCl detection was successfully developed and its potential applications in aqueous solution, in vitro and in vivo were demonstrated. By exploring the LRET principle, the Ru@UCNPs nanoprobe was developed by functionalizing HOCl-responsive Ru-DNPH (acceptor) to the surface of Tm doped UCNPs (donor). Due to the effective ET process, the blue emission of Ru@UCNPs nanoprobe was initially quenched, but gradually switched on in the presence of increased HOCl. The Ru@UCNPs nanoprobe exhibited a rapid luminescence response and a high selectivity for background-free HOCl detection. Paper-based test was conducted for the detection of HOCl in a simple and easy-to-use method. Ru@UCNPs as the nanoprobe were found to sensitively detect and image HOCl in cells and at the mouse inflammation site. The successful development of the new NIR-excitable nanoprobe thus provides a robust way for HOCl detection as well as a new strategy for the design of UCL-based nanoprobe.

4. Experimental Section

Materials and Characterization: YCl₃·6H₂O (99.99%), YbCl₃·6H₂O (99.99%), TmCl₃·6H₂O (99.99%), NaOH (>98%), NH₄F (99.99%), OA (90%), 1-octadecene (ODE, 90%), and NaOCl (4-4.99%), LPS, and Whatman qualitative filter paper were all purchased from Sigma-Aldrich. α-CD was purchased from Alfa Aesar Reagent Company. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Dulbecco’s Modified Eagle Medium, fetal bovine serum, α-glutamine, penicillin, and streptomycin sulphate were purchased from Life Technologies (Australia). 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-methyl-1-triazene (NOC-13),[11] Ru-DNPH, and [Ru(bpy)₃(COOH-bpy)](PF₆)₂ were synthesized by following the reported methods.[14] Nude mice (6–8 weeks) were obtained from Experimental Animal Center of Dalian Medical University, China. All the experiments on nude mice were performed in compliance with the relevant local laws and institute guidelines, and approved by the institution committee of Dalian Medical University. All chemical materials were purchased from commercial sources and used without further purification. Deionized distilled water was used throughout.

The morphology and size of OA-UCNPs, α-CD-UCNPs, and Ru@UCNPs particles were characterized using TEM (JEOL-JEM-1010 TEM) operated at an acceleration voltage of 100 kV. High-resolution TEM images were obtained on a Philips Tecnai F20 FEG-S/TEM. The samples for TEM images were dispersed in cyclohexane or water and then dropped on a copper grid. X-ray powder diffraction (XRD) patterns were collected on a PAN analytical X’Pert Pro MPD X-ray diffractometer using Cu Ka radiation (40 kV, 40 mA, λ = 0.15418 nm). The particle size distribution and zeta potential of α-CD-UCNPs and Ru@UCNPs in aqueous suspension were measured on a Nano Zeta-Sizer (Malvern instruments). The upconversion luminescence spectra were recorded in a Fluorolog-Tau-3 spectrometer (Jobin Yvon-Horiba) equipped with an external 980 nm CW diode laser at the pump power of 1.2 W. Absorption spectra were measured on a Perkin-Elmer Lambda 35 UV–vis spectrometer. FTIR spectra were measured on a Nicolet iS10 (Thermo Fisher Scientific Inc., USA) spectrometer at a resolution of 4 cm⁻¹ for 32 scans. Confocal luminescence imaging was performed on an inverted Zeiss LSM 880 laser scanning confocal microscope using a ×40 oil-immersion objective. The microscope was equipped with a fiber-coupled diode 978 nm laser to illuminate the UCNPs. The images were analyzed by ImageJ software version 1.44p. Imaging of HOCl in mice was performed on a SPECTRAL Ami Imaging Systems (Spectral Instruments Imaging, LLC, Tucson, AZ) with an excitation filter of 465 nm and an emission filter of 610 nm for Ru(II) complex luminescence, and excitation of 980 nm laser and emission filter of 460 nm for UCL.

SYNTHESIS OF OA-COATED UCNPS (NaYF₄:Yb, Tm): NaYF₄·Yb, Tm upconversion nanoparticles were synthesized using the solvothermal decomposition method reported previously.[14] Typically, under argon atmosphere, YCl₃ (0.795 mmol), YbCl₃ (0.200 mmol), and TmCl₃ (0.005 mmol) in 5 mL methanol solution were added into a 100 mL three-necked flask containing 6 mL OA and 15 mL ODE. The resulting mixture was heated to 150 °C for 30 min to form a homogenous solution. After cooling down to room temperature, 10 mL of methanol solution containing NaOH (2.5 mmol) and NH₄F (4 mmol) was added dropwise into the flask, and stirred at room temperature for another 45 min. The mixture was slowly heated to 120 °C to remove the methanol, and then quickly heated to 310 °C and aged for 1 h. After cooling down, the nanoparticles were precipitated by adding ethanol, and collected by centrifugation at 5000 rpm for 5 min. The NaYF₄·Yb, Tm UCNPs were redispersed in 10 mL cyclohexane for further utilizations after washing with ethanol for three times.
Preparation of α-CD Functionalized UCNPs: (α-CD-UCNPs): Dried OA-UCNPs (20 mg) were added into an aqueous solution containing 20 mg mL$^{-1}$ of α-CD. The mixture was ultrasonically treated for 30 min at room temperature. The resultant mixture was then centrifugally separated, and α-CD-UCNPs were collected and washed with water for three times. The isolated α-CD-UCNPs were dried in vacuum for further use.

Preparation of Ru@UCNPs: In a typical process, α-CD-UCNPs (20 mg) were added into an acetonitrile solution of Ru-DNPH (5 mL, 25× 10$^{-6}$ to 1000 × 10$^{-6}$ M). The mixture was ultrasonically treated for 10 min, and then stirred at room temperature overnight. The formed Ru@UCNPs were collected via centrifugation at 10000 rpm for 10 min and washed with acetonitrile and water each for three times. The Ru@UCNPs were dried in vacuum for further use.

Detection of HOCl in Aqueous Solution: For detection of HOCl in aqueous solution, 0.1 mg mL$^{-1}$ of Ru@UCNPs was treated with HOCl at concentrations of 0 × 10$^{-6}$ to 20 × 10$^{-6}$ M for 10 min at room temperature and then subjected to spectrometric analysis. The calibration curve was derived from the ratios of the upconversion luminescence intensity at 450 and 475 nm to that at 800 nm.

Ru@UCNPs-Loaded Test Paper for the Detection of HOCl: Whatman filter paper was used as the solid substrate for loading Ru@UCNPs and sensing HOCl. Circular filter paper disks (6 mm diameter) were prepared by cutting filter paper using a standard paper hole-punch, and then adding 10 µL of Ru@UCNPs in water (5 mg mL$^{-1}$), followed by drying at room temperature overnight. For detection of HOCl, 5 µL of HOCl aqueous solution at concentrations of 0 × 10$^{-3}$ to 1 × 10$^{-3}$ M was applied onto each test paper, and dried in the fume cupboard for a while before light irradiation and image recording.

Confocal Imaging of Internalized Ru@UCNPs and HOCl in Live Cells: MDA-MB-231 cells were typically seeded at a density of 3 × 10$^{5}$ cells per cm$^{-2}$ in 22 mm coverglass bottom culture dishes (ProSciTech Australia) for the confocal microscope imaging. After incubation for 12 h, 50 µg mL$^{-1}$ of Ru@UCNPs was added to the cell culture medium and incubated for another 4 h. The cells were washed three times with PBS. Then, NaOCl (5 × 10$^{-6}$, 10 × 10$^{-6}$ M) in PBS solution (about 50% transferred to HOCl in PBS) was added to the cells and incubated for another 30 min, followed by washing three times with fresh cell culture medium. Finally, the cells were imaged using an inverted Zeiss LSM 880 laser scanning confocal microscope. The Ru(II) complex luminescence was excited with a 458 nm laser and the emission detected by a PMT with acetonitrile and water each for three times. The Ru@UCNPs were centrifugally separated, and dried in vacuum for further use.

Detection of HOCl in Nude Mice: All the animals were provided by the Experimental Animal Centre of Dalian Medical University and used according to local institute guidelines (Dalian Medical University). All animal studies were carried out in agreement with the guidelines of the Institutional Animal Care (No. ZD110037000000860) approved by the Animal Ethical and Welfare Committee (AEWC). The nude mice (6–8 week old) were anesthetized by isoflurane in a flow of oxygen during the whole experiment. The mice were rinsed with PBS (3 × 2 mL per dish) and then subjected to luminescence imaging measurements on the confocal microscope. Imaging was performed at 5 min interval until 20 min. For imaging of endogenous HOCl generation in an inflammation mode of mice, the mice were subcutaneously injected with LPS (5 µg mL$^{-1}$, 100 µL) and after 4 h, Ru@UCNPs (1.0 mg mL$^{-1}$, 100 µL) was injected in the same area, followed by similar imaging procedure.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Keywords
bioassay and imaging, luminescence resonance energy transfer, nanoprobe, paper-based test strip, selective HOCl detection and imaging

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Preparation of reactive oxygen species.

For the upconversion luminescence response specificity measurements, Ru@UCNPs was treated with different reactive oxygen species (ROS) for 10 min at R. T., and then subjected to luminescence analysis. Hydrogen peroxide (H$_2$O$_2$) was diluted immediately from a stabilized 30% solution, and was assayed by using its molar absorption coefficient of 43.6 M$^{-1}$cm$^{-1}$ at 240 nm.$^1$ Hydroxyl radical (•OH) was generated in the Fenton system from ferrous ammonium sulfate and hydrogen peroxide.$^2$ Peroxynitrite was generated by using SIN-1 as an ONOO$^-$ donor. Singlet oxygen was chemically generated from the Na$_2$MoO$_4$-H$_2$O$_2$ system in 0.1 M carbonate buffer of pH 10.5.$^3$ Nitric oxide (NO) was generated by using NOC-13 as a NO donor. The stock solution of hypochlorite was used throughout. The concentration of HOCl was determined by using its molar extinction coefficient of 391 M$^{-1}$cm$^{-1}$ at 292 nm
t-BuO· was produced by Fenton reaction, i.e., 10 equiv, of ferrous sulfate was added to the solution of tert-butyl hydroperoxide aqueous solutions.

**Cell line and cell culture.**

Human breast adenocarcinoma cell, MDA-MB-231 (ATCC® HTB-26™) and mouse macrophage, J774A.1 (ATCC® TIB-67™) were obtained from American Type Cell Collection. MCF-7 cells were cultured in RPMI-1640, supplemented with 10% FBS, 1% penicillin, 1% streptomycin sulfate in a humidified 5% CO₂/95% air incubator at 37 °C. J774A.1 cells were maintained in RPMI 1640 medium, supported with 10% FBS, 100 U/mL penicillin, and 1% L-glutamine in a humidified 5% CO₂/95% air incubator at 37 °C. The growth medium was changed every two days. MDA-MB-231 cells were routinely subcultured with trypsin-EDTA solution and growth to 80% confluence prior to experiments. For J774A.1 macrophage cells, cells were dislodged from flask substrate using a cell scraper, and then aspirated and dispensed for subculture.

**MTT cell viability assays.**

The MTT assay involves the reduction of a yellow tetrazolium salt, [3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide] tetrazolium to an insoluble formazan crystal by the metabolic activity of living cells. In this work, MTT assay was utilized to investigate the cytotoxicity of Ru@UCNPs. MDA-MB-231 cells were seeded at a density of 5000 cells per well in a 96-well micro-assay culture plate. After 12 h incubation at 37 °C in a 5% CO₂ incubator, the cells were treated with cell culture medium dispersion of Ru@UCNPs at various concentrations of 0, 12.5, 25, 50, 75, 100, 200 and 400 µg/mL. Control wells were prepared by the addition of culture medium, and wells containing culture media without cells were used as blanks. After incubation at 37 °C in a 5% CO₂ incubator for 24 h in dark, cell culture medium was removed and cells were washed three times with PBS. Then, 100 µL, 0.5
mg/mL MTT solution in PBS was added to each well, and the cells were incubated for another 4 h. The excess MTT solution was then carefully removed from each well, and the formed formazan was dissolved in 150 μL of DMSO (dimethyl sulfoxide). The optical density of each well was then measured at a wavelength of 520 nm using a PHERAstar microplate reader. The results from the five individual experiments were averaged. The following formula was used to calculate the viability of cell growth: Viability (%) = (mean of absorbance value of treatment group–blank)/(mean absorbance value of control–blank) × 100.

**Figure S1.** X-ray diffraction (XRD) patterns of the UCNPs (NaYF₄: 20 mol % Yb, 0.5 mol % Tm) nanoparticles.
**Figure S2.** FTIR spectra of OA-UCNPs (black line) and α-CD-UCNPs (red line).

**Figure S3.** Different absorption at $\lambda_{\text{max}}$ (478 nm) of as-prepared Ru@UCNPs complexes (0.02 mg/mL) with varied concentrations of Ru-DNPH.
Figure S4. UCL spectra of OA-UCNPs dispersed in cyclohexane (1 mg/mL, red line), α-CD-UCNPs dispersed in water (1 mg/mL, blue line), and Ru@UCNPs dispersed in water (1 mg/mL, black line). Pink line represents the luminescence spectrum of Ru-DNPH (10 μM) under 980 nm excitation.

Figure S5. Quenching efficiency of upconversion luminescence at 450 nm (black) and 475 nm (red) against the concentration of Ru-DNPH in Ru@UCNPs.
Figure S6. Lifetime of $\alpha$-CD-UCNPs and Ru@UCNPs.

Figure S7. Stability of the prepared Ru@UCNPs complexes in the aqueous solution.
Figure S8. (A) Emission spectra of Ru@UCNPs in aqueous solution with different pH from 5.0 to 9.0 and in cell culture medium; (B) diameter changes of Ru@UCNPs at different conditions (R: diameter of Ru@UCNPs at different conditions; R₀: diameter of Ru@UCNPs in water).

Figure S9. Ratiometric value of Ru@UCNPs at I₄₅₀/I₈₀₀ and I₄₇₅/I₈₀₀ of Ru@UCNPs in aqueous solution at continuous laser irradiation (980 nm).
**Figure S10.** Enhancement in UCL intensity of Ru@UCNPs in aqueous solution after the addition of HOCl (20 µM) for different time (0-180 s).

**Figure S11.** Changes in ratiometric values of $I_{450}/I_{800}$ (pink) and $I_{475}/I_{800}$ (cyan) of Ru@UCNPs in aqueous solution the presence of various metal ions, anions, and biomolecules, including a) blank, b) HOCl, c) t-BuOOH, d) Cu$^{2+}$, e) Na$^+$, f) K$^+$, g) HS$^-$, h) HSO$_3^-$, i) Cys, j) GSH, k) Cu$^+$, l) Fe$^{3+}$, m) mixed species, n) mixed species and HOCl.
**Figure S12.** Preparation of paper-based test strip for HOCl detection.

**Figure S13.** MDA-MB-231 human breast cancer (A) and J774A.1 macrophage cell (B) viability values (%) assessed using an MTT proliferation test versus incubation concentrations of Ru@UCNPs.
**Figure S14.** (A) Bright-field, ULC, and merged images of MDA-MB-231 cells stained with Ru@UCNPs (50 µg/mL) for 4 h and then treated with HOCl at different concentration for 30 min. Scale bar: 5 μm. (B) Relative mean UCL intensity of MDA-MB-231 cells of images.

**Figure S15.** (A) Ru@UCNPs (1.0 mg/mL, 100 μL) was subcutaneously injected (area of blue arrow), followed by imaging at 5 (B), 10 (C), 15 (D), and 20 min (E). (F) represents the data of imaging analysis of Ru luminescence channel (blue bars) and UCL channel (red bars), respectively. Background fluorescence emission of mouse showing in the area of red arrow. Excitation filter of 465 nm and an emission filter of 610 nm for Ru(II) complex luminescence, and excitation of 980 nm laser and emission filter of 460 nm for upconversion luminescence.
Table S1. Some recently reported fluorescent probes for HOCl detection.

<table>
<thead>
<tr>
<th>Probes</th>
<th>Detection limit</th>
<th>Ex/Em (nm)</th>
<th>Response time</th>
<th>Intracellular analysis</th>
<th>In vivo/ex vivo sensing</th>
<th>Other applications</th>
<th>Ref.</th>
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<td>Molecular probes</td>
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<td>RHHP</td>
<td>1.40 nM</td>
<td>520/580</td>
<td>&lt; 30 s</td>
<td>Exogenous HOCl in HeLa cells</td>
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<td>Paper-based analysis of HOCl</td>
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<td>RSTPP</td>
<td>9.00 nM</td>
<td>553/580</td>
<td>2 min</td>
<td>Endogenous HOCl in mitochondria of macrophage</td>
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<td>BiTClO</td>
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<td>560/575</td>
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<tr>
<td>Flu-1</td>
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<td>1</td>
<td>480/542</td>
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<td>MPhSeO-BOD</td>
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<td>460/510</td>
<td>5 min</td>
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<td>1b</td>
<td>0.356 µM</td>
<td>398/455</td>
<td>5 min</td>
<td>Exogenous HOCl in HepG2 cells</td>
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<td>PZ-Py</td>
<td>17.9 nM</td>
<td>400/562</td>
<td>A few seconds</td>
<td>Exogenous HOCl in mitochondria HeLa, endogenous HOCl in mitochondria of RAW 264.7</td>
<td>(In vivo) nude mice (exogenous HOCl)</td>
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<td>Ptz-AO</td>
<td>2.7 nM</td>
<td>475/540</td>
<td>5 s</td>
<td>Exogenous HOCl in INS-1 β-islet cells and endogenous HOCl in macrophage</td>
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<td>UCNP nanoprobe</td>
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<td>hCy3-csUCNP:DD</td>
<td>27 ppb</td>
<td>808/540,655</td>
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<td>UCL images of exogenous HOCl in HeLa cells</td>
<td>UCL imaging of the arthritis living mouse</td>
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<td>RBH1-UCNPs</td>
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<td>Zn(DZ)3-UCNs</td>
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<td>980/544, 659</td>
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<td>Ru@UCNPs</td>
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<td>980/450, 475, 800</td>
<td>&lt;1 min</td>
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<td>UCL imaging of exogenous/endogenous HOCl in nude mice</td>
<td>Paper-based analysis of HOCl</td>
<td>This work</td>
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endogenous
HOCl
generation in
inflamed
J774A.1
macrophage
cells

References


