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Functional vesicles formed by anticancer drug assembly

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ABSTRACT

In this Letter, a new type of nitrogen mustard conjugate vesicles is developed to improve the stability and efficiency of anticancer drug. Benzoic acid nitrogen mustard–peptide (AAAK) conjugate was designed and synthesized, which was found to self-assemble into vesicles in water. The formation of the vesicles was confirmed by dynamic light scattering (DLS), transmission electron microscopy (TEM) and circular dichroism (CD). The degradation data revealed that the benzoic acid nitrogen mustard peptide (AAAK) conjugate vesicles are more stable than the parent drug in aqueous solution. Furthermore, MTT assay revealed that the free drug conjugate has similar antitumor activity against MCF-7, Hela, HepG-2 cell lines compared with the parent drug. The benzoic acid nitrogen mustard–peptide conjugate vesicles may have potential in the treatment of cancers.

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In the past several decades, nitrogen mustards have been widely used in the treatment of various cancers including chronic leukemia, breast cancer, Hodgkin's disease, non-Hodgkin's lymphoma, lung, and ovarian.[1–6] Nitrogen mustards act as nonspecific alkylating agents with DNA and induce subsequent DNA interstrand crosslinking.[7,8] Because of their high reactivity, nitrogen mustards are unstable and lack of selectivity with high toxicity to normal tissues. They are usually poorly soluble in water and undergo rapid hydrolysis in physiological solution.[9] It is reported that nitrogen mustards usually have a hydrolysis half-life less than 30 min in water and a half-life of 1.5 h in vivo circulation.[10,11] In order to address these drawbacks, many efforts have been made to improve their therapeutic potential.

One of the most important strategies is to create hybrid molecules of nitrogen mustards combining with functional groups. Ferlin et al designed the derivative of nitrogen mustards having DNA targeting groups to increase the drug concentration around DNA for enhancing selectivity.[12] Boens et al used a natural pyrimidine to modify nitrogen mustards leading to encouraging results in biological assays.[13] Other groups prepared a series of amide-coupled benzoic acid nitrogen mustard derivatives as kinase inhibitors.[14–16] Fonseca et al reported several peptide-chlorambucil conjugates to reduce drug resistance.[17] Alkylating agent carried by steroid is also an important method to improve toxicity and selectivity. The enhanced distribution of steroid in tumor offers the possibility of developing a targeted chemotherapy specific to certain tumors.[18] Bartzatt et al synthesized antineoplastic nitrogen mustard agent using a D-amino acid as a drug carrier.[19] However, these modifications often alter significantly the pharmacokinetic profile of nitrogen mustards.[20,21]

Vesicles are effective carriers for the delivery of hydrophobic drugs, providing a concentrated multivalent display of drugs[22] and protecting drugs from degradation.[23] Vesicles-based drugs have made the successful translation from academic labs to clinical trials or applications.[24–27] However, conventional vesicular delivery systems mainly rely on the encapsulation of hydrophobic drugs, having inherent drawbacks of low drug loading as well as noticeable leakage. Recently, amphiphilic peptides attracted much attention,[28] which could self-assemble to form micro- or nanostructures in water, such as, vesicles,[29] micelles,[30] and nanofibers.[31–33] The amphiphilic peptide-vesicles of a high aspect ratio have shown advantageous properties as drug carriers of prolonged blood circulation time,[34] broader biodistribution,[35] increased targeting efficiency[36] with enhanced cellular uptake.[37] Most recently, amphiphilic drug–peptide conjugates have been created by conjugating hydrophobic anticancer drugs to a short peptide segment, which could form self-assembled nanostructures directly[38] with extremely high drug loading, completely devoid of drug leakage.

Herein, we proposed an approach of conjugating nitrogen mustard with a short amphipathic peptide which could form vesicles in aqueous solution. The hybrid conjugate of nitrogen mustard combining with peptide will not only be a cargo of prodrug but also used as an effective vesicular carrier. To achieve this goal, benzoic acid nitrogen mustard derivative carrying alanine

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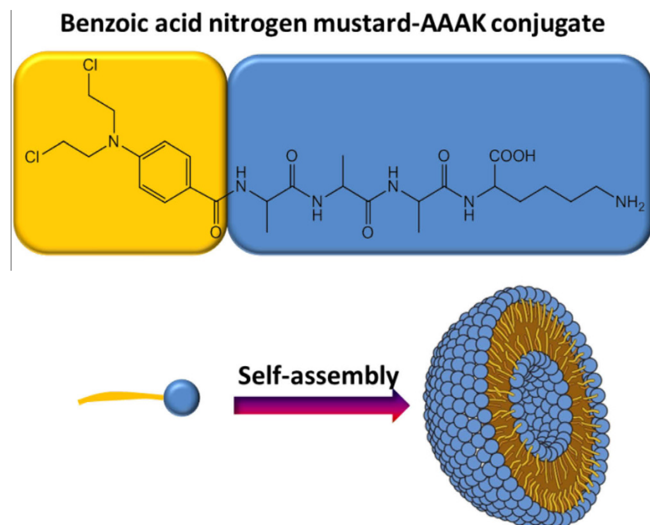

Figure 1. Chemical structure and schematic illustration of self-assembly of benzoic acid nitrogen mustard–AAAK conjugate.

(A)-alanine (A)-alanine (A)-lysine (K) (AAAK) peptide was designed as showed in Figure 1. Starting from Fmoc-amino acids, the conjugate was synthesized with solid-phase peptide synthesis (SPPS) and identified by mass spectrometry. After physiochemical tests, the conjugate vesicles were prepared by dried thin film technique (Supporting information) followed by subsequent characterization.

The dependence of the conductivity of benzoic acid mustard–AAAK conjugate aqueous solution on the concentration is shown in Figure 2. It was found that the conductivity has an upward trend with the increase of the conjugate concentration. In addition, the turning point at about 0.23 mM was determined to be the critical micelle concentration (CMC) of benzoic acid nitrogen mustard–AAAK conjugate. It is apparently that the existence of CMC for benzoic acid nitrogen mustard–AAAK conjugate demonstrated the formation of micelles by self-assembly.

The structure of benzoic acid nitrogen mustard–AAAK conjugate in aqueous solution was further validated by circular dichorism (CD). As shown in Figure 3, the conjugate exhibited a pronounced trough of emission at 190–200 nm ($n\pi^*$ transition), indicating the random coil conformation of the conjugate. The positive peak of emission at 230–240 nm ($\pi\pi^*$ transition) may indicate the π–π interaction of phenyl groups of nitrogen mustards.

Dynamic light scattering (DLS) and transmission electron microscopy (TEM) were used to investigate the physicochemical

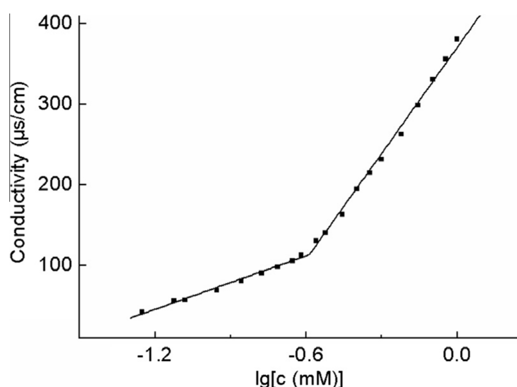

Figure 2. The conductivity of benzoic acid nitrogen mustard–peptide conjugate aqueous solution.

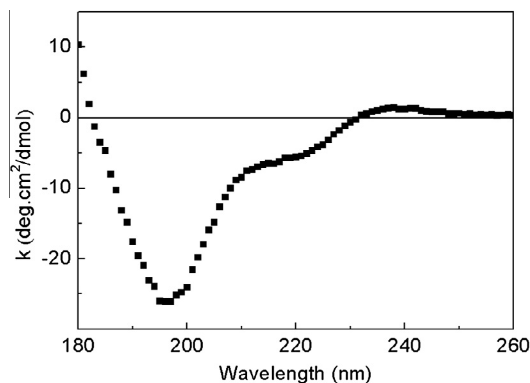

Figure 3. Circular dichroism of benzoic acid nitrogen mustard–AAAK conjugate in aqueous solution with the concentration of 0.03 mM.

and structural properties of benzoic acid nitrogen mustard–AAAK conjugate vesicles in aqueous solution. As shown in Figure 4, the prepared vesicles gave a single-diameter distribution with a mean at approximately 266 nm. TEM investigations showed that the conjugate formed globular shaped particles with uniform diameters ranging from 200 to 400 nm (Fig. 5), consistent with the data obtained by DLS. By contrast, benzoic acid nitrogen mustard–KKK conjugate did not show a diameter distribution by DLS (data not shown). According to these results, it could be assumed that benzoic acid nitrogen mustard–AAAK conjugate form bilayer vesicles: the lysine heads are exposed to surrounding water, sequestering the benzoic acid nitrogen mustard tails within the bilayer.[26] As a comparison, benzoic acid nitrogen mustard conjugated with short hydrophilic peptide (KKK) didn't self-assemble to form vesicles, but dissolved in PBS to form true solution.

To demonstrate the stability of benzoic acid nitrogen mustard–AAAK conjugate vesicles in aqueous medium, the vesicles degradation was carried out at 37 °C in two different media: 0.01 M PBS buffer (pH 7.4) and 0.01 M PBS buffer containing 10% fetal bovine serum (FBS), which provide a basic idea of the drug release in physiological system. As shown in Figure 6, the degradation data was fitted to an exponential equation assuming first-order kinetics, and the hydrolytic kinetic constant was obtained as the slope of the line. In PBS buffer, the conjugate vesicles is degraded for 30%, while most of free drug benzoic acid nitrogen mustard is degraded within the first 2 h. The kinetic constant in PBS was found to be 0.0022 min^{-1} for benzoic acid nitrogen mustard–AAAK conjugate vesicles and 0.017 min^{-1} for benzoic acid nitrogen mustard free drug. Notably, the similar phenomenon was observed in the medium containing serum: the kinetic constant was found to be 0.0005 min^{-1} for benzoic acid nitrogen mustard–AAAK conjugate vesicles and 0.0072 min^{-1} for benzoic acid nitrogen mustard free drug. However, the benzoic acid nitrogen mustard–AAAK conjugate dissolved in media without forming vesicles has kinetic constants of 0.016 min^{-1} in PBS and 0.0068 min^{-1} in serum, which is similar to benzoic acid nitrogen mustard free drug. The result showed that the benzoic acid nitrogen mustard–AAAK conjugate vesicles have significant stability compared with free drug in true solution. It may be ascribed that benzoic acid nitrogen mustard groups was sequestered within the bilayer of the conjugate vesicles isolating from aqueous solution. The stability in both PBS buffer and serum media suggests that the benzoic acid nitrogen mustard–AAAK conjugate vesicles are stable in vivo, which is critical for drug delivery application. Moreover, the conjugate vesicles and free drug in PBS containing serum are more stable than those in PBS without serum. It is probably because protein-binding in serum reduces the degradation of the drugs as reported in the literature.[9,39]

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