Functional targeting nanomicelles with ph-triggered peptide activation for improved drug delivery in lung cancer

Yuehua Wang¹,², Fei Jia²,³, Chunyan Yue²,³, Jian Sun¹*, Weizhi Wang²*

¹School of Pharmaceutical Science and Technology, Health Science Platform, Tianjin University, Tianjin 300072, China;
²CAS Key Laboratory of Standardization and Measurement for Nanotechnology, CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, CAS Center for Excellence in Nanoscience, National Center for Nanoscience and Technology of China, Beijing 100190, P. R. China
³University of Chinese Academy of Sciences, Beijing 100049, P. R. China

Email: wangyh@nanoctr.cn

Abstract. Mild acidic environment and tumor angiogenesis are two typical features. The specific response toward lower pH may increase the targeting ability both for drug delivery. Herein, we present a responding self-assembled nanoparticle that is tumor angiogenesis targeting and pH triggered based on a novel recognition peptide DP. DP is pH-responsive as well as the affinity ligand of tumor marker EGFR. Efficient drug delivery and in vitro therapy in cell level could be accelerated in the conditions of EGFR overexpression and a mild acidic environment. We believed that such responsive materials will open a new way to turn on target imaging diagnostics and specific cancer therapies.

1. Introduction
Lung cancer, as one of the severest cancers all over the world, shows a rising trend in incidence rate for the past few years, bringing patients and their family great suffering and economic pressure.[1] However, traditional chemotherapeutic drugs are lack of specificity for swollen and painful cells, and it is difficult to achieve effective concentration inside the tumor.[2] While killing tumor cells, they will also cause damage to some normal tissues and cells in the body, and have disadvantages.[3] Hence, a series of tumor-targeting therapy have been studied currently. These studies show that there are a lot of differences in micro-environment between tumors and normal tissues, such as angiogenesis, permeability, and the extracellular pH differences, which can be used for drugs to recognize tumors specifically.[4] The pH in tumor microenvironment varies with site of tumors, as 6.0-7.0, while pH in blood and normal tissues is about 7.4.[5] All studies support that low pH promotes the development and tumor metastasis.[6] And high rate of glycolysis results in low pH at hypoxic tumor cells, which can be a target for tumor therapy.[7]

Herein, micelle is a new form of targeted drug delivery system and is a hot research object in the field of nanometer. It is a micelle formed with the hydrophilic end of amphiphilic polymer as the shell and the hydrophobic end as the core, which can selectively deliver drugs to the tumor site to play a therapeutic role without affecting the function of normal cells, tissues or organs, so as to improve the efficacy and reduce the toxic and side effects.[8] With a small particle size between 10-80 nm, the drug accumulates in tumor tissues through EPR effect to achieve passive targeting.[9] The critical micelle concentration was low and the dilution stability was good after administration in vivo. When
the shell is connected with the specific ligand, the physiological effect of the drug can be improved by active targeting.[10] So, micelles target EGFR, which is highly expressing in non-small cell lung cancer, for targeting tumor tissues and lowering drug toxicity.

2. Results and discussion

2.1 Design of the self-assembled DENMEI

As shown in Scheme 1, the amphiphilic self-assembling nanostructures were first constructed. In this work, a switchable and functional nanoparticle system was established based on nanomicelles encapsulating Erlotinib (EI). The micelles were functionalized by one type of peptide DP (DP: CHLWEFDHA; Scheme 1a) with both pH-responsive and EGFR-targeting ability. This novel peptide shows high specificity toward EGFR in a mild acidic microenvironment. DENM with EI encapsulated in it (DENM\textsuperscript{EI}) exhibits high efficiency of targeting, release, and penetration in the tumor microenvironment toward EGFR in vitro (Scheme 1b). As an environment-triggered carrier, DENM\textsuperscript{EI} could perform with the self-assembling ability that was of significance for cellular delivery.

Figure 1. (Left) Schematic illustration of the preparation and application of DENM\textsuperscript{EI}. (Right) a. Size distribution of MENM\textsuperscript{EI}. b. Size distribution of DENM\textsuperscript{EI}. c. TEM graph of MENM\textsuperscript{EI}. d. TEM graph of DENM\textsuperscript{EI}.

2.2 Synthesis and characterization of DENM\textsuperscript{EI} and MENM\textsuperscript{EI}.

The distribution of size, surface potential and the TEM images of formed micelles were presented in Figure 1. As shown in Figure 1a and b, the average diameter of micelles were determined by DLS to be 36.3 ± 3.85 nm (PDI = 0.283) for MENM\textsuperscript{EI} and 67.4 ± 2.53 nm (PDI = 0.247) for DENM\textsuperscript{EI} in acidic environment. When hydrophobic drug was further entrapped into the hydrophobic core, the micelle size was always larger than blank micelles. These might result from the stretch of PEG chains and hydrophilous peptide DP on the surface of micelle toward water phase. The change in the size explained that modification might be achieved. As shown in Figure 1c and d, zeta potential measurements revealed an overall neutral charge for both micelles, with a charge of -0.99 ± 0.17 mv for MENM\textsuperscript{EI} and -5.76 ± 0.64 mv for DENM\textsuperscript{EI}, respectively. The absolute values of the \( \zeta \) potential revealed that both MENM\textsuperscript{EI} and DENM\textsuperscript{EI} exhibited good dispersion stability in acidic environment, which was suitable for the subsequent experiment. Moreover, the morphologies of MENM\textsuperscript{EI} and DENM\textsuperscript{EI} were observed by transmission electron microscopy. As shown in Figure 1e and f, the
morphology feature demonstrated that DENM\textsuperscript{EI} could self-assemble to form uniformly spherical nanoparticles.

2.3 The prepared nanomicelles were characterized by FTIR analysis.

The targeted peptide DP modified CMC micelle was prepared using MAI-PEG-NHS as a linker. PEG was assembled to CMC by the reaction between NHS and NH\textsubscript{2}, following sulphydryl group of peptides grafted to maleimide functional sites of PEG at pH 6.5. Peptide-free mPEG-CMC was chosen as a control, and both micelles were assessed by using FTIR (Fourier Transform infrared spectroscopy). As shown in figure 2, the FTIR spectra showed mPEG grafted carboxymethyl chitosan with characteristic peaks at 1102 cm\textsuperscript{-1}(C-O-C stretching), and 2870 cm\textsuperscript{-1} (aliphatic CH\textsubscript{2} stretching) belonging to mPEG. The characteristic peaks at 1622 cm\textsuperscript{-1} (N-H stretching vibration) and 1385 cm\textsuperscript{-1} (C-N stretching) also indicated the link of mPEG onto CMC by reacting to NH\textsubscript{2} (1602 cm\textsuperscript{-1}). In the IR spectrum of DP-PEG-CMC, a new peak at 1710 cm\textsuperscript{-1} might correspond to the C=O stretching of carboxyl groups belonging to peptides and the peak at 1344 cm\textsuperscript{-1} could attributed to the C-S stretching, indicating the grafting of peptides to PEG.

![Figure 2. FTIR spectra of BCNM (control), MENM and DENM at pH 6.5.](image)

2.4 Cell viability and drug release of the Erlotinib-loaded micelles on PC cells

MTT assays were performed to investigate the cytotoxicity of the MENM and DENM polymer and it loaded with Erlotinib. As shown in Figure 3a, the results proved that cell apoptosis of DENM\textsuperscript{EI} group at pH 6.5 was more significant than that of DENM\textsuperscript{EI} group at pH 7.4 and other groups with increasing concentrations, and especially at high concentrations. Furthermore, the DENM and MENM group showed no apparent difference at all concentrations in terms of cytotoxicity under the condition of pH 7.4 and pH 6.5. This might be caused by the better cellular uptake of the DP-linked DENM\textsuperscript{EI} complexes when applied to the EGFR-positive PC9 cells, and this was further supported by the results of the cellular uptake assays.

Controlled and sustained drug release is very important in drug delivery systems. During the study, the rate of drug release in both pH 6.5 and pH 7.4 was evaluated by dialysis method. Erlotinib (EI) was packaged on the above vector (DENM and MENM) for detection and identification. As shown in Figure 3b, the rate of Erlotinib release of DENM\textsuperscript{EI} at pH 7.4 was lower than at pH 6.5 (90\% and 94\%, respectively). At the same time, the release rate of MENM\textsuperscript{EI} at pH 7.4 was lower than that of the system at pH 6.5 (80\% and 86\%, respectively). When the pH is lowered from 7.4 to 6.5, the dissociation of Erlotinib increases due to the positive charge repulsion between the drug molecules. At the same time, DSPE-PEG\textsubscript{2000} changes from the original negative charge into a positive charge and weakens the electrostatic attraction between Erlotinib and nanomicelle. Therefore, DENM\textsuperscript{EI} may be accelerated by endogenous lysosomes under acidic conditions after endocytosis by tumor cells.
Figure 3. a. MTT assay for PC9 cells incubated with different Erlotinib formulations at different concentrations. b. In vitro release of Erlotinib from DENM\textsuperscript{EI} and MENM\textsuperscript{EI} at pH 7.4 and pH 6.5 (n=3).

2.5 Specific recognition of DENM\textsuperscript{EI} at pH 6.5 in living cells
To evaluate the targeting delivery efficiency of DENM\textsuperscript{EI} toward EGFR positive cells at different pH environments, the human lung adenocarcinoma cell line PC9 was employed. As shown in Figure 4a, the nucleus of fluorescence signals were observed at the blue (Hoechst 33342, ex, 405 nm; em, 488 nm) and yellow (Dil; ex, 549 nm; em, 565 nm) channels in order to trace the interaction between nanomicelles and cells. The results indicated that PC9 cells treated with DENM\textsuperscript{EI} demonstrated much stronger fluorescent intensity than MENM at pH 6.5. Dil was successfully combined with the cell membrane. On the contrary, at pH 7.4, lower fluorescence signals were observed in PC9 cells incubated with DENM\textsuperscript{EI}. This revealed that DENM\textsuperscript{EI} is inactive at neutral condition. It manifested higher uptake efficiency of DENM\textsuperscript{EI} in the PC9 cell line at pH 6.5 since the overexpression of EGFR, which indicated that targeting binding achieved.

Figure 4. Characterization of the targeted recognition DENM\textsuperscript{EI} in cellular levels. a. CLSM images of PC9 cells incubated with MENM\textsuperscript{EI} or DENM\textsuperscript{EI} at pH 6.5 or pH 7.4 for 1 h (63×, oil-immersion objective). b. Flow cytometric analyses of the cellular uptake of MENM\textsuperscript{EI} or DENM\textsuperscript{EI} at pH 6.5 or pH 7.4.

3. Conclusion
This study systematically demonstrated a switchable micelle drug-delivery system, which functionalized by pH-triggered and EGFR-targeting peptide DP. The nanocarriers could enhance the antitumor effect at acidic tumor microenvironment and are expected to be potential candidates for lung cancer therapy. Our further work may pay more attention to the stability, specificity, and targeting affinity of the nanomicelles in vivo, in order to develop the enhanced synergy functional nanocarriers system, which could be prospectively applied in lung cancer therapy.
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