Epidermal growth factor-ferritin H-chain nanoparticles as nanocarrier of doxorubicin for overcoming drug resistance in human breast cancer cells

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ABSTRACT

The emergence of drug-resistant cancer cells together with the side effects of chemotherapy agents are the major obstacle in cancer treatment. We focused on providing a new pattern of protein-based nanoparticle as drug delivery system. FTH1 is a kind of protein that exist in human body which has high bio-safety while EGFR is overexpressed in many cancer cells and it has been used as therapeutic target for cancer treatment. Thus, we used genetic methods for integrating EGF onto the surface of FTH1 and found out a more simple way to express the fusion proteins. To enhance the loading capacity, we first inserted a linker containing more sulfydryl sites (cysteine) into EGF-FTH1 and then adopted chemistry way to covalent bonding the doxorubicin to the nanoparticles. It was found that 72 of DOX molecular could be loaded to one nanoparticle, *in vitro* they could be released from the nanoparticles at pH5.0. These results demonstrate that the DOX/EGF-XCys-FTH1 could be a very promising drug delivery system.

Keywords: Drug delivery system,ferritin Hchain,Epidermal growth factor

1 INTRODUCTION

To date, cancer remains one of the leading causes of death in the world. Although the rapid developments in medicinal and pharmaceutical chemistry, the treatment to the cancer patient is still a challenge[1]. Traditional chemotherapy is not a good way for cancertherapy now because when the free cancer drug administered into bloodstream, the primarily renal clearance will clear most of them away and they will also distribution in non-target tissues, these process not merely reduce the concentration of the drug at the tumor site but also result in unwanted side effects. The better way to improve the problem is use nanoparticles loaded with cancer drug as drug delivery systems, which can deliver the drugs to the site of the tumor so as to increase therapeutic benefits, reduce side effects and enhance patient compliance. While a variety of functional nanomaterials, like polymers, silicon nanowires, gold/silver nanoparticle,quantum dots,dendrimers,lipsomes have been developed as cancer drug delivery system [2-3], not many researchers focused on the development of proteins as drug delivery system. In this study, we mainly concentrated on using ferritin as carrier,ferritin is a family of proteins found in different forms in most living organisms so as it has high biosafty. Each ferritin is made of 24 subunits which can self-assemble to form a cagelike nanostructure[4-5]. We use this protein carrying model cancer drug doxorubicin for targeting human breast cancer cell line MCF-7/ADR. The specific bonding to this cell line is based on the interaction of EGF and EGFR on the surface of the cells, which has been used for cancer treatment[6]. Thus, we used genetic methods for integrating EGF onto the surface of FTH1 and found out a more simple way to express the fusion chimeric proteins. Moreover, a similar strategy could be applied to generate other protein-based nanoparticles with surface-attached polypeptied in an effcient and reliable manner suitable for industrial production. However, the interior space of the FTH1 is too small to load many doxorubicin; this feature is the main block for minisize nanoparticles used as drug delivery systems. To solve the problem we first inserted peptides containing more sulfydryl sits (cysteine) into EGF-FTH1 and then adopt chemistry way to covalent bonding the doxorubicin (DOX) to the nanoparticles to enhance the loading capacity[7]. Finally, we tested the resulted nanoparticles on the breast cancer MCF-7/ADR cells Since doxorubicin loaded EGF-FTH1nanoparticles are not the substrate of the P-gp, thus it might lead to more doxorubicin delivered into the cells

2 RESULTS

2.1 Preparation and Characterization of EGF-5Cys-FTH1 Nanoparticles

Since we had demonstrated EGF-FTH1 nanoparticles showed the targeting ability to tumors, the EGF-5cys-FTH1 nanoparticles, which bearing cysteine for conjugation of doxorubicin, are prepared by using EGF-FTH1 as templet. First, the plasmid of EGF-5cys-FTH1 was constucted. A sequence containing 5 cystine and a G4S linker (5'TGC GGT TCT TGC GGT TCT TGC GGT TCT TGC GGT TCT TGC 3') were inserted between the sequence of EGF and FTH1. Then the fusion protein was expressed in *Escherichia coli BL21(DE3)* cells. Isopropyl-β-D-

thiogalactoside(IPTG) could induce high expression of EGF-5cys-FTH1 at 37°C, but most of the expressed fusion protein was insoluble in the cytoplasm. Thus, the insoluble macro-aggregates of EGF-5cys-FTH1were recovered and solubilized in buffer containing 8 M urea. After that, the urea in the solution was removed by a stepwise dialysis procedure, which resulted in active EGF-5cys-FTH1 proteins (shown in Figure1). Native gel electrophoresis showed the protein with a molecular weight(MW) of 696kD that illustrating the EGF-5cys-FTH1 subunits could refolded and assembled together *in vitro*.

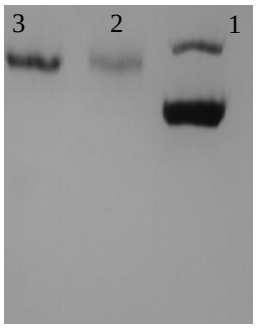

Figure 1: EGF-5cys-FTH1 nanoparticles analyzed by 7% native PAGE and stained with Coomassie blue. Lame 1: apoferritin from equine spleen (440 kD); Lane2,3: EGF-5cys-FTH1(different concentration).

Transmission electron microscopy (TEM) were also used to analyze the structure of the prepared nanoparticles. As shown in Figure 2, a typical image of the EGF-5cys-FTH1 nanoparticles exhibited a highly uniform with a distinctive cage-like structure. The diameter of the nanoparticles was about 12 nm.

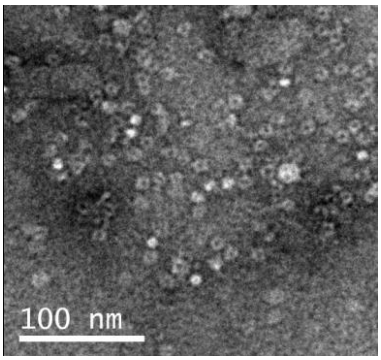

Figure 2: A typical TEM image of EGF-5cys-FTH1 nanoparticles.

2.2 Binding Activity of EGF-5cys-FTH1 Nanoparticles to MDA-MB-468 Cells

To test the cell binding ability of EGF-5cys-FTH1 nanoparticles, fluorescence-activated cell sorting (FACS) analysis was performed using fluorescein(FAM)-labeled nanoparticles. Figure 3 shows a typical FACS analysis of binding of fluorescein-labeled EGF-5-Cys-FTH1 nanoparticles to MDA-MB-468 breast cancer cells that express high level of endogenous EGFR. MDA-MB-468 cells had very low autofluorescence with a geometric mean fluorescence intensity value of 331. However, after incubation with high concentration of fluorescein-labeled EGF-5Cys-FTH1 nanoparticles, the geometric mean fluorescence intensity value of 21,879 were archieved. In the same way, the fluorescence intensity did not increase too much when the same concentration of EGF-5Cys-FTH1 nanoparticles were treated to human normal breast epithelia MCF-10A cells. It could be also found that the binding could largely blocked by treatment of EGF ligands (data not shown). It meant that EGF-5cys-FTH1 can specific targeting to the cancer cells expressing EGFR and could be used as a nanocarrier for drugs to target the tumors.

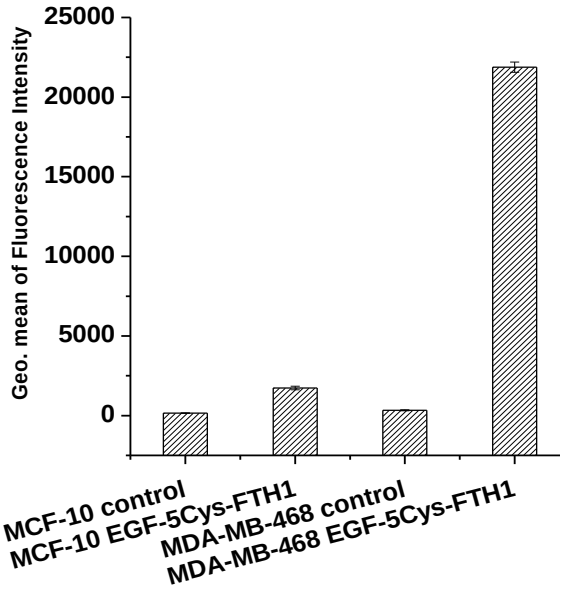

Figure 3: Binding of cages to cells measured by FACS. 5×10^5 cells were incubated with fluorescein labeled nanoparticles at 4°C for 1 h. Nanoparticles were normalized to 10 μM fluorescein. The decreased level of fluorescence intensity for EGF-5-Cys-FTH1 nanoparticles indicates their extremely weak binding to the MCF-10 cells due to the low expression of EGFR. Data represent mean ± SD, n = 3.

2.3 EGF-5Cys-FTH1 Nanoparticles Loaded with Doxorubicin

Since the interior space of the EGF-5cys-FTH1 is too small to load many doxorubicin; this feature is the main block for minisize nanoparticles used as drug delivery systems. To overcome this problem, chemical method was used for loading the doxorubicin (DOX) to sulfydryl sits of

cystine on the surface of the nanoparticles. N-[ε-Maleimidocaproic acid) hydrazide, trifluoroacetic acid salt (EMCH) are heterobifunctional crosslinkers containing maleimide and hydrazide moieties. Maleimides can react with free sulfhydryls (-SH) to form stable thioether bonds, while hydrazide groups react with carbonyls (aldehydes and ketones) to form stable hydrazone bonds. Here, DOX was first reacted with EMCH form hydrazone bonds which is also very sensitive to low pH enviroment. The EMCH linked to DOX was conformed by mass spectrum, the molar weight of the substance was 751.4 in accordance with the reference. Then, the resulted substance of DOX-EMCH were further reacted with EGF-5cys-FTH1 nanoparticles. Finally, the DOX loaded EGF-5cys-FTH1 nanoparticles could be obtained and analyzed by Native-PAGE. As shown in Figure 4, both DOX loaded and unloaded EGF-5cys-FTH1 nanoparticles were separated by 7% Native-PAGE. Only one red bind for EGF-5cys-FTH1 nanoparticles loaded with DOX could be observed (Figure 4A, line 1), and this bind could be further stained by Coomassie blue (Figure 4B, line 1). It meant that DOX molecules were successfully conjugated to the nanoparticles. It could be also determined that there were 72 DOX molecules loaded into the EGF-5cys-FTH1 nanoparticles.

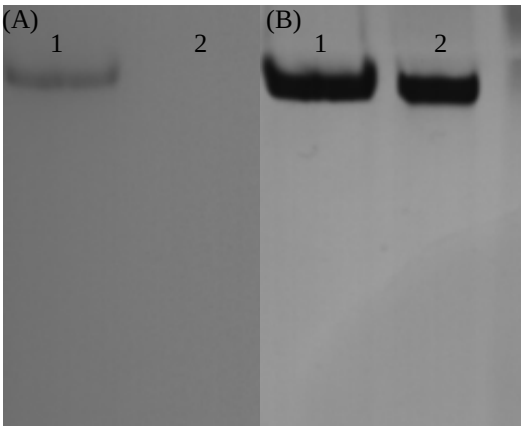

Figure 4: Confirmation of EGF-5Cys-FTH1 conjugate by 7% native PAGE using A) white light detection or B) staining with Coomassie blue. Lane 1, DOX conjugated EGF-5Cys-FTH1 nanoparticles; lane 2, EGF-5Cys-FTH1 nanoparticles.

2.4 *In Vitro* Release Behavior of EGF-5-Cys-FTH1-DOX Nanoparticles

It is important for DOX conjugates to release DOX in a controlled maner. Since the formed hydrazone bonds in the DOX-EGF-5Cys-FTH1 conjugate is very sensitive to low pH enviroment, DOX melecules might be released at low pH. To demonstrate it, *in vitro* release of doxorubicin from EGF-5cys-FTH1-DOX nanoparticles was examined in phosphate-buffered solutions (PBS) at different pH. PBS (pH7.0) was simulated normal physiological enviroment, while PBS (pH5.0) was simulated the lysosome conditions of cancer cells. Figure 5 showed that at pH 5 the release of DOX from the nanoparticles became much faster and the cumulative release of DOX could achieve to 80% within 40h. However, only a very small amount of DOX was released from the nanoparticles at very slow rate in PBS (pH 7.0). These results suggested that EGF-5cys-FTH1-DOX chould release DOX in a controlled release manner and demonstrated a pH-responsive drug release character.

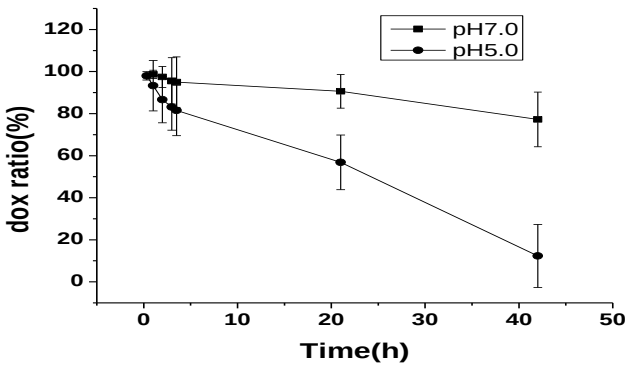

Figure 5: *In vitro* DOX release of EGF-5Cys-FTH1 nanoparticles loaded with DOX in PBS at different pH.

2.5 Internalization of EGF-FTH1-DOX to Human Breast Cancer Cells

To determine whether EGF-5Cys-FTH1-DOX can delieve the DOX into the drug resistance MCF-7/ADR cells, the internalization of different conjugates to cancer cells was evaluated. The association and subcellular distribution of ferritin-based nanoparticles and free doxorubicin were analyzed after 3h of incubation with MCF-7/ADR cells by using fluorescence microscopy. As shown in Figure 6, Apoferritin-DOX nanoparticles were barely transported into the cytosol of breast cancer MCF-7/ADR. Meanwhile, treatment of cells with free doxorubicin also lead to few amount of DOX taken up by cells. However, EGF-FTH1-DOX can be largely taken up into the cytosol of cells. These results suggested that EGF-FTH1-DOX were transported into the cytosol based on the interaction between the EGF and EGFR. Besides, free drugs were pumped out of the cells due to the drug resistance mechanism of P-gp in the MCF-7/ADR, but it would not happen to EGF-FTH1-DOX nanoparticles. All together, it means EGF-FTH1 can be used as nanocarrers to deliver the drugs into the cells, and more importantly more drugs in the cytosol of cells could be obtained which means that this nanocarrier might overcoming the drug resistance.

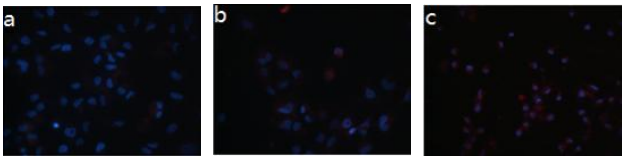

Figure 6:Typical fluorescence microscopic images of ferritin-based nanoparticles and free doxorubicin for internalization to MCF-7/ADR. a) Apoferritin-DOX; b) free doxorubicin; c) EGF-FTH1-DOX. Nano-particles were normalized to 5μg/ml DOX.

3 DISCUSSION

In this study, we demonstrated the use of epidermal growth factor-ferritin H-chain nanoparticles as nanocarrier to overcome drug resistance in MDR human breast cancer MCF-7/ADR. Our previous data has demonstrated that EGF-FTH1 nanoparticles could be prepared by using a genetic method. The generated EGF-FTH1 nanoparticles can be specifically taken up by EGFR-positive breast cancer cells depending on the targeted EGF units on the surface of the nanoparticles, and *in vivo* tumor accumulation can be also found. However, loading drugs into these nanoparticles is very difficult due to their tiny interior size. Thus, in this study a simple method has been developed for higher DOX loading amounts by inserting peptides containing 5 cysteine into the nanoparticles. Our prelimited results show that highly stable EGF-5Cys-FTH1 nanoparticles could be prepared by genetic method, and higher loading capacity of 72 DOX/cage could be also be achieved by using chemically modification. In addition, hydrazone bonds, which are very sensitive to low pH enviroment, introduced into the DOX conjugated EGF-5Cys-FTH1 nanoparticles when EMCH is used as a linker. The resulted DOX loaded EGF-5Cys-FTH1 nanoparticles can release DOX *in vitro* in a pH-responsive release manner.

The obstacle to effective cancer treatment is the development of multidrug resistance(MDR). The major mechanism of drug resistant is overexpression of P-gp[8-9]. Doxorubicin loaded EGF-FTH1 nanoparticles are not the substrate of the P-gp, thus more doxorubicin (DOX) can delivered into the cells compared with free DOX and non-targeted apoferritin loaded with DOX. It seems that DOX molecules conjugated to the nanoparticles were avoided to be pulled out from the cells. However, the mechanism is still under being carried out.

4 CONCLUSION

EGF-5Cys-FTH1 nanoparticles have been prepared by using a genetic method. The developed nanoparticles are allowed a higher DOX loading. When EMCH was used as linker for DOX molecules conjugated to EGF-5Cys-FTH1 nanoparticles, the resulted EGF-5Cys-FTH1-DOX nanoparticles show a pH-responsive controlled-release manner *in vitro*. This method can also be used to incorporate some drugs with active amino groups onto the exterior surface of the ferritin H-chain based-nanoparticles. The method is very simple and low-cost, and robust for higher loading of drugs, thus might be applied to industrial production. The EGF-5Cys-FTH1 nanoparticles generated here show a precisely assembled nanometer-scale structure, and small size with narrow size distribution. The nanoparticles can be specifically taken up by EGFR-positive breast cancer cells depending on the targeted EGF units on the surface of the nanoparticles. It is also found that more DOX molecules could be delivered into the cells compared with free DOX drugs. All together, it means that EGF-5Cys-FTH1 nanoparticles might be used as nanocarrier to overcome drug resistance in MDR human breast cancer MCF-7/ADR.

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