Research on selective uptake of photosensitizer C$_3$N$_4$@RP by different cancer cells

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**Abstract**

Photodynamic therapy (PDT), as a clinical treatment, can remove malignant cells upon laser irradiation by selective uptake of photosensitizer (PS). The relative contribution of these antitumor effects depends largely on the dose and uptake of PS. In this study, C$_3$N$_4$@RP was chosen as a candidate for selective uptake studies of different cancer cells. C$_3$N$_4$@RP has been proved to possess excellent properties, including absorption edges extending up to 700 nm, efficient cellular uptake, low cytotoxicity, and favorable intracellular fluorescence localization. Considering the optimal therapeutic effect, we first incubated different concentrations of PS with A549 cells and HeLa cells to observe the uptake efficiency at different times. At a concentration of 20 μg ml$^{-1}$, the cellular uptake by A549 and HeLa showed a time-dependent accumulation. The increasing accumulation for cancer cells at the most effective cellular uptake for 24 h follows an order of HeLa > A549. These results suggest that different types of cancer cells have different uptake saturation times for the same PS. All of the presented results support the idea that a properly designed PS is suitable for specific cancer at a specific time to achieve the best therapeutic effect.

**1. Introduction**

It is well known that cancer is a leading cause of death worldwide [1, 2]. Clinically, most cancer patients respond effectively to chemotherapy, radiotherapy, and adjuvant therapy [3]. However, the development of chemo-resistance, radio-sensitivity, recurrence, and non-specific targeting results in the shortening of survival time and limits these traditional treatments [4]. Photodynamic therapy (PDT), as a modern and non-invasive form of therapy, is based on the local or systemic application of photosensitive compounds, namely, photosensitizers (PSs), which are intensely accumulated in pathological tissues through osmosis effect, subsequent penetrates into tumor tissue to reach tumor cells, and produce internalization into those cells [5]. The PS molecules absorb light at appropriate wavelengths, initiating activation processes that lead to the selective destruction of inappropriate cells [6–8]. Recently, the development of fluorescence detection technology of PSs accumulated in tumor tissue has attracted people’s attention. PS fluorescence can be used as a diagnostic index for malignant tumor detection [9]. However, whether PSs can be used to distinguish different tumor cells remains a question.

Polymeric carbon nitride (CN) is a semiconductor-based photocatalyst. Due to its low toxicity and demonstrated affinity for tumor tissue, metal-free CN has been developed as a highly efficient photocatalyst for tumor cells destruction [10]. However, its activity is extremely low under irradiation with a wavelength beyond 400 nm [11, 12]. More importantly, red phosphorus (RP) has emerged as a new class of photocatalyst, whose visible light absorption edge extends up to 700 nm [13–15]. Creating heterostructure of CN and RP is an effective way to increase the visible light absorption, upshift the conduction band edge, and accelerate the photogenerated electron transfer, which is beneficial to the PDT properties of PSs.
Herein, we evaluated the structure, dark cytotoxicity, cellular uptake, and subcellular localization of C$_3$N$_4$@RP nanosheets (Scheme 1). Furthermore, cell counting kit-8 and flow cytometry analysis provided powerful methods for assessing the optimal time and amount of uptake of C$_3$N$_4$@RP by different types of cancer cells. The cellular uptake by A549 and HeLa showed a time-dependent accumulation at the concentration of 20 μg ml$^{-1}$. The increasing accumulation for cancer cells at the most effective cellular uptake for 24 h follows an order of HeLa > A549. The research can help screen new photosensitive materials C$_3$N$_4$@RP for treating different types of cancer.

2. Experimental section

2.1. Characterization
The synthesis of C$_3$N$_4$@RP samples referred to the phosphorus-doped polymeric carbon nitride nanosheets previously synthesized by our group [16]. The morphology of the samples was observed by a scanning electron microscope (FEI Magellan 400, America) and a transmission electron microscope (EXALENS HT7700, China). Fluorescence data were acquired using a fluorescence spectrophotometer (F-280, China). A laser confocal microscope (Leica SPE, Germany) was used for the fluorescence imaging experiment at 405, 488, and 532 nm. Particle size distribution and zeta potential of C$_3$N$_4$@RP were measured by a dynamic light scattering analyzer (Microtrac Nanotrac Wave II, Japan).

2.2. Cell culture
Human lung adenocarcinoma (A549) and cervical cancer (HeLa) cell lines were acquired from Procell Life Science&Technology Co., Ltd (Wuhan, China). The HeLa cell donor has deceased prior to the research and therefore lack of consent. The cells were authenticated by short tandem repeat analysis. All the procedures complied with the guidelines of Medical Ethics Committee of Affiliated Hospital of Qingdao University. Cell lines were cultured in Dulbecco’s modified eagle medium (DMEM) containing 10% FBS, 100 U ml$^{-1}$ Penicillin and 100 μg ml$^{-1}$ Streptomycin in an incubator (5% CO$_2$) at 37°C. All experiments were performed on cells in the logarithmic growth phase. The culture media was replaced every two days, and the cells were subcultured using 0.25% trypsin-EDTA.

2.3. Scratch assay [17] and phosphorylated apoptosis factor assay [18–20]
Cells were plated in a 12-well plate at a concentration of 3 × 10$^5$ cells per well, which were counted by a cell counter (Olympus Model R1, Japan). After incubation for 24 h, serum-free DMEM was incubated with
DMEM solution containing 0.1% FBS was replaced every 24 h.

Observation, and digital images of cancer cell growth were automatically captured every 24 h with DMEM. IncuCyte S3 Live-Cell Analysis System free DMEM three times. An approximately 700 μm wide scratch was created in each well and washed three times with DMEM. The cancer cells were seeded overnight on a 96-well plate. Then, the original medium was exchanged and washed with serum-free DMEM, and cultured in the dark for 24 h [21, 22]. The DMEM solution containing 0.1% FBS was replaced every 24 h.

Cells were seeded on microscopic slides at a density of 3 × 10⁵ cells per well overnight. Then, the original medium was replaced with a solution of C₃N₄@RP at a concentration of 20 μg ml⁻¹, and cultured in the dark for 24 h. After being washed with PBS, the cells were fixed with 4% paraformaldehyde. Next, pro-caspase-3 (ABCAM, England) and 4, 6-diamidino-2-phenylindole (DAPI; 10 μg ml⁻¹ in PBS) antibody staining were performed for routine immunofluorescence. Finally, the slide was transferred to a microscope, and the cells were observed under a confocal microscope.

2.4. Cellular uptake in vitro [7, 8]

The cancer cells were seeded overnight on a 96-well fluorescent plate at a density of 1 × 10⁴ cells per well. Then, the original medium was replaced with C₃N₄@RP with different components in serum-free DMEM, and incubated in the dark for 4, 8, 24, and 48 h. The whole mixture was discarded from the original medium, washed three times with PBS, and added 100 μl DMEM. The retention of cell-associated C₃N₄@RP was detected by fluorescence measurement using a multifunctional microplate reader (TANGEN SAFIRE II, China).

Cells were seeded on microscopic slides at a density of 3 × 10⁵ cells per well overnight. Then, the C₃N₄@RP of different components, which in serum-free DMEM, exchanged the original medium, and cultured in the dark for 24 h. After being washed with PBS, the cells were fixed with 4% paraformaldehyde. Finally, the slide was transferred to a microscope, and the cells were observed under a confocal microscope based on C₃N₄@RP blue fluorescence.

The cellular uptake of the studied PSs was also determined using flow cytometry and quantified based on C₃N₄@RP orange fluorescence. Cancer cells were cultured overnight in a 6-well plate at a density of 3 × 10⁵ cells per well. Then, the original medium was replaced with 20 μg ml⁻¹ C₃N₄@RP, and incubated with cancer cells for 24 h. The excess particles were washed off with PBS, and the cells were digested with trypsin. NP uptake was then quantified by flow cytometry (APOGEE A50-MICR0, England).

GraphPad Prism 8 software was used for statistical analysis. One-way and two-way analyses of variance with Tukey’s test were used to compare the multiple groups. The results were expressed as the mean ± standard deviation (SD) of at least three independent experiments. P < 0.05 was considered statistically significant, and *, ** and *** in the quantitative images were P < 0.05, P < 0.01, and P < 0.001, respectively.

3. Results and discussion

3.1. Characterization of C₃N₄@RP

To track and investigate the uptake process precisely, the morphology of C₃N₄@RP was observed by SEM and TEM. As shown in figure 1(A), C₃N₄@RP is a typical stacked layered structure. The TEM image in figure 1(B) further confirms the nanosheet morphology of C₃N₄@RP. The interactions between nanoparticles and cancer cells are significant, the properties of nanomaterials such as size, shape, and composition are closely related to the ability of cell uptake [23]. The dimensions are a strong determinant of total cell uptake, particles with a size of ~100 nm are suitable for the tumor-specific enhanced permeability and retention effect and possess high nanomaterial retention rate, and tend to accumulate in tumor site [24]. Combined with the particle size of C₃N₄@RP in figure 2(A), this result indicates that C₃N₄@RP nanosheets has been successfully constructed, and make them good candidates for cancer cell uptake.

Figure 1. Characterization of C₃N₄@RP. (A) SEM image and (B) TEM image of C₃N₄@RP.
To investigate whether the functionalized C$_3$N$_4$@RP PS can remain stable fluorescence properties, the hydrodynamic diameter and Zeta potential were studied by dynamic light scattering. Figure 2(A) shows that C$_3$N$_4$@RP has a diameter of about 110 nm. He et al studied the effects of particle size on cellular uptake of polymeric nanoparticles [25]. They found that nanoparticles size in the range $\leq$150 nm was tended to accumulate in tumor more efficiently. We therefore conclude that the C$_3$N$_4$@RP PS in this study can potentially be taken up by tumor cells. Zeta potential is generally used to evaluate the physical stability of particle dispersion systems [26]. Generally, zeta potential data is associated with colloidal stability [27]. As reported by the previous report [28], zeta potential values of $\pm$10–10 mV are classified as highly unstable, $\pm$10–20 mV as relatively stable, $\pm$20–30 mV as moderately stable, and $\geq$30 mV as highly stable. According to figure 2(B), the zeta potential of C$_3$N$_4$@RP is about 31 mV, demonstrating the high stability of colloidal dispersion containing C$_3$N$_4$@RP photosensitizer in the tumor microenvironment. To employ the fluorescent potential of PS during cellular uptake, the excitation and emission fluorescence spectra were obtained by exposing the solution of C$_3$N$_4$@RP to UV light ($\lambda = 370 \pm 50$ nm). As shown in figure 2(C), the solution displays intense fluorescence with optimal emission in the blue range ($\lambda = 500 \pm 50$ nm), which makes C$_3$N$_4$@RP a good candidate as a fluorescent probe in bioimaging [29].

Further, the fluorescence imaging function of C$_3$N$_4$@RP was investigated [30]. A confocal laser scanning microscope was used for showing that PS can be displayed different colors under different excitation wavelengths in figure 3, which demonstrated C$_3$N$_4$@RP nanosheets could be used for tumor bioimaging in cellular uptake, this property lays the foundation for further exploration of PDT in different tumor cells.

3.2. Evaluation of biocompatibility

A scratch assay was adopted to investigate the effect of the PS prepared on cell migration. Human lung adenocarcinoma cells (A549) were determined after co-incubation with various C$_3$N$_4$@RP for four days (figures 4(A) and (B)). The results indicated that A549 cells maintained intact scratch closure (about 100% of the cell coverage area) under the condition of no more than 50 $\mu$g ml$^{-1}$ PS. There was no significant difference between the different concentration groups and the control groups. Cervical cancer cells (HeLa) were determined after co-incubation with various C$_3$N$_4$@RP NRs for six days (figures 4(C) and (D)). The intact scratch closure of HeLa cells was consistent with that of A549 cells. The results indicated that both kinds of cells maintained high viability under the condition of no more than 50 $\mu$g ml$^{-1}$ PS. The excellent biocompatibility of C$_3$N$_4$@RP was beneficial to therapeutic applications.

Pro-caspase-3 is a key enzyme in cell apoptosis [31]. Pro-caspase-3, as its proenzyme form, is a crucial effector enzyme molecule in the signal transduction pathway of cell apoptosis, which can activate endonuclease, degrade DNA, and lead to cell apoptosis. Based on the above physicochemical and biological evaluations of C$_3$N$_4$@RP, we also measured the expression of substrate pro-caspase-3 in HeLa cells treated with or without 20 $\mu$g ml$^{-1}$ C$_3$N$_4$@RP. The results showed that after 24 h of co-incubation, the expression of pro-caspase-3 was
measured according to the average fluorescence intensity (figures 5(A) and (B)) [32]. The result showed that there was no significant difference in the expression of pro-caspase-3 between the C3N4@RP treated groups and the untreated groups. Taken together, C3N4@RP showed good biocompatibility.

3.3. Cellular uptake in vitro

According to the previous reports [8], the effective accumulation of uptake means PS inside the tumor cells, which can be evaluated via fluorescence microplate. The fluorescence intensity reflects the effective accumulation of PS. To confirm the uptake of C3N4@RP-NPs by tumor cells, we incubated different concentrations of PS with A549 cells and HeLa cells to observe uptake efficiency at different times [14]. As presented in figure 6(A), the cellular uptake of A549 cells was time-dependent at a concentration of 20 μg ml⁻¹, and an effective accumulation of uptake was achieved after 4 h of incubation. The cellular uptake of HeLa cells was also time-dependent at the concentration of 20 μg ml⁻¹. However, effective accumulation of uptake was achieved after 24 h of co-incubation (figure 6(B)). These results suggested that different types of cancer cells have different saturated concentrations and saturated times of uptake for the same PS. The uptake tendency of C3N4@RP-NPs by tumor cells is consistent with Xia and coworkers, who investigated the uptake of folate-based
nanoparticles by HeLa cells and A549 cells, and they found that cellular uptake behavior of folate-based nanoparticles depended on incubation time and folate concentration [33].

In addition, we confirmed these trends in A549 cells and HeLa cells by flow cytometry analysis. The results showed that PS exhibited more efficient cellular uptake after incubation with HeLa cells for 24 h. And median fluorescence intensity by flow cytometry showed that there was significant difference between the uptake of HeLa cells and A549 cells (figure 7). According to Santos et al. report [34], the NP enter different cell types via different uptake mechanisms. They found that in the HeLa cell line, actin filaments play a decisive role in the uptake process of NPs, while microtubules are mainly involved in NP uptake in the A549 cell line. Therefore, it is supposed that the uptake mechanisms of NP may affect the uptake difference of C3N4@RP. Similarly, from previous report it has been mentioned that A549 cell expresses various stem cell markers and drug resistance proteins, HeLa cells culture have differentially expressed drug resistance related markers, which also proved to be related to cellular uptake and various protein expression [35].

It is important to determine the initial target of PS in PDT by intracellular localization [36]. To precisely evaluate the intracellular localization of PS, the internalization of different cancer cells was observed by confocal laser scanning microscopy (figure 8). It was further confirmed that increased uptake accumulation of HeLa cells was higher than that of A549 cells when incubated at 20 μg ml⁻¹ for 24 h. These observations are consistent with the results of the flow cytometry assay (figure 7), suggesting that PS has efficient internalization. A similar approach has been used by Gong et al. [37], who also utilize fluorescence of ICG-nanoparticles to assess cellular uptake and localization of nanoparticles. Confocal fluorescence microscopy showed obvious fluorescence in the cytoplasm of cancer cells, indicating effective uptake and intracellular distribution of nanomaterials.

4. Conclusion

The design and development of new PSs have always been a hot topic [30, 38]. In this work, the uptake of a PS nanomaterial by different kinds of cancer cells was studied. The PS nanomaterial we selected did not affect the proliferation and migration ability of cells or promote the apoptosis of cells, showing the advantages of low cytotoxicity, good stability, and great biocompatibility. Moreover, different types of cancer cells have different uptake saturation times for the same PS nanomaterials. This will be helpful for screening safer photosensitive
nanomaterials in the future. It also provides help for drug loading, improvement of PS, cancer diagnosis and combination therapy.

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Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

Ethics approval

Regrettably, donor of HeLa cells has deceased prior to the research and is unable to grant consent but is still identifiable, therefore we added cells origin, short tandem repeat analysis and lack of consent in the manuscript. All the procedures complied with the guidelines of Medical Ethics Committee of Affiliated Hospital of Qingdao University.

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