Chitosan Nanoparticle Combined with Ultrasound for Cervical Cancer Detection

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Abstract

Photodynamic medicine is a novel approach for cancer detection and treatment via different photosensitizers and suitable light sources. The 5-Aminolevulinic acid (5-ALA) can be converted into protoporphyrin IX (PpIX) that can be utilized as a fluorescent probe for tumor detection. However, the fluorescent quantity of PpIX is sometimes too low for detection. In this study, 5-ALA was encapsulated in chitosan nanoparticles, and then pushed into HeLa cancer cells by ultrasound to increase the fluorescent quantity of PpIX. Chitosan nanoparticles loaded with 5-ALA (CNA) was prepared by ionic gelation method after mixing chitosan with 5-ALA.The size and zeta potential of CNA, measured by transmission electron microscope and zetasizer, was 102.4 nm and 25.0 mV, respectively, which were enough to prevent particles from aggregating. The loading efficiency of 5-ALA in CNA was up to 90%. The amounts of CNA uptaken by HeLa cells and the PpIX concentration in cells were significantly enhanced by ultrasound. Based on the above results, a novel photodynamic detection system might be designed to enhance the diagnostic accuracy of early cervical cancer.

Keywords: Photodynamic medicine, 5-Aminolevulinic acid, chitosan, nanoparticle and ultrasound.

1. Introduction

Cervix cancer is the second most common cancer in female worldwide. Although most cervical cancers are squamous in origin, adenocarcinomas appear to be increasingly common recently. Adenocarcinomas include many more histological subtypes than squamous cancers. Early-stage disease may be asymptomatic, but can be recognized by a smear or large loop excision of the transformation zone (LLETZ). A biopsy and a histopathological examine by an experienced gynecological pathologist is required to diagnose cervix cancer. However, the biopsy needs a large enough tissue block to demonstrate invasion or not, but often only a small LLETZ can be used.[1] Therefore, a new and more convenient method to detect cervical cancer needs be developed.

Photo-diagnosis is one of promising and non-invasive methods to detect malignant or premalignant tissue.[2] Photo-diagnosis generally uses fluorescence to help detection and painting of abnormal tissue regions, in which exogeneous chromophore is excited by optima light to generate fluorescence to detect the cancer lesions.[3] 5-ALA is a precursor of heme synthesis in human body, and totally degraded in cells after converted to protoporphyrin IX (PpIX). PpIX is one of photosensitive fluorophores which can be used as fluorescence source to detect cancer lesion due to the different decomposition rate between PpIX in cancer cells and normal cells.[4,5] Generally, PpIX can be totally degraded within 2 - 4 hours in normal cells but 12 - 24 hours in cancer cells.[6] However, 5-ALA could be easily engulfed by the bacteria in vagina that leads to miss interpretation of observation. Therefore, a carrier is needed to prevent 5-ALA from bacteria uptake and to enhance 5-ALA passing through the lipophilic barrier and entering mitochondria to form PpIX, which serves as the photo source for photo-diagnosis.[7]

Chitosan is a natural polymer. Comparing with other biological polymers, chitosan bears positive charges to approach cell membrane conveniently and has muco-adhesive property to prolong the retention time of

chitosan in the interested locations.[8,9] Additionally, chitosan is biocompatible and induces no allergic reactions or immuno-rejections. It can be utilized as an adsorbent for toxic metals and possesses antibacterial properties, which can prevent from bacteria uptake in the vagina.[10] Furthermore, chitosan is a linear polyamine containing a number of free amino groups which are available for crosslink, and its cationic nature also allows for ionic crosslink with multivalent anions.[11]

It is believed that ultrasonic mechanical waves or other subsequent interacting mechanisms can alter the cell membrane permeability and might thus increase the uptake of macromolecules.[12] Cavitation is typically generated through the activation of small dissolved gas nuclei in the presence of an acoustic pressure field. It is further believed that cavitation can make some "temporal holes" on cell membranes that make them transiently permeable to large molecules.

In this study, we designed a chitosan nanoparticle to encapsulate 5-ALA and combined with ultrasound to enhance its uptake by cervical cancer cells.

2. Materials and Methods

2.1 Preparation of chitosan nanoparticles loaded with 5-ALA (CNA)

The preparation of chitosan nanoparticles loaded with 5-ALA is based on an ionic gelation interaction between positively charged chitosan and negatively charged tripolyphosphate or ionized 5-ALA at room temperature.

Chitosan was dissolved to a concentration of 0.5mg/ml in 0.01M acetic acid solution under the pH of 4.0. 5-ALA was dissolved to a concentration of 1.5 mg/ml in 0.5mg/ml sodium tripolyphosphate solution and the pH was adjusted to 7.4. Two ml of 5-ALA STPP PBS solution was dropped slowly in 5 ml of chitosan solution under a flowing rate of 0.5 ml/min. Nanoparticles were used without further purification for all studies.

2.2 Ultrasound apparatus setup

The ultrasound setup used in the experiments is shown in Figure 1. All experiments were performed in a tank containing degassed water at 35~37 ºC (O_2 saturation in water of less than 3 ppm). The ultrasound field was generated by a 1-MHz plane piezoelectric transducer that was 3.8 cm in diameter and 20 cm in the focal length (model A392S, Panametrics, Waltham, MA, USA). The 24-well plate was placed 5.4 cm above the transducer, and only one well at a time was exposed to ultrasound by aligning the center of the exposed well with the center of the transmitting transducer. Programs written by Labview (v. 7.1, National Instruments, Austin, TX, USA) were used to control the ultrasound exposure time, pulse repetition frequency (PRF), and output voltage of the function generators (model 271, Wavetek, San Diego, CA, USA). The output signals from the function generators were amplified by a power amplifier (model A100, Amplifier Research, Souderton, PA, USA) and monitored by a power meter (model 4421, BIRD Electronic Corporation, Solon, OH, USA). The input power to the transducer was set to 55 W. A needle hydrophone (Onda, Sunnyvale, CA, USA) was used to measure the ultrasound pressure. In order to avoid the influence of reflected waves, a 20-cycle short pulse was transmitted from the transducer to the needle hydrophone. Under the above conditions, the peak-to-peak pressure obtained was 0.361 MPa. The attenuation of the 24-well plate was about 9%, and the average acoustic intensity at the bottom of the exposure well was calculated to be about 0.54 W/cm^2.

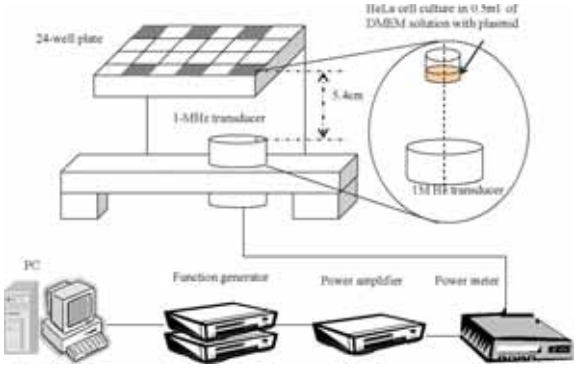

Figure 1. The ultrasound apparatus.

2.3 Enhancement of HeLa cells uptake by ultrasound

HeLa cells were seeded on 24-well culture plates. After the HeLa cells were cultured for 24 hours, the medium was replaced with fresh medium with either CNA or 5-ALA. HeLa cells were exposed to ultrasound of 50% duty cycle (for a total ultrasound exposure of 20 s), and on- and off-times of 5 ms each. After exposing to ultrasound, HeLa cells were further cultured for 05, 1, 3, 6, 12 and 24 hours to allow the uptake of nanoparticles and the conversion of 5-ALA to PpIX. To quantify the amount of PpIX, the PpIX were extracted by using DMSO and its concentration was determined by the GEMINE XS (Molecular Device SPECTRA MAX GEMINE XS) with the excitation wavelength of 405 nm and the emission wavelength at 635 nm.

For in situ observation, the cultured medium was removed, and then the glass plate was washed with PBS three times. HeLa cells were fixed by 10 % formalin and examined under fluorescence microscopy in which green laser was used to excite PpIX to emit red fluorescence.

3. Results and Discussions

3.1 Enhancement of HeLa cells uptake by ultrasound

Figure 2 shows the OD value of PpIX, which was converted by HeLa cells from 5-ALA with (ALA+US) or without (ALA only, positive control) ultrasound exposure. From this figure, when the incubation period of cells with 5-ALA increases, the amount of PpIX in cells, also increases. The concentration of PpIX in HeLa cells is significantly higher in groups receiving ultrasound exposure. It might be due to holes produced by acoustic cavitation on cell membranes, and 5-ALA could diffuse into cell successfully via these holes.

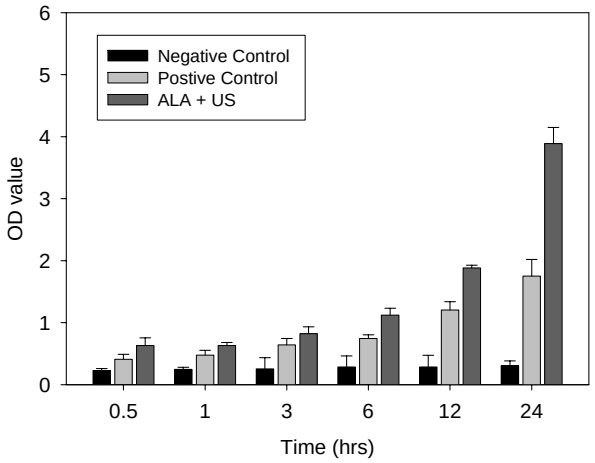

Figure 2. The OD values of PpIX converted by HeLa cells from 5-ALA.

The OD value of PpIX, which was converted by HeLa cells from 5-ALA loading in CNA with (CNA+US) or without (CAN only, positive control) ultrasound exposure , is shown in figure 3. When the incubation period of cells with CAN increases, the concentration of PpIX increases. From these results, it was found that the content of CNA, which was uptaken by HeLa cells, could be enhanced by ultrasound exposure. It might also be due to the holes produced by acoustic cavitation on cell membranes, and CNA could diffuse into cell successfully via these holes.

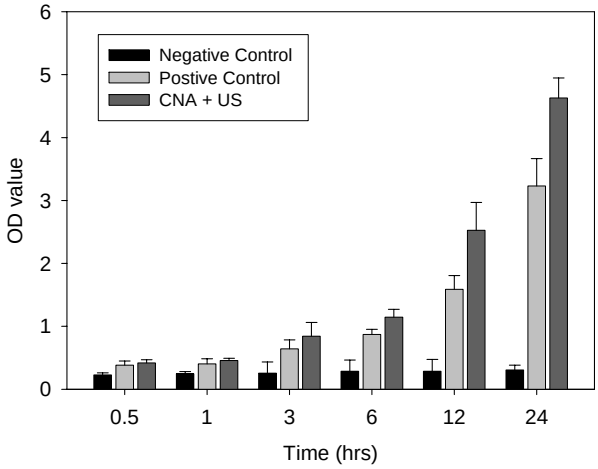

Figure3. The OD values of PpIX converted by HeLa cells from 5-ALA loading in CNA.

Comparing figure 2 with figure 3, we could find that the OD values of PpIX in CAN+US groups are

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higher than 5-ALA+US groups after 6 hours of incubation. Since 5-ALA was a hydrophilic and zwitterionic drug at physiological condition, it could poorly cross biological lipophilic barriers. However, when 5-ALA was encapsulated, it bears positive charges on surface (25mV) and has stronger interactions with HeLa cell membranes,, resulting in higher efficacy of internalization.

The photo images of red fluorescence of PpIX excited by green laser under fluorescence microscope are showed in figure 4. From these photo images, we could find that the uptake of 5-ALA or CNA by HeLa cells was both enhanced by ultrasound exposure. 5-ALA or CNA could diffuse into cell easily via holes produced by ultrasound.

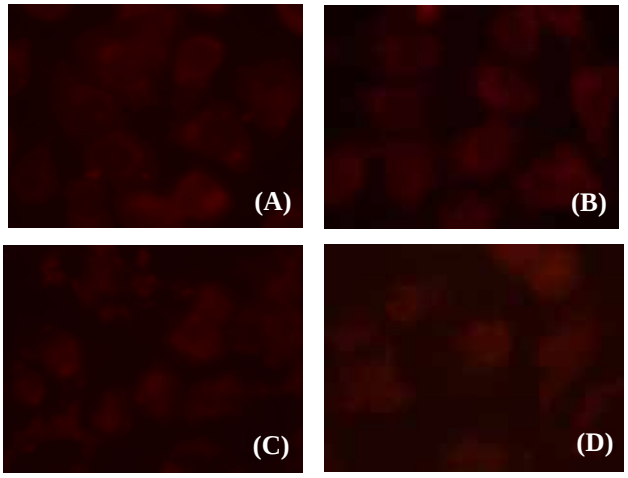

Figure 4. Red fluorescence of PpIX excited by green laser and observed by fluorescence microscope. (A) the bright field of HeLa cells fed with 5-ALA, (B) the fluorescence picture of HeLa cells fed with 5-ALA and exposed to ultrasound, (C) the bright field of HeLa cells fed with CNA, (D) the fluorescence picture of HeLa cells fed with CNA and exposed to ultrasound.

4. Conclusion

By ionic gelation method, chitosan nanoparticles loaded with 5-ALA were prepared and the average sizes were about 100 nm. The zeta-potential of particle was 25 mV. Bioassy results of CNA for HeLa cell revealed that HeLa cell could uptake CNA and the amounts of CNA uptaken by cell could be enhanced greatly by ultrasound. According to this concept, a novel photodynamic detection system could be designed to enhance the diagnostic accuracy of early cervical cancer.

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