論 文 の 内 容 の 要 旨

論文題目　Development of Enzymatically Activated Nanocarriers Directed to Enhanced Anticancer Therapy
（抗がん治療の強化を志向した酵素活性化ナノキャリアの開発）

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In recent years, several drug-loaded nanocarriers have been developed for tumor therapy, including liposomes, nanoparticles and polymeric micelles [1]. Some of these formulations have shown enhanced antitumor efficacy and negligible toxicity [2]. Among them, polymeric micelles are attracting much interest due to their easy preparation, based on the self-assembly in aqueous solution of amphiphilic block polymers, and the controllable modification of their properties [3]. The mechanism for specific tumor targeting of these nanocarriers takes advantage of the so-called enhanced permeability and retention (EPR) effect, which is due to leaky blood vasculature and impaired lymph drainage of solid tumors [4]. Moreover, the targeting of nanocarriers can be further improved by modifying their surface with specific ligands having high binding ability against receptors overexpressed on cancer cells [5]. In addition, the specific stimuli in the microenvironment of tumor tissues can be used for tumor selective release of the loaded drugs [6]. Thus, by increasing the specificity of drug delivery to tumor tissues, nanocarriers can minimize the off-targeting effects to and maximize the efficacy of the treatments.

As one type of nanocarrier, polymeric micelles, which commonly have a size range of 10-100 nm, are formed of amphiphilic polymers in aqueous solution due to self-assembly [7]. The hydrophobic segments in polymers compose the compact micelle core and the hydrophilic segments form the micelle shell. The outer hydrophilic shells are usually made up of poly (ethylene glycol) (PEG). These hydrophilic micelle shells are important for drug delivery due to the stealth properties, which protect them from taking up by the RES and elongate blood circulation time. The hydrophobic segment has more diversity and can be finely adjusted to give polymer specific properties, such as stimuli responsibility, micelle cross-linking and drug loading. The polymeric micelles have attracted the interest of researchers due to their advantages over other nanocarriers, such as easy formation due to self-assembly and flexible design, which allows diverse drug conjugation and different drug release pathways. Block polymers based on PEG-poly(amino acids) are a good example of amphiphilic polymer for making polymeric micelles for drug delivery due to their biocompatibility of poly (amino acids) segment. In fact, many polymeric micelle formulations based on this polymer design have shown good bioactivities and now are under clinic trials [8].

The established thinking about tumor targeting consider that these nanocarriers take advantage of the so-called enhanced permeability and retention (EPR) effect, which is due to the leaky blood vasculature and impaired lymph drainage of solid tumors. However, it is already reported that EPR effect alone may be not enough for specific targeting, and toxicities caused by off-targeting effects are frequently reported. One solution for this is installation of ligands on the nanocarriers' surface which can specifically bind with targets to minimize undesired accumulation. The common ligands used for surface modification of nanocarriers include antibodies, transferrin, aptamer, cRGD, galactose, hyaluronic acid, folic acid, and so on [9]. As the binding affinity of ligand and receptor is high, ligand-installed nanocarriers would bind to the target cells once encounter the receptor. Then, the cells would take up the bound nanocarriers through receptor-mediated endocytosis [10]. As a total effect, the intracellular delivery into cancer cells can be improved even though the

tumor accumulation level is the same that under the EPR effect.

Besides ligand installation on nanocarriers, another way of minimizing off-target toxicity is using specific stimuli for triggered drug release. Due to the abnormal growth of cancer cells, there are some intrinsic stimuli in the microenvironment of tumor tissues, such as low oxygen level, acidity, high reduction potential and overexpressed enzymes, can be used for tumor selective release of the loaded drugs. In addition, other external stimuli, such as temperature, magnetic field, ultrasound and light, which can be exerted in a focused area surrounding tumor tissue, are also reported to be suitable trigger for specific drug release [11]. With careful nanocarrier design, the covalently bound or physically entrapped drugs can be released with response these stimuli. Thus, the triggered drug release is confined into stimuli positive part, which can reduce off-targeting toxicity by minimizing drug release in undesired tissues.

As a successful combination of ligand mediated targeting drug delivery and stimuli mediated drug activation, antibody-drug conjugates (ADCs) have achieved many big triumphs [12]. The basic design of ADCs is conjugating potent drugs on monoclonal antibodies for targeted cancer cell killing. Some ADCs have shown high therapeutic effects and have been approved for clinical use, like Brentuximab-vedotin and Trastuzumab-metansine, and many others are under clinical trials. Even though there are a lot of good reports about ADCs, there are still some issues to be solved. One problem is the prodrug activation profile. In previous report, the amount of activated prodrug are usually checked by HPLC, which is an end-point method and impossible for continuous surveillance of prodrug activation [13]. In addition, this method only offer an overall extent of prodrug activation in one tissue and the microdistribution information of activated drug cannot be obtained. This issue is of importance because some tissues, especially tumor, are not homogeneous. Thus, a new method for understanding prodrug activation process and microdistribution of activated drug is needed. Another problem of ADC is the drug loading capacity. In conventional ADCs, the drug antibody ratio is limited to 8 drugs per antibody, with a higher drug loading would interfere binding ability of antibody against antigen [14]. To further increase the loading capacity and decreased the amount of antibody needed for drug delivery, another vehicle is indispensable. Taking advantage of the high loading capacity and diverse design of polymeric micelles, drugs covalently conjugating on carrier polymer and released under tumor-specific stimuli may be a good solution for this problem. Also, the further understanding of ADC working flow chart, for example, the temporal prodrug activation profile and the prodrug linkage degradation, could supply information for future ADC design. However, it is challenging to investigate these issues due to the inherent property of drugs used in ADC, such as the lack of strong and unique UV-Vis absorbance peak for drug concentration determination or fluorescence emission for evaluation of drug microdistribution, and the leakage of prodrugs in conventional bioconjugation chemistry of ADCs.

This research is intended to develop polymeric micelle platforms capable of solving these problems for delivering potent prodrugs. Compared with previous results, this research got the following achievements, which can push the front line of cancer therapy forward, as follows:

First, the unspecific release effect happening in conventional ADCs is solved by loading maleimide-functionalized prodrug through a different bioconjugation method. Here, the conjugation chemistry was chosen to be the Diels-Alder cycloaddition reaction between maleimide moieties of prodrugs and furan moieties on carrier polymers. In previous ADC designs, the bioconjugation chemistry for prodrug loading on antibody is the Michael addition reaction between thiol on cysteines and maleimide on prodrugs. However, it is already known that the retro-Michael addition reaction readily occurs under physiological condition, especially under high concentration of glutathione [15], leading to unspecific cleavage of the bonds and leakage of the drugs. On the contrary, the happening of reverse Diels-Alder reaction needs high temperature, which is less impossible in

physiological condition [16]. Thus, this conjugation method supply another possibility to make stable nanocarrier for preventing offloading effect of prodrugs.

Second, Hoechst prodrugs are used for visualizing of prodrug activation and microdistribution of activated drugs. These Hoechst prodrugs have the same linkers which are used in ADCs containing monomethyl auristatin E (MMAE). However, unlike MMAE, Hoechst has inherent fluorescence emission and the maximum emission wavelength can be largely shifted with stronger emission intensity after intercalating with DNA. On the other hand, conjugated Hoechst molecules are not in good size for passing through cell nucleus envelop and intercalating with DNA. Thus, Hoechst can work as perfect DNA targeting probe for investigation of linkage degradation and prodrug activation. In this study, continuous tracking of Hoechst prodrug activation is achieved both *in vitro* and *in vivo*, which could supply valuable information about screening of suitable prodrug linker and microdistribution of activated drugs for future ADC designs. It was found that valine-citrulline peptide linker can be degraded in a controllable way compared with valine-alanine peptide linker, even though both of them are used as enzyme-responsive prodrug linkers for MMAE ADCs.

Third, the ligand density effect of Fab on micelle surface is studied. Even though it is well known that antibody ligand can affect the bioactivity of nanocarriers, a systematic research about the ligand density against micelle binding and cellular uptake is not reported before, partly due to the challenge of installing antibody at high density (limited at 30%) [17]. Herein, this research reports a method for controllable Fab installation on micelle surface. It is found that the Fab installation efficiency can be controlled by simply changing the PEG length in cross linkers. It was proved that when the PEG length is larger than 7 units in the cross-linker, a theoretically maximum Fab installation density could be achieved. On the contrary, when using cross-linker with 4 units of PEG, the maximum installation ratio is limited at 50% under the Fab/azide feed ratio used in this research. It was also confirmed that 60% Fab installed micelle had similar micelle binding ability with 30% Fab installed micelle. However, the cellular uptake was interfered when the Fab density increased from 30% to 60%, as proved by the decreased micelle internalization and weakened cytotoxicity of 60% Fab installed micelles.

Fourth, polymeric micelles are used for increasing loading capacity of MMAE prodrug. By conjugating MMAE prodrug on carrier polymer and form micelles in aqueous solution, one micelle could load around 200 MMAE prodrug molecules. After modification of Fab on micelle surface, the drug/Fab ratio is found to have a 40-fold augment compared with conventional MMAE ADC. The Fab-modified micelles have higher cellular uptake than naked micelle and can achieve the same level of cytotoxicity as ADC. Also, compared with naked micelle, Fab-MMAE micelles could extend retention time of micelles in tumor tissues, enhance degradation of enzyme-responsive linker and prodrug activation in tumor, thereby, retarding the growth of tumors.

Finally, the introduction of stimulus responsibility into micelle is explored to accelerate prodrug activation process after cellular internalization. A general problem of using enzymatically responsive nanocarriers for drug delivery is the hampered enzyme access to its substrate caused by the PEG shell [18]. This problem is solved in this study by introducing imidazole moieties on carrier polymer side chains. As imidazole groups get protonated in lysosomal pH and transit from hydrophobic in physiological pH into hydrophilic in lysosome, the critical micelle concentration can be shifted to 10-fold higher level. Thus, there are more prodrug-loaded unimers in lysosome for enzymatic activation. The results of this research confirmed that after introduction of pH sensitivity to the prodrug carrier polymer, the activation of Hoechst prodrug loaded by pH-sensitive micelles is faster than that loaded by non pH-sensitive micelle. Also, regarding the *in vitro* cytotoxicity, SN38 prodrug-loaded pH-sensitive micelles showed lower 50% inhibitory concentration

(IC50) against PC3 cells than that loaded in non-pH sensitive micelles.

As conclusion of this research, a systematic study has been done to develop a platform polymeric micelle for delivering enzymatically-activated prodrugs. With this micelle platform, the prodrug activation process and microdistribution of activated free drug can be easily tracked through fluorescence microscopy. Also, this micelle platform can be controllably modified on the surface with Fab to enhance targeting specificity. Fab installed micelles have higher cell binding and cellular uptake in receptor over-expressed cell lines, which leads to longer tumor retention, increased prodrug activation and improved antitumor efficacy. In addition, the introduction of other functional groups on the polymer could offer micelle further stimuli responsiveness, which could adjust prodrug activation process under desired conditions. This research supplies a novel way, besides ADCs, to deliver potent prodrugs with enzymatically active peptide linkers, opening a window for broadening the application of polymeric micelles in drug delivery with promising application in future cancer therapy.

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