# Antibacterial Polypeptide-Grafted Chitosan-Based Nanocapsules As an "Armed" Carrier of Anticancer and Antiepileptic Drugs

Chuncai Zhou, Mingzhi Wang, Kaidian Zou, Jing Chen, Yunqing Zhu, and Jianzhong Du*

School of Materials Science and Engineering, Tongji University, 4800 Caoan Road, Shanghai, 201804, China

Ⓢ Supporting Information

ABSTRACT: Antibacterial polypeptides as ancient immune defense systems are effective against bacteria. Here we report a novel kind of "armed" carrier: an antibacterial polypeptide-grafted chitosan-based nanocapsule with an excellent anti-bacterial efficacy against both Gram-positive and Gram-negative bacteria. This nanocapsule also has excellent blood compatibility and low cytotoxicity. Patients after tumor surgery may benefit from this "armed" carrier because it is highly anti-inflammation and is able to deliver anticancer and antiepileptic drugs simultaneously.
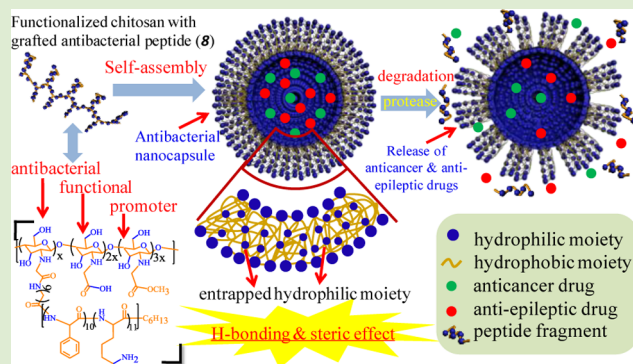
Bacterial infections during chemotherapy may be lethal for some cancer patients due to their lower resistance to them.[1] Therefore, it is necessary to administrate antibiotics, anticancer drugs and even antiepileptic after brain tumor surgery. However, some traditional antibiotics may cause serious side effects for those patients after tumor surgery. Furthermore, abuses of antibiotics have led to the emergence of more resistant and virulent strains of pathogens.[2] Therefore, it is necessary to develop next-generation multifunctional antibacterial agents using new design principles. Antibacterial peptide, as an ancient immune system for animals and plants, is a promising candidate. We report herein an antibacterial polypeptide-based polymeric nanocapsule with an excellent antibacterial efficacy as an "armed" drug carrier, which may benefit patients who are being treated by multiple drugs due to its likely less drug interactions compared to administrating antibiotics with other drugs simultaneously.

Natural peptides, such as defensins, cathelicidins (LL-37), magainins, and so on, are usually small proteins with cationic charge involved in host innate immune defense, showing broad antimicrobial activities against bacteria, fungi, and some type of virus with less bacterial resistance.[3] However, they are usually obtained by separation from nature, which cannot meet the clinical demand. Moreover, the high cost of natural antibacterial peptides is a major obstacle to their widespread use as antibacterial agents.[4] Fortunately, recent advances in polymerization techniques have facilitated the controlled polymerization of N-carboxyanhydrides (NCAs),[5] which offers a propensity to synthesize antibacterial polypeptides in a large scale and at a low cost.[11]

We recently reported the significantly enhanced antibacterial efficacy when an individual antibacterial polymer chain self-assembled into polymer micelles or vesicles due to concentration of local positive charges.[6] It is well-known that polymeric nanosized capsules are excellent drug carriers, which can be used to deliver anticancer drugs such as doxorubicin (DOX) to decrease its toxicity, slow down its release rate, and prolong its circulation time in bloodstream.[7] They can also release drugs at a given location and time when triggered by chemical and physical stimuli.[8]

Herein, we report an antibacterial polypeptide-grafted chitosan-based nanocapsule with an excellent antibacterial efficacy and blood compatibility as an "armed" drug carrier (Scheme 1). An acid-functionalized chitosan was selected as the backbone of the building polymer because it is a widely used biocompatible natural polymer with functional −COOH groups. The nanocapsules are formed by self-assembly of $[poly(Lys_{11}\text{-}stat\text{-}Phe_{10})\text{-}g\text{-}Cs]_x\text{-}stat\text{-}Cs_{2x}\text{-}stat\text{-}ECs_{3x}$ (polymer **8**, Schemes 1 and 2) in aqueous media. The antibacterial $poly(Lys_{11}\text{-}stat\text{-}Phe_{10})$ was synthesized by random copolymerization of protected NCA-lysine and NCA-phenylalanine monomers, which was then statistically grafted into the acid-functionalized chitosan backbone to afford the $[poly(Z\text{-}Lys_{11}\text{-}stat\text{-}Phe_{10})\text{-}g\text{-}Cs]_x$ unit, serving as the antibacterial component after deprotection of Z group. Half −COOH groups were statistically esterified by methanol to promote the antibacterial activity (ECs_{3x}). One third of residual −COOH groups in the acid-functionalized chitosan (Cs_{2x}) can be used for further functionalization, such as conjugation of prodrugs. The nanocapsule is able to simultaneously deliver anticancer drug such as doxorubicin (DOX) and antiepileptic such as Dilantin.

Scheme 1. Antibacterial Polypeptide-Grafted Chitosan-Based Nanocapsule as an "Armed" Carrier of Drugs[a]
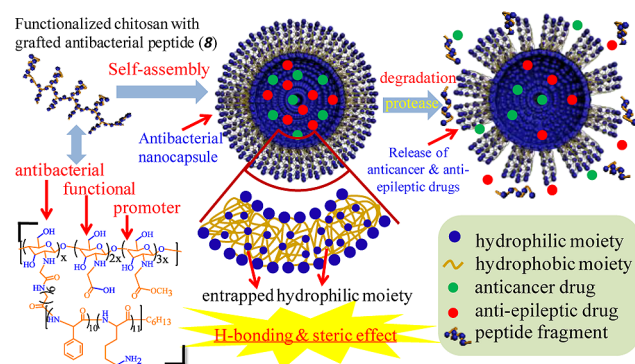
[a]Nanocapsules from $[poly(Lys_{11}\text{-}stat\text{-}Phe_{10})\text{-}g\text{-}Cs]_x\text{-}stat\text{-}Cs_{2x}\text{-}stat\text{-}ECs_{3x}$ (polymer **8**) have excellent antibacterial activity, while they are capable of delivering anticancer and antiepileptic drugs simultaneously. One sixth of −COOH groups in the acid-functionalized chitosan were grafted by an antibacterial peptide [poly(Lys$_{11}$-stat-Phe$_{10}$)], providing an excellent antibacterial efficacy for nanocapsules. Half −COOH groups in the acid-functionalized chitosan were esterified by methanol for enhancement of antibacterial activity. One third of residual −COOH groups in the acid-functionalized chitosan can be further functionalized when necessary. The membrane of capsule is composed of both hydrophobic and entrapped hydrophilic moieties. Drugs are released faster in the presence of protease due to biodegradation of polypeptide.

Deming and co-workers developed transition metal initiators that allow controlled polymerization of α-amino acid N-carboxyanhydrides (NCA) to afford polypeptide.[9] Zhou *et al.* prepared wide spectrum and highly antibacterial polypeptide by using a Ni(COD)$_2$ initiator.[10] However, it showed high toxicity to blood cells due to the residual catalyst. In this paper, a novel antibacterial polypeptide-grafted chitosan-based polymer, $[poly(Lys_{11}\text{-}stat\text{-}Phe_{10})\text{-}g\text{-}Cs]_x\text{-}stat\text{-}Cs_{2x}\text{-}stat\text{-}ECs_{3x}$ (**8**), with excellent antibacterial activity but low toxicity to blood cells has been synthesized in five steps (Scheme 2): (a) The antibacterial polypeptide (**3**), poly(Z-Lys$_{11}$-stat-Phe$_{10}$), with a −NH$_2$ end group was synthesized by vacuum NCA copolymerization of hydrophilic lysine (**1**) and hydrophobic phenylalanine (**2**; Figures S1−S3 in the Supporting Information for ^{1}H NMR analysis); (b) Polypeptide **3** was modified by hexamethylene diisocyanate (HDI) at room temperature to form poly(Z-Lys$_{11}$-stat-Phe$_{10}$)-NCO (**5**) with a reactive −NCO end group; (c) Polypeptide (**5**) was statistically grafted to acid-functionalized chitosan to afford $[poly(Z\text{-}Lys_{11}\text{-}stat\text{-}Phe_{10})\text{-}g\text{-}Cs]_x\text{-}stat\text{-}Cs_{5x}$ (**6**; Figure S5); (d) The −COOH groups in polymer **6** were partially esterified by methanol using H$_2$SO$_4$ (95%) as the catalyst to afford $[poly(Z\text{-}Lys_{11}\text{-}stat\text{-}Phe_{10})\text{-}g\text{-}Cs]_x\text{-}stat\text{-}Cs_{2x}\text{-}stat\text{-}ECs_{3x}$ (**7**) to enhance the antibacterial activity with an esterification degree of about 50% (Figure S6); (e) The Z group in polymer **7** was deprotected in the presence of HBr/CH$_3$COOH to afford the final polymer for preparation of nanocapsules: $[poly(Lys_{11}\text{-}stat\text{-}Phe_{10})\text{-}g\text{-}Cs]_x\text{-}stat\text{-}Cs_{2x}\text{-}stat\text{-}ECs_{3x}$ (**8**). The ^{1}H NMR and GPC analyses of the key polymers were presented and discussed in the Supporting Information.

Nanocapsules were self-assembled by polymer **8** in THF/H$_2$O (1:4, v/v), following a dialysis in pure water to remove THF. ^{1}H NMR spectrum of the nanocapsules in D$_2$O revealed significantly attenuated signals of polymer **8** chains due to

aggregation (Figure S7). The structure of nanocapsules is rather complex due to the fuzzy boundary between hydrophobic and hydrophilic domains, which is similar to the case in our recently reported homopolymer vesicles.[11] As shown in Scheme 1, in principle, partial hydrophilic moieties should form the nanocapsule corona, whereas both the hydrophobic and embedded hydrophilic moieties form the complex membrane due to the H-bonding and steric effect. Therefore, it is difficult to get a perfect transmission electron microscopy (TEM) image of nanocapsule (Figure S8). DLS study (Figure 1) revealed that the nanocapsules have a Z-averaged hydrodynamic diameter of about 230 nm. AFM studies (Figure 1) suggested a diameter of about 160 nm, which is reasonably in agreement with the DLS analysis. The height profile of AFM study also confirmed a hollow structure of nanocapsule, rather than a solid particle.[12]

Unlike microbe-killing mechanism of traditional antibiotics, antibacterial peptides insert into cell membranes, interacting with one another to form pores that disrupt membrane function, leading to cell killing.[13] Thus, it is difficult for pathogens to develop resistance to antibacterial peptides.[3a,14]

To confirm the enhancement of the antibacterial efficacy of $[poly(Lys_{11}\text{-}stat\text{-}Phe_{10})\text{-}g\text{-}Cs]_x\text{-}stat\text{-}Cs_{2x}\text{-}stat\text{-}ECs_{3x}$ (**8**) nanocapsules compared to poly(Lys$_{11}$-stat-Phe$_{10}$) (**4**) chain (which is the effective antibacterial component in **8**), we evaluated their minimum inhibitory concentrations (MICs) against both Gram-negative *E. coli* and Gram-positive *S. aureus* (Figure 2): 16 μg/mL (polymer **8** nanocapsules) and 31 μg/mL (polymer **4** chain, which is not in any assembled state). This is partially due to a higher local positive charge density in nanocapsules.[6] Furthermore, the esterification of −COOH groups in the acid-functionalized chitosan facilitates the antibacterial activity of nanocapsules (ECs promoter in **8**). For example, polymer **9** (by deprotection of **6**) without the esterification of −COOH showed a less active antibacterial behavior (see Scheme S1 and Table S1 in the Supporting Information).

Table S1 shows the MIC values of polymer **4** chains (the effective antibacterial component in polymer **8**), polymer **9** chains (without esterification compared with polymer **8**) and polymer **8** nanocapsules (the "armed" carrier). Polymer **4** chains have a highly antibacterial activity against both Gram-negative and Gram-positive bacteria. However, when polymer **4** was grafted into acid-functionalized chitosan to form polymer **9**, its antibacterial activity decreased obviously due to the residual −COOH groups in the acid-functionalized chitosan chain, which are negatively charged in aqueous solution. According to the bacteria-killing mechanism, the positively charged peptide is absorbed onto the surface of negatively charged bacteria membrane. Therefore, the negative charge from the residual −COOH groups in the acid-functionalized chitosan counteracts partial positive charge in the peptides, leading to a decrease in the antibacterial activity for polymer **9**. However, when 50% −COOH groups (relative to the entire −COOH groups in the acid-functionalized chitosan) were esterified by methanol to form polymer **8** and then self-assembled into nanocapsules, their antibacterial activity against *E. coli* increased by one time.

It is noteworthy that the direct comparison in the antibacterial activities between polymer **4** chains, polymer **9** chains, and polymer **8** chains is not possible because polymer **8** chains form nanocapsules in aqueous solution above their MIC, which is usually higher than their critical concentration for the nanocapsule formation. According to our previous study, the antibacterial activity of polymer **8** chains should be enhanced

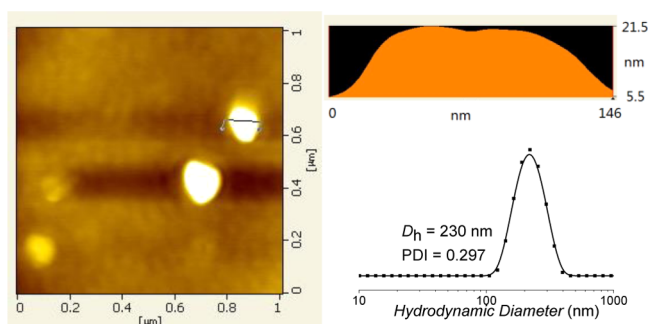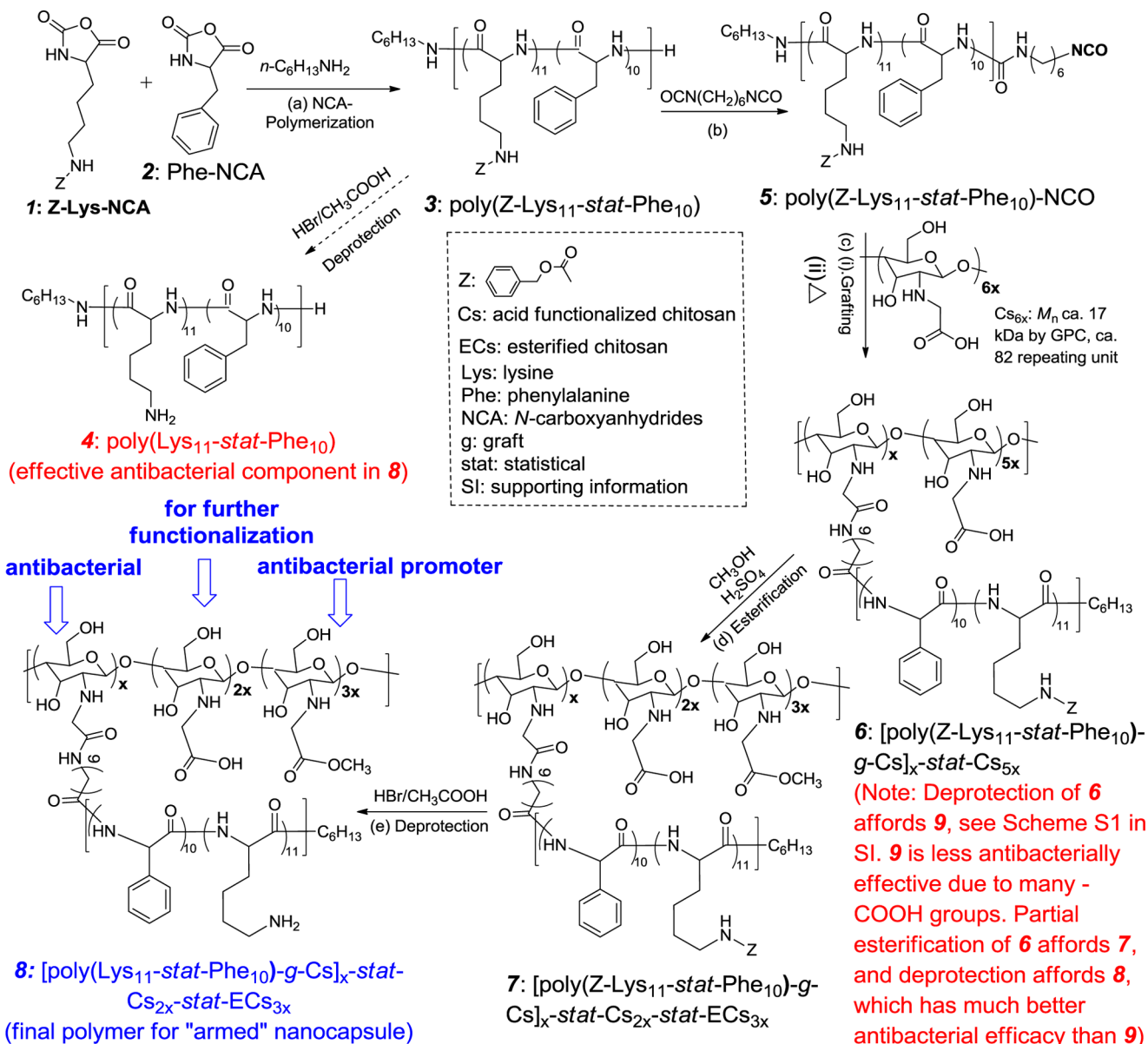**Scheme 2. Synthetic Strategy toward Antibacterial Peptide-Grafted Chitosan-Based Polymer**

Z: [benzyl carbamate structure]

Cs: acid functionalized chitosan

ECs: esterified chitosan
Lys: lysine
Phe: phenylalanine
NCA: *N*-carboxyanhydrides
g: graft
stat: statistical
SI: supporting information

1: Z-Lys-NCA

2: Phe-NCA

3: poly(Z-Lys₁₁-*stat*-Phe₁₀)

4: poly(Lys₁₁-*stat*-Phe₁₀)
(effective antibacterial component in **8**)

5: poly(Z-Lys₁₁-*stat*-Phe₁₀)-NCO

Cs₆ₓ: M_n ca. 17 kDa by GPC, ca. 82 repeating unit

for further functionalization

antibacterial   **antibacterial promoter**

6: [poly(Z-Lys₁₁-*stat*-Phe₁₀)-*g*-Cs]ₓ-*stat*-Cs₅ₓ
(Note: Deprotection of **6** affords **9**, see Scheme S1 in SI. **9** is less antibacterially effective due to many -COOH groups. Partial esterification of **6** affords **7**, and deprotection affords **8**, which has much better antibacterial efficacy than **9**)

7: [poly(Z-Lys₁₁-*stat*-Phe₁₀)-*g*-Cs]ₓ-*stat*-Cs₂ₓ-*stat*-ECs₃ₓ

8: [poly(Lys₁₁-*stat*-Phe₁₀)-*g*-Cs]ₓ-*stat*-Cs₂ₓ-*stat*-ECs₃ₓ
(final polymer for "armed" nanocapsule)

Figure 1. Polymer **8** nanocapsules: (A) Height contrast of atomic force microscopy (AFM) images; (B) The corresponding height profile reveals an aspect ratio of **9**, suggesting a capsular structure. (C) Size distribution by DLS.

when self-assembled into nanocapsules due to the increase of local concentration of positive charge. Furthermore, according to the above analysis, the esterification of −COOH groups

facilitates the enhancement of its antibacterial activity. Therefore, the improvement of antibacterial activity of polymer **8** nanocapsules is ascribed to both the self-assembly of the individual polymer **8** chain and the esterification of partial −COOH groups.

To compare their blood compatibilities (one of the most important properties for antibacterial materials) between polymer **8** nanocapsules and polymer **4** chains, we evaluated their abilities to lyse red blood cells in terms of the quantity H_{50}, which is defined as the minimum peptide concentration that produces 50% hemolysis using 0.1% Triton X-100 as a standard (Figure 3). Their H_{50}'s are 700 and 110 μg/mL, respectively, indicating much better blood compatibility and much lower toxicity to eukaryotic cells of polymer **8** nanocapsules than polymer **4** chains. Their corresponding selectivities (the values of H_{50}/MIC) are 44 (700/16) and 3.4 (110/32) to *E. coli*, respectively, indicating that the incorporation of biocompatible acid-functionalized chitosan into polymer **8** and the self-assembled structure significantly
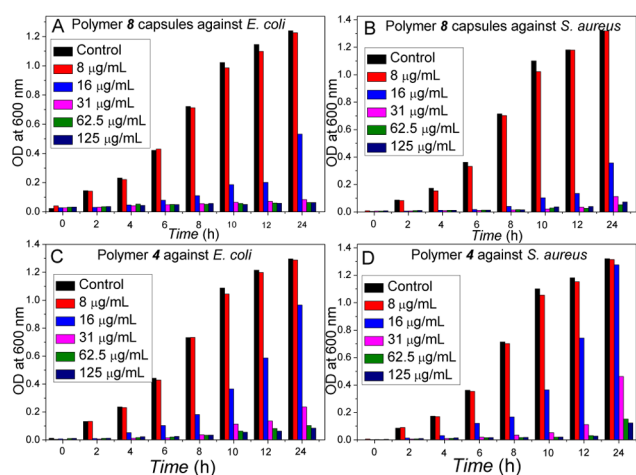
Figure 2. Dose-dependent growth inhibitions of typical gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria in the presence of polymer **8** nanocapsules and polymer **4** chains. OD: optical density.
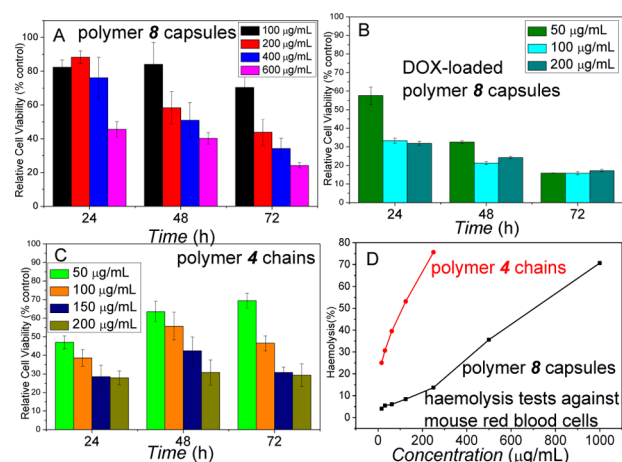

Figure 3. Cell viability (A-C, CCK-8 assays, $n = 5$) and hemolysis tests (D) of polymer **8** nanocapsules and polymer **4** chains.

simultaneously encapsulated into the nanocapsule. The drug loading content (DLC) and drug loading efficiency (DLE) of DOX were calculated according to the following equations.

$$DLC(\%) = \frac{(\text{weight of drug encapsulated in nanocapsules}) \times 100\%}{\text{weight of polymer}}$$

$$= \frac{31.1 \ \mu g/mL \times 20.68 \ mL \times 100\%}{15.0 \ mg} = 4.29\%$$

$$DLE(\%) = \frac{(\text{weight of drug encapsulated in nanocapsules}) \times 100\%}{\text{weight of drug in feed}}$$

$$= \frac{31.1 \ \mu g/mL \times 20.68 \ mL \times 100\%}{3.0 \ mg} = 21.4\%$$

The DLC and DLE of Dilantin are 10.3 and 51.7%, respectively, as calculated according to a similar procedure (see Supporting Information). The higher DLC and DLE of Dilantin than that of DOX is because the stronger interaction between the nanocapsules and Dilantin than DOX.

At 37 °C, both DOX (Figure 4A) and Dilantin (Figure 4B) showed a retarded release due to the membrane barrier of

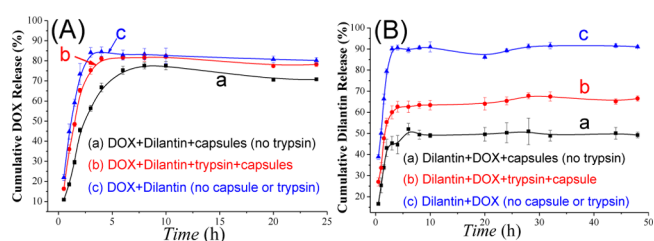**Figure 4.** Cumulative release profile of DOX and Dilantin-loaded polymer **8** nanocapsules at 37 °C in 0.01 M tris buffer and pH 7.4 [concentrations (if any): trypsin 6.0 mg mL^{-1}, nanocapsule solution 0.36 mg mL^{-1}]: (A) DOX release profile determined by fluorescence spectrometer; (B) Dilantin release profile determined by UV−vis spectroscopy.

lower its toxicity compared to polymer **4**. In addition, the higher selectivity of polymer **8** nanocapsules than many other synthetic and natural antibacterial peptides allows it a wide range of applications in human and animals.

Self-assembled structure and incorporating antibacterial peptide into acid-functionalized chitosan lower cytotoxicity of nanocapsule. The cytotoxicity of polymer **8** nanocapsules and polymer **4** chains (effective antibacterial component in **8**) were evaluated using CCK-8 assay (Figure 3). There are no significant differences in the relative cell viabilities between polymer **8** nanocapsules-treated human HCCLM3 liver cancer cells and the controls in vitro, even after 72 h treatment at 100 μg/mL (Figure 3A). In contrast, polymer **4** chains showed much higher cytotoxicity even at a lower concentration of 50 μg/mL (Figure 3B). Moreover, the nanocapsule has little effect on the toxicity of an anticancer drug, DOX, to tumor cells (Figure 3C).

The antibacterial nanocapsule showed enzyme response due to peptide chains. In the presence of 5.83 mg mL^{-1} of trypsin, about 40% of polymer **8** nanocapsule had been degraded within 4 h (Figure S10). To evaluate its drug release ability in the presence of proteases such as trypsin, an antiepileptic drug (Dilantin), and an anticancer drug (DOX) had been

nanocapsules. In the presence of an enzyme (trypsin), the release rates of both DOX and Dilantin were accelerated in the first four hours, and the final release content increased compared to that in the absence of trypsin.

In summary, we have successfully developed a novel antibacterial nanocapsule as an "armed" drug carrier which exhibits excellent efficacy for killing both Gram-positive and Gram-negative bacteria. A synthetic antibacterial peptide, poly(Lys$_{11}$-*stat*-Phe$_{10}$), has been grafted into acid-functionalized chitosan to serve as a highly efficient bacterial killer. The esterification of half −COOH groups in the acid-functionalized chitosan, together with the self-assembly of [poly(Lys$_{11}$-*stat*-Phe$_{10}$)-*g*-Cs]$_x$-*stat*-Cs$_{2x}$-*stat*-ECs$_{3x}$ to form a capsular nanostructure, has significantly enhanced its antibacterial activity. The residual −COOH groups in the nanocapsule may be further functionalized such as for conjugation of prodrugs. Importantly, the "armed" nanocapsule has excellent blood compatibility and low cytotoxicity. Finally, such multifunctional antibacterial nanocapsules are able to simultaneously encapsulate both anticancer and antiepileptic drugs and show an enzyme-triggered faster release profile, which suggests their potential application as "armed" drug carriers, and may benefit patients after tumor surgery.

■ ASSOCIATED CONTENT

S Supporting Information

Full synthetic and characterization details. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: jzdu@tongji.edu.cn.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

■ REFERENCES

(1) (a) Bressler, A. M.; Kaye, K. S.; LiPuma, J. J.; Alexander, B. D.; Moore, C. M.; Reller, L. B.; Woods, C. W. *Infect. Control. Hosp. Epidemiol.* **2007**, *28*, 951−958. (b) Leleu, G.; Aegerter, P.; Guidet, B. *J. Crit. Care* **2002**, *17*, 168−175. (c) Lai, C. C.; Teng, L. J.; Hsueh, P. R.; Yuan, A.; Tsai, K. C.; Tang, J. L.; Tien, H. F. *Clin. Infect. Dis.* **2004**, *38*, 149−153.

(2) Zhang, L. J.; Parente, J.; Harris, S. A.; Woods, D. E.; Hancock, R. E. W.; Fallal, T. J. *Antimicrob. Agents Chemother.* **2005**, *49*, 2921−2927.

(3) (a) Hoffmann, J. A.; Kafatos, F. C.; Janeway, C. A.; Ezekowitz, R. A. B. *Science* **1999**, *284*, 1313−1318. (b) Baroni, A.; Donnarumma, G.; Paoletti, I.; Longanesi-Cattani, I.; Bifulco, K.; Tufano, M. A.; Carriero, M. V. *Peptides* **2009**, *30*, 267−272. (c) Pistolesi, S.; Pogni, R.; Feix, J. B. *Biophys. J.* **2007**, *93*, 1651−1660. (d) Kristiansen, P. E.; Fimland, G.; Mantzilas, D.; Nissen-Meyer, J. *J. Biol. Chem.* **2005**, *280*, 22945−22950.

(4) (a) Tew, G. N.; Scott, R. W.; Klein, M. L.; Degrado, W. F. *Acc. Chem. Res.* **2010**, *43*, 30−39. (b) Choi, S.; Isaacs, A.; Clements, D.; Liu, D. H.; Kim, H.; Scott, R. W.; Winkler, J. D.; DeGrado, W. F. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 6968−6973.

(5) (a) Pickel, D. L.; Politakos, N.; Avgeropoulos, A.; Messman, J. M. *Macromolecules* **2009**, *42*, 7781−7788. (b) Aliferis, T.; Iatrou, H.; Hadjichristidis, N. *Biomacromolecules* **2004**, *5*, 1653−1656.

(6) (a) Yuan, W. Z.; Wei, J. R.; Lu, H.; Fan, L.; Du, J. Z. *Chem. Commun.* **2012**, *48*, 6857−6859. (b) Zhang, C.; Zhu, Y. Q.; Zhou, C. C.; Yuan, W. Z.; Du, J. Z. *Polym. Chem.* **2013**, *4*, 255−259. (c) Zhu, H. S.; Geng, Q. R.; Chen, W. Q.; Zhu, Y. Q.; Chen, J.; Du, J. Z. *J. Mater. Chem. B* **2013**, *1*, 5496−5504.

(7) (a) Mane, S. R.; Rao, N. V.; Chaterjee, K.; Dinda, H.; Nag, S.; Kishore, A.; Das Sarma, J.; Shunmugam, R. *Macromolecules* **2012**, *45*, 8037−8042. (b) Stuart, M. A. C.; Huck, W. T. S.; Genzer, J.; Muller, M.; Ober, C.; Stamm, M.; Sukhorukov, G. B.; Szleifer, I.; Tsukruk, V. V.; Urban, M.; Winnik, F.; Zauscher, S.; Luzinov, I.; Minko, S. *Nat. Mater.* **2010**, *9*, 101−113.

(8) Chen, W. Q.; Du, J. Z. *Sci. Rep* **2013**, *3*, 2162 DOI: 2110.1038/srep02162.

(9) (a) Deming, T. J. *Nature* **1997**, *390*, 386−389. (b) Wyrsta, M. D.; Cogen, A. L.; Deming, T. J. *J. Am. Chem. Soc.* **2001**, *123*, 12919−12920.

(10) Zhou, C. C.; Qi, X. B.; Li, P.; Chen, W. N.; Mouad, L.; Chang, M. W.; Leong, S. S. J.; Chan-Park, M. B. *Biomacromolecules* **2010**, *11*, 60−67.

(11) (a) Zhu, Y. Q.; Liu, L.; Du, J. Z. *Macromolecules* **2013**, *46*, 194−203. (b) Fan, L.; Lu, H.; Zou, K. D.; Chen, J.; Du, J. Z. *Chem. Commun.* **2013**, DOI: 10.1039/C1033CC45873C.

(12) Huang, J.; Bonduelle, C.; Thevenot, J.; Lecommandoux, S.; Heise, A. *J. Am. Chem. Soc.* **2012**, *134*, 119−122.

(13) (a) Karlsson, A. J.; Pomerantz, W. C.; Weisblum, B.; Gellman, S. H.; Palecek, S. P. *J. Am. Chem. Soc.* **2006**, *128*, 12630−12631. (b) Epand, R. M.; Vogel, H. J. *Biochim. Biophys. Acta, Biomembr.* **1999**, *1462*, 11−28.

(14) (a) Shai, Y. *Biopolymers* **2002**, *66*, 236−248. (b) Hancock, R. E. W.; Scott, M. G. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8856−8861.