Co$_3$O$_4$-cored carbon dots were prepared to induce chemiluminescence from the carbon dots in the presence of hydrogen peroxide at neutral pH. Neither a strong oxidative agent nor alkaline was needed as a co-reactant, which permitted for the first time the application of CDs for such chemiluminescence analysis of cells. The detection limit of hydrogen peroxide was determined to be 10 nM. Due to their good cellular permeability and low cytotoxicity, these nanoparticles were loaded into cells. The greater luminescence observed in cells with elevated intracellular hydrogen peroxide levels showed that our probe could measure the change in concentration of hydrogen peroxide inside cells.

Carbon dots (CDs) are new fluorescent semiconducting quantum dots in bio-imaging and display low cellular toxicity, good cell permeability and excellent photoluminescence. Recent studies have shown CDs to generate chemiluminescence in the presence of strong oxidative agents such as HSO$_3^-$, I$_2O_4^-$, and concentrated alkaline solutions. A possible mechanism for this chemiluminescence involves the production of a reactive oxygen radical in the solution and the energy transfer from the oxygen radical to the CDs, resulting in the production of excited-state CDs (CD$^*$) followed by a return to the ground state and emission of photons. CDs have therefore been applied as a new chemiluminescent probe for the analysis of hydrogen peroxide or oxygen radicals in biological samples such as living cells and tissues. Although fluorescent indicators have been fashioned for assaying intracellular hydrogen peroxide, the luminescent CD probes have shown the advantage of low toxicity, good cell permeability and the potential ability to be applied in large mammalian animals. Applying CDs to chemiluminescence assays in biological samples, however, has been impeded by disadvantageous properties of the strong oxidative or alkaline co-reactants used, in particular their toxicity for cells and the inability to introduce them into cells through solid materials. As a result, the development of novel CDs generating luminescence with hydrogen peroxide in the absence of any oxidative agents or alkalines is needed, and is described in this communication.

Co$_3$O$_4$ nanomaterials have been shown to be excellent catalysts of the decomposition of hydrogen peroxide into OH radicals under neutral or alkaline pH conditions. Since CDs display luminescence in the presence of the OH radical, it was reasonable to propose that combining Co$_3$O$_4$ nanomaterials and CDs could generate luminescence with hydrogen peroxide in neutral buffers, which would afford a chemiluminescence analysis of intracellular hydrogen peroxide. However, Co$_3$O$_4$ nanomaterials are typically toxic for cells and cannot be introduced into the cells directly. We thought, however, that the decoration of Co$_3$O$_4$ nanoparticles with CDs, might create novel core@shell nanomaterials that couple the advantages of the low cytotoxicity of CDs and the high catalysis of Co$_3$O$_4$ nanoparticles and that can be of practical use for the luminescence analysis of hydrogen peroxide. During the analysis, hydrogen peroxide would be expected to diffuse into the nanomaterials and react with Co$_3$O$_4$ nanoparticles in the core to generate OH radicals, which would collide with the surrounding CDs to produce excited-state CDs and subsequent photon emission indicative of the luminescence, as shown in Fig. 1.

![Fig. 1](https://example.com/fig1.png)
Fig. 1 shows the process used to prepare Co3O4@CD nanoparticles (Co3O4@CDs). Co3O4 nanoparticles were produced by mixing Co(CH3COO)2 and an ammonia solution at 150 °C for 3 h. Since the heating of polyacrylamide has been shown to generate fluorescent CDs,† the prepared Co3O4 nanoparticles were mixed with a large amount of polyacrylamide solution at 260 °C. The generated CDs wrapped the Co3O4 nanoparticles to form Co3O4@CD nanoparticles. Extra CDs were removed by centrifugation. The details of the preparation process are shown in the ESI.†

The prepared Co3O4 and Co3O4@CD nanoparticles were imaged using transmission electron microscopy (TEM), as shown in Fig. 2A and B. The average size of the Co3O4 nanoparticles was 30.2 ± 5.8 nm. After the introduction of polyacrylamide and heating, the average size of the nanoparticles, as shown in Fig. 2B, increased to 38.6 ± 8.7 nm. Small dots were observed to surround the Co3O4 nanoparticles, as shown in the inset of Fig. 2B. Considering that the size of CDs prepared using polyacrylamide was previously shown to be ~8 nm,† the small dots surrounding the Co3O4 nanoparticles might be CDs. A complete coverage of each Co3O4 nanoparticle by one layer of CDs should decrease the toxicity of the Co3O4 nanoparticles for cells.

To confirm the formation of Co3O4@CD nanoparticles, an infrared spectrum of these nanoparticles was recorded and compared to those of CDs and Co3O4 nanoparticles, as shown in Fig. 2C. The peaks at 2926 and 2853 cm⁻¹ observed from the Co3O4@CD nanoparticles were associated with the stretching vibration of C–H in the CDs, and the peak at 1653 cm⁻¹ from these nanoparticles were due to the stretching vibration of C=O in the CDs.‡ Meanwhile, peaks at 683 cm⁻¹ were observed from both the Co3O4@CDs and Co3O4 nanoparticles, and were ascribed to the stretching vibrations of Co–O in Co3O4.‡ XRD spectra of Co3O4@CDs and Co3O4 nanoparticles (Fig. S1 in ESI†) exhibited similar peaks.‡ All of these results provided evidence for the co-existence of CDs and Co3O4 in the Co3O4@CD nanoparticles. The elementary analysis of Co3O4@CD nanoparticles and CDs yielded percentages of N of 1.61% and 8.68%, respectively, which suggested that the percentages of CDs and Co3O4 in the Co3O4@CD nanoparticles were 18.5% and 81.5%.

Since CDs are fluorescent and were observed on the outside surface of the core@shell nanomaterials, Co3O4@CD nanoparticles should be fluorescent. To compare the fluorescence efficiency levels of Co3O4@CD nanoparticles and CDs in solution, the concentration of Co3O4@CD nanoparticles was adjusted so that the same mass concentration of CDs was achieved for both samples. Similar peak wavelengths and fluorescence intensities in the emission spectra of the Co3O4@CD nanoparticles and the CDs confirmed the fluorescence property and the mass composition of the Co3O4@CD nanoparticles (Fig. 2D). The fluorescence facilitated the observation of the nanoparticles loading into the cells using fluorescence microscopy.

The luminescence of Co3O4@CDs (0.1 mg mL⁻¹) was observed in 10 mM phosphate saline buffer (PBS, pH 7.4) containing 1 mM hydrogen peroxide. As shown in Fig. 3A, the background luminescence was near zero, indicating no luminescence from the nanoparticles by themselves. After the addition of hydrogen peroxide into the buffer, a rapid increase in luminescence followed by a slow decay was observed. In the control experiments, CDs or purely Co3O4 nanoparticles, each with the same mass concentration as their respective counterparts in the Co3O4@CD nanoparticles, were mixed with hydrogen peroxide. No or little luminescence was recorded, confirming that the coordination of CDs, Co3O4 nanoparticles and hydrogen peroxide was critical to induce luminescence. The wavelength of the luminescence was determined to be near 450 nm (Fig. S2 in ESI†). To understand the how the luminescence was generated, acidic, neutral and alkaline buffers were used,
and the luminescence in each of these buffers was measured, as shown in Fig. 3B. Under the acidic condition, almost no luminescence was observed; while more luminescence was produced in the solutions with higher pH values. Since Co₃O₄ nanoparticles could generate more OH radicals from hydrogen peroxide at alkaline conditions and OH radicals have been shown to induce luminescence from CDs, the dependence of the luminescence on pH that we observed suggested that OH radicals were involved in the generation of the luminescence.

To confirm such generation of OH radicals, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was used as a spin trap for OH radicals, and characterized using electron paramagnetic resonance (EPR). Fig. 3C shows a quartet of signals with relative intensities of 1:2:2:1 from the mixture of Co₃O₄@CD nanoparticles and hydrogen peroxide, which suggested the formation of DMPO-OH. As compared with the small signal from the mixture of CDs and hydrogen peroxide, the EPR signal illustrated the generation of OH radicals in the presence of Co₃O₄ nanoparticles. All of these results supported the proposal that the production of the luminescence involved the generation of OH radicals through the Co₃O₄ nanoparticles and the luminescence arising from the CDs and the radicals. The ability to generate luminescence at neutral pH permitted the application of these nanoparticles to the analysis of intracellular hydrogen peroxide.

The correlation of the luminescence intensity with the concentration of hydrogen peroxide in PBS buffer (pH 7.4) was investigated. The luminescence traces are shown in Fig. 3D. Enhanced luminescence was observed when the concentration of hydrogen peroxide was increased from 10 μM to 1 mM, as shown in Fig. S3 (ESI†). The correlation could be attributed to the greater decomposition of hydrogen peroxide to OH radicals at higher hydrogen peroxide concentrations. The detection limit was determined to be 10 μM (S/N 3). The lifetime of the luminescence was characterized to be as long as 20 min, as shown in Fig. S4 (ESI†). The relatively long lifetime suggested that CDs outside the nanoparticles were abundantly available for the consumption of OH radicals, which was important for the following assaying of hydrogen peroxide inside the cells.

To measure intracellular hydrogen peroxide, we attempted to load Co₃O₄@CD nanoparticles into MCF 7 cells through the incubation of cells in a medium consisting of Co₃O₄@CD nanoparticles (0.1 mg mL⁻¹) overnight. Fig. 4A and B show the bright-field and fluorescence images of the cells after the loading of the nanoparticles. Green fluorescence was observed from all of the cells, confirming the accumulation of the nanoparticles into these cells. To exclude the possibility that the nanoparticles adsorbed on the cell surface, the cells were further treated with 0.25% trypsin for half an hour to remove the surface proteins. The fluorescence remained, confirming that the nanoparticles were inside the cells. An MTT assay, as shown in Fig. 4C, revealed that Co₃O₄@CD nanoparticles with a concentration less than 0.1 mg mL⁻¹ were not significantly toxic to the cells. In contrast, half of the cells exposed to Co₃O₄ nanoparticles (containing the same mass concentration of Co₃O₄ as in the Co₃O₄@CD nanoparticles) were found to be dead. These results suggested that an envelopment of the Co₃O₄ nanoparticles by the CDs decreased the cytotoxic effects of the Co₃O₄ and induced the permeability of the cells to the nanoparticles so that they could be used to analyse the intracellular hydrogen peroxide.

For the intracellular hydrogen peroxide assay, 10⁵ cells were pre-loaded with the nanoparticles. After monitoring background luminescence, the cells were exposed to a buffer with 10 mM hydrogen peroxide. Immediately after this exposure, the aqueous hydrogen peroxide diffused into the cell to elevate the intracellular hydrogen peroxide level to near 10 mM. As shown in Fig. 4D, the introduction of hydrogen peroxide into the buffer triggered an increase in luminescence, which showed that our probe could measure the change in concentration of intracellular hydrogen peroxide. Since the concentration of intracellular hydrogen peroxide remained nearly constant, the slow decay in the luminescence was likely caused by the consumption of Co₃O₄@CD nanoparticles inside the cells.

The same analysis process was performed on cells of another cancer cell line, HeLa cells, using the Co₃O₄@CD nanoparticles. As shown in Fig. S5 (ESI†), similar results including those of the cell viability in the presence of Co₃O₄@CD nanoparticles and the analysis of intracellular hydrogen peroxide were obtained. More analyses on other cell lines are being carried out in the laboratory to provide evidence for a general ability to carry out such a chemiluminescence analysis of cells using Co₃O₄@CD nanoparticles.

In summary, Co₃O₄-cored CDs were prepared to induce chemiluminescence from the carbon dots in the presence of hydrogen peroxide at neutral pH. Due to their good cellular permeability and low cytotoxicity, these nanoparticles could serve as novel luminescent probes for the analysis of intracellular hydrogen peroxide. A continuous improvement in the structures of Co₃O₄@CDs is expected to offer higher...
luminescence efficiency so that the intracellular hydrogen peroxide could be imaged.

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Notes and references