Controllable Preparation and Anti-tumor Studies of Upconversion Nanomaterials

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Research Background

Along with the increasing of population aging and the prevailing of smoking, unhealthy diet and obesity around the world, the number of new cancer diagnoses keeps growing. According to the data of 2020 global cancer burden from WHO's International Agency for Research on Cancer (IARC) illustrating the latest morbidity, mortality and development trends of 36 types of cancers in 185 countries across the world, there were totally 19.29 million new cancer cases including 10.06 million males and 9.23 million females, and 9.96 million cancer deaths including 5.53 million males and 4.43 million females in 2020. It is estimated that the global burden of cancer in 2040 will increase 50% compared with 2020 dramatically in the countries undergoing social and economic transition. There will be nearly 30 million new cancer cases worldwide by then(1).

There are certain limitations in most traditional cancer treatments such as chemotherapy, radio therapy and operative therapy. For example, besides the operating difficulty in some sites, operations may cause irreversible damages as well; without specificity in killing cancer cells, normal cells will be destroyed as well during the chemotherapy; with expensive price, long treatment cycle and more complications, radiotherapy can also reduce patients' survival duration. These treatments share one trait that cancer cells cannot be eradicated and thus may cause the recurrence and metastasis of cancers in a certain period of time.

In a bid to overcome these shortcomings, the emerging nanotechnology has been used in the treatment of cancers, contributing a lot to medical development and bringing a new dawn for the improvement of human health. Due to the toxicity of nanomaterials, high dosage of nanoparticles as anticancer drugs can hurt human bodies, and thus the studies of upconversion luminescent materials and spectroscopic properties have aroused wide

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public concern.

Upconversion luminescence refers to the anti-Stokes luminescence or anti-Stokes nonlinear process in which ground-state ions (activator ions) transiting to the high-energy metastable state after continuous absorption of two or more low-energy photonsemit high-energy photons when returning to the ground state after radiative and non-radiative relaxation processes. Upconversion luminescent materials take place in the compounds doped with rare-earth ions mainly including fluorides, oxides, sulfur compounds, oxyfluorides and halogenides (2).

Upconversion luminescent materials have obvious advantages. For instance, comparing with semiconductor quantum dots and organic dyes, UC fluorides have the properties of profound depth of tissue penetration, no spontaneous fluorescence interference, low biotoxicity, high photostability and targeting ability. For one thing, nanomaterials can be applied to locate the sites of tumors through tumor acidic microenvironment. The drugs loaded nanomaterials act on genes or molecules, and cause the specific deaths of tumor cells with less influence on normal body tissues. The targeted therapy of nanomaterials can increase the dissolution and absorption rates of poorly soluble drugs, improve the drug effects and decrease the toxic side effects while releasing drugs at specific sites, times, and speeds with the purpose of reducing the damages of drugs to human bodies (3).

For another thing, cancer cells tend to phagocytize nanoparticles and the phagocytized upconversion materials make the tumor cells fluoresce, which will thus achieve the passive targeting effects. The imaging of fluorescent tumors can help to observe the sites and trajectories of cancer cells and anti-cancer drugs in human bodies more precisely.

Furthermore, magnetic resonance imaging, in connection with the application of upconversion materials, is one of the important means for analysis and diagnosis, combined treatment and medical research in modern biomedicine. Non-radioactive damages can provide multi-dimensional visual images with high resolution for biomedical researches. In recent years, there are some studies on the radiosensitizer of gadolinium-based nanoparticles, because the gold nanoparticle, relatively mature for radiation therapy, cannot be used for clinical treatment due to its major damage to human bodies. Therefore, the rare-earth upconversion nanoparticles, such as gadolinium, are expected to be the materials of radiation treatment with the functions of radiosensitizer and contrast agent (4).

Hence, upconversion nanomaterials, in addition to maximizing the drug effects and shortening the treatment cycles, can kill cancer cells more thoroughly and reduce the damages of drugs to other tissues of human body.

Experiments

Firstly, solvothermal synthesis technique was used in the preparation of upconversion nanomaterials, which needed some reagents such as Y(NO3) ·6H2O, Yb(NO3) ·5H2O, Er (NO3) ·5H2O, ethylene glycol (EG), NH4F and Nacl, and some instruments such as a centrifuge, scale and magnetic stirrer. Put 0.298g Y(NO3) ·6H2O, 0.089g Yb (NO3) ·5H2O and 0.008g Er (NO3) ·5H2O into a beaker, add ethylene glycol (EG) and stir for 10 minutes. Then, put 0.45g NH4F and 0.0584g NaCl, add water and continue to stir for 20 minutes. After being treated by ultrasonic wave for 2 minutes, keep it at a hydrothermal temperature of 200°c for 12 hours. Freeze dry after water washing and alcohol washing. Then, prepare solvent

mixtures of four different proportions: pure EG30ml; EG25ml, water 5ml; EG20ml, water 10ml; and EG15ml, water 15ml.

Secondly, the anti-cancer drug of doxorubicin (DOX) needed to be loaded on the upconversion materials in advance. Prepare PBS solution of DOX (1mg/mL), adjust the pH value to around 8.0, put the weighted 10mg-20mg upconversion powder into 3ml of the above-mentioned PBS-DOX solution, add 1mL PEI with the concentration of 5mg/mL (molecular weight: 25000). Keep it away from light at room temperature and stir for about 12 hours overnight.

Thirdly, use the scanning electron microscope of F4800 to observe the structures of upconversion nanomaterials, which are mainly composed of nanoparticles and nanorods with the diameter accurate to nanometers. Next, take photos under different luminous intensities. The upconversion nanomaterial solvent could be seen glowing under the irradiation of 980 laser in the darkroom. Then, observe the ultraviolet absorption spectrums of pure upconversion nanomaterial solution, pure doxorubicin solution and the upconversion material solution loading with doxorubicin, compared their absorption peaks, and deduced whether the upconversion materials succeeded in drug loading.

The first experiment was cell passage cultivation, a prerequisite for cell experiment. The first step was to prepare the clean bench, complete medium (DMEM/RPMI+10%FBS+1%P/S) and PBS, put the opened bottle of pancreatin beside the alcohol lamp (roasting before and after opening) for use, and keep the instruments sterile to avoid the interference with the experiment. The second step was to take cells from the incubator to observe the cell density under a microscope and start the operation of cell passage when it reached 80-90%. After that, put the cell culture flask on the clean bench, unscrew the cap for roasting after roasting the flask mouth, suck out the waste culture medium from the flask with a pipet, wash the cells twice with PBS, add 1mL pancreatin and put them in the incubator to digest for 3 minutes (the digestion time was determined according to the type of culture cell).

After the digestion time, observe the cell state under a microscope. If the cells crumpled into a ball, it suggested a good digestion. If there were a mass ofadherent cells, it needed more time for digestion or much pancreatin should be added in the previous step. Add the digested cells to the complete medium immediately to terminate the cell digestion, and gently blow the cells adherent to the wall of the culture flask into the culture medium with a straw. Then, transfer the cell suspension to a centrifuge tube of 15mLfor the centrifugation at 1200rpm for 3 minutes.

Discard the supernatant after the centrifugation, add about 3mL new complete medium, stir and blow it gently to disperse the cells. Suck up 1mL cell suspension and put into the culture flask, add again 4mL complete medium, stir and blew it gently to disperse the cells.

Tighten the cap of the culture flask after roasting its mouth and cap, put it flat on the clean bench, and shake it gently from side to side to disperse the cells evenly in the culture flask. In the end, observe whether the cells were dispersed uniformly under a microscope, and, if needed, repeat the previous step to achieve the uniform dispersion before putting it into the incubator for further cultivation.

The second experiment was to conduct the experiment on cell viability by the use of CCK-8, the abbreviation of Cell Counting Kit-8, the WST-8-based high-sensitivity and rapid test kits

widely used for testing cell proliferation and cytotoxicity. In the presence of electron coupling reagent, WST8 could generate orange-colored formazan during its reduction by some dehydrogenases in mitochondria. The more and faster the cells proliferated, the darker they became in color; the greater the cell cytotoxicity was, the lighter they became in color.

Prepare the clean bench, complete medium(DMEM/RPMI+10%FBS+1%P/S), PBS, pancreatin, 96-well plate and cell counting plate first, and then take cells from the incubator, wash them twice with PBS and add 1mL pancreatin for digestion (the digestion time was determined according to the type of culture cell).

The following was to prepare the cell suspension. Terminate the digestion of the digested cells with the complete medium, transfer the cell suspension to a centrifuge tube of 15mL for the centrifugation at 1200rpm for 3 minutes, discard the supernatant containing pancreatin, and add new complete medium to prepare the cell suspension. The next step was to conduct the cell counting. Pipet 10uL of the well-mixed cell suspension, drop onto the cell counting plate, and insert the cell counter to read the data (the traditional method is to pipet 10uL of the well-mixed cell suspension, drop onto the blood counting chamber and then read the data under a microscope). The cell density was 5000-8000 cells per 100uL during normal cytotoxicity tests (the cell density was determined in accordance with the type, size and multiplication rate of the tested cell).

The next was the dilution of cell suspension. Dilute the cell suspension with complete medium to reach the cell density suitable for the cell culture plate according to the data of cell counting.

In the following process of cell seeding, a 96-well plate was prepared in advance. Add equal volume of PBS into the peripheral holes without growing cells to prevent the evaporation of the medium of cell culture, and put the cell plate into the incubator for 24 hours (determined in accordance with the cell adherence) after adding the cell suspension. After 24-hour culture, suck out the culture medium from the pore plate, add with materials of different concentrations and gradients as shown in the graph and put it in the incubator for 24 hours (the incubation time could be 36 or 48 hours according to the experimental requirements).

After the incubation, prepare 10% CCK-8 solution (complete medium as the solvent), and put it on the clean bench away from light for use. Then, take out the pore plate, suck out the culture medium from the pore plate, add the prepared CCK-8 solution, and put it in the incubator for 0.5-4 hours (the incubation time was determined according to the experimental requirements, and one-hour incubation typically could bring the most obvious effect).

The following was ELISA test. Take out the pore plate from the incubator and keep it away from light for centrifugation at 1200rpm for 10 minutes. Pipet 70% of the supernatant into another pore plate for ELISA test (detection wave length=450nm)

The third experiment was to conduct the experiment of live/dead cell staining to compare and contrast the killing effects of drugs on cancer cells at different concentrations and different times. The reagents used in live/dead cell staining were Calcein AM (CA) and Propidium Iodide (PI). CA is a stain which can be used for the fluorescent labelling of live cells based on the operating principle that the calcein formed by its shearing by intracellular esterase after penetrating the cell membrane retains in

the cell and emits bright green fluorescence. PI, a nuclear stain which cannot penetrate the living cell membrane, could only enter the cell nucleus across the disordered regions of dead cells, embed itself in DNA double helix and thus emit red fluorescence. Since CA and PI couldn't be excited by 490nm simultaneously, live and dead cells would be observed under a fluorescence microscope at the same time.

Prepare the clean bench, complete medium, PBS, pancreatin, CA/PI stain and 96-well plate before the experiment.

Take out the cells from the incubator after digestion, wash twice with PBS, and add 1mL pancreatin for digestion (the digestion time was determined according to the type of the culture cell).

Terminate the digestion of the digested cells with the complete medium, transfer the cell suspension to a centrifuge tube of 15mL for the centrifugation of 3 minutes at 1200rpm, discard the supernatant containing pancreatin, and add new complete medium to prepare the cell suspension.

The process of cell counting was to pipet the well-mixed cell suspension, drop onto the cell counting plate, and insert the cell counter to read the data (the traditional method is to pipet the well-mixed cell suspension, drop onto the blood counting chamber and then read the data under a microscope). The cell density usually used in 6-well plate is 100,000-150,000 cells per mL (the cell density was determined in accordance with the type, size and multiplication rate of the tested cell).

The following was the dilution of cell suspension. Dilute the cell suspension with complete medium to reach the cell density suitable for the cell culture plate according to the data of cell counting. Add the diluted cell suspension onto the pore plate and shake it gently from side to side to disperse the cells evenly.

Put the cell plate into the incubator for 24 hours (determined in accordance with the time of cell adherence). After the cells adhered to the wall, replace the complete medium with culture medium containing materials, and put it into the incubator for some time (determined in accordance with the cellular uptake of materials).

The next step was the X-ray irradiation of the incubated cells. Put the cells into the incubator for 3 hours after the X-ray irradiation as required.

Prepare the solution for co-staining after the incubation time (adding 5ml of PBS with 100pL CA and 15uL PI stock solution, mixing well to prepare the working fluid, and conducting cell staining at the 2M concentration of CA and 4.5M concentration of PI).

The following was cell digestion. Suck out the culture medium containing materials, wash twice with PBS, and add trypsin digestive cells over the cells in every pore.

Collect cells by centrifugation after terminating the digestion, and wash the cells twice with PBS to deactivate the esterase in the culture medium; in the following cellular staining, add 1mL of the prepared solution for cellular staining into the centrifuged cells, stir and blow gently with a pipette to make them mix well. And then put them into the incubator for 15 minutes.

Transfer the cell suspension to the pore plate after incubation, observe the live and dead cells under a fluorescence microscope (observing the

live cells of green fluorescence at 488nm and observing the dead cells of red fluorescence at 561nm).

Results and Discussions

Firstly, the aforementioned upconversion nanomaterial solvents of four different proportions needing to be prepared were pure EG30ml; EG25ml, water 5ml; EG20ml, water 10ml; and EG15ml, water15ml respectively. Rodlike structure and globular structure were observed under scanning electron microscope. According to the observation results, it could be concluded that the larger the proportion of water in solvent is, the more rodlike structures there are in the nanomaterials under the electron microscope, and the luminescence phenomena will be more obvious. Figure 1 was the structure of nanoparticle and nanorod in nanomaterials, and it was clear that the luminous intensity of nanorod was stronger than that of nanoparticle when being placed together.

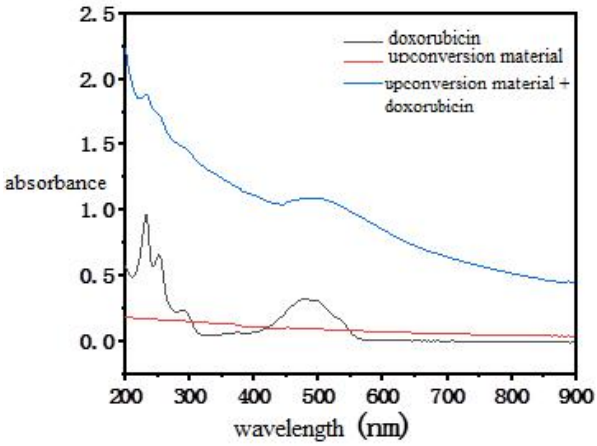
Figure 1 Structure of Nanoparticle and Nanorod in Nanomaterials

As shown in Figure 1, it was obvious that nanorods outnumbered nanoparticles and had stronger luminous intensity.

Secondly, according to the observation of the UV-visible absorption spectra of the nanomaterial after loading with doxorubicin during the in vitro studies of drug loading performance, there were the same peak values with the UV absorption spectrum of pure doxorubicin, illustrating that the upconversion nanomaterial has loaded doxorubicin successfully.

In Figure 2, the red line is the UV absorption spectrogram of pure upconversion nanomaterial, the black line is the absorption spectrogram of pure doxorubicin, and the blue line is the spectrogram of upconversion nanomaterial loading with doxorubicin, in which it can be observed that the upconversion nanomaterial successfully loading with doxorubicin had showed the absorption peaks of the pure doxorubicin.

Figure 2 The Comparison of Three Peak Values



With regard to the studies of drug-loading upconversion materials' toxic effect on tumor cells, the drugs were divided into two groups on a 96-well plate. In the first group, the experiment aimed to observe the numbers of cancer cell deaths after mixing cancer cells with the same concentration of different drugs for 4 hours and 24 hours respectively. Figure 3 are the pictures of them 4 hours after the mixture. It could be seen that there were almost no deaths

of tumor cells without adding any reagent, some deaths of cancer cells when adding upconversion materials only, and more deaths of cancer cells when adding upconversion materials loading with doxorubicin, which, however, can still illustrate that upconversion materials themselves are toxic.

Figure 3 Killing of cancer cells after 4-hour mixture

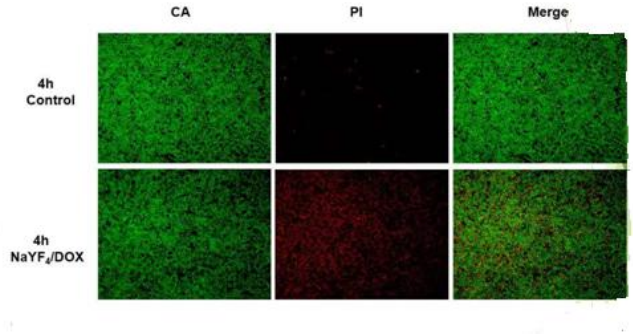
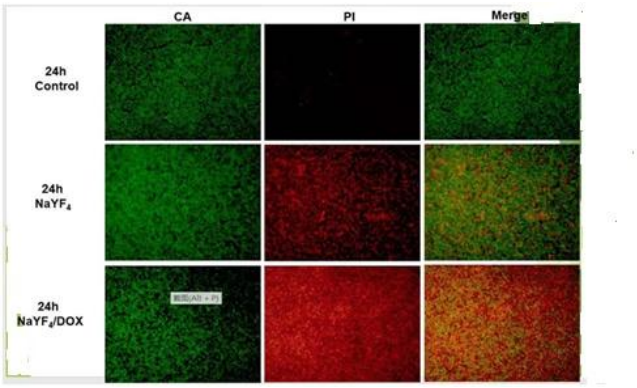


Figure 4 showed the numbers of the surviving cells 24 hours after the mixture of cancer cells with various concentrations of different drugs. It could be seen that there were a tiny number of deaths of cancer cells without any intervention, some deaths of cancer cells when adding upconversion materials only, and more deaths of cancer cells when adding upconversion materials loading with doxorubicin.

Figure 4 Killing of cancer cells after 24-hour mixture



Comparing with 4 hours after mixing with cell solutions, the number of deaths of cancer cells increased significantly 24 hours after the mixture. It could be deduced that the solution had the strongest killing effect on cancer cells when adding upconversion materials loading with doxorubicin, and the killing effect became much stronger when the time of mixing the drugs with cancer cells increased.

In the second group, the upconversion nanomaterials loading with doxorubicin were mixed at different concentrations for the same time, respectively 400, 200, 100, 50, 25, 12.5, 6.2, 3.1, 1.5 and 0 ug/ml, and then added 10% CCK-8 solution prepared away from light (the mixture time was 0.5-4hours, and the optimum time was 1 hour). The more cells survived, the darker the solution appeared. Through observing the 96-well plate, it could be found that there were almost no deaths of cancer cells without adding any reagent, and higher drug concentration exerted stronger killing effects on cancer cells and made less of them survive when the cell solution became yellow and lighter after adding the same drug with different concentrations to mix for the same time.

Figure 5 Killing rate of cancer cells under the action of various concentrations of drugs

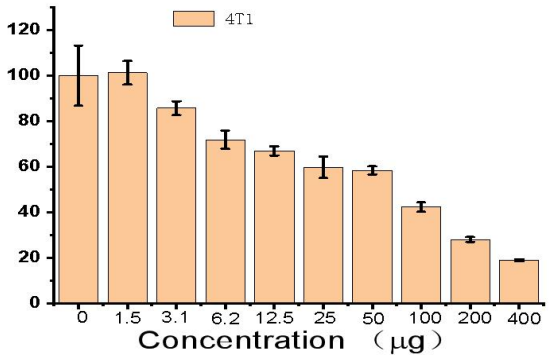
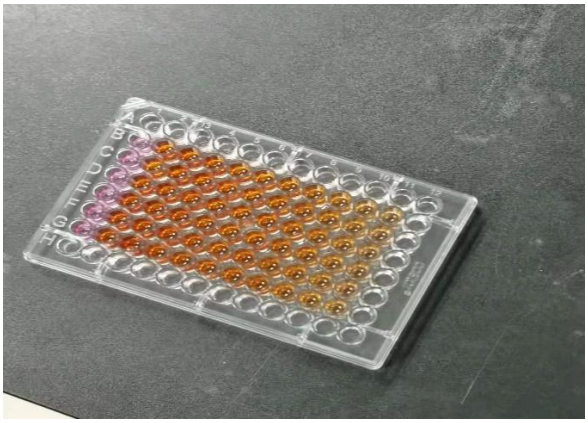


Figure 5 was the experimental data, from which it could be seen that lower concentration could bring lower killing rate. When the concentration of upconversion nanomaterials loading with

doxorubicin reached 400ug/ml, its killing rate for tumor cells was about 80%.

As shown in Figure 6, CCK-8 solution was added into the cell solutions injected with drugs, and the concentrations of the upconversion nanomaterials loading with doxorubicin increased gradually from the left to the right. After adding CCK-8 solution for 1 hour, the colors became lighter gradually from the left to the right. The lighter the color of cell solution appeared, the less cells survived.

Figure 6 Colors after adding CCK-8 solution



Conclusions and Outlooks

For one thing, there are safety and follow-up problems when using nanomaterials for direct treatment of cancer or as drug carriers to release drugs after approaching the tumor cells.

For instance, gold nanoparticles can hurt human bodies in the direct treatment of cancers, and high dosage of gold nanoparticles in serious condition of the tumor can do harm to human health due to its toxicity.

Besides, how to take out and control nanomaterials after its being put in human bodies as drug carriers remains to be resolved properly. According to the introduction in 2004 Nature magazine, the experiment on lab rats, conducted by some researchers of University of Rochester in New York, USA, showed that the 35nm-diameter carbon nanoparticles could appear rapidly in the olfactory bulbs (regions for olfactory processing in the brain) after entering the rat body through the breathing, and their continuous accumulation ultimately caused the immediate deaths of lab rats. Thus, the safety of using nanomaterials for cancer treatment needs to be further considered. (5)

For another thing, the application of upconversion nanomaterials in in-vivo imaging increases the targeting ability of nanomaterials as carriers loading anti-cancer drugs, which can reduce the damage of drugs to other tissues in human bodies and help to achieve multifunctional and integrated diagnosis and treatment. Furthermore, in terms of zero-damage clinical treatment, the materials should possess good compatibility, and there is need to make breakthroughs in crystal sizeandsynthesis. At present, theories on upconversion become more and more consummate, and new products are emerging and flourishing day by day. With energy conservation and environmental protection becoming the mainstream of development, rare-earth materials are drawing more and more concerns. If there are thorough studies of the charge transfer band of rare-earth ions, which can be used to excite the broadband absorption of photon energy and the energy transfer of rare-earth ions, luminous efficiency can be then improved significantly with bright prospects. (4)

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