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pH 调控法制备载羟基喜树碱棒状纳米靶向给药系统及性能研究

Preparation and Evaluation of HCPT-loaded, Targeted Nanorods Induced by pH Switch

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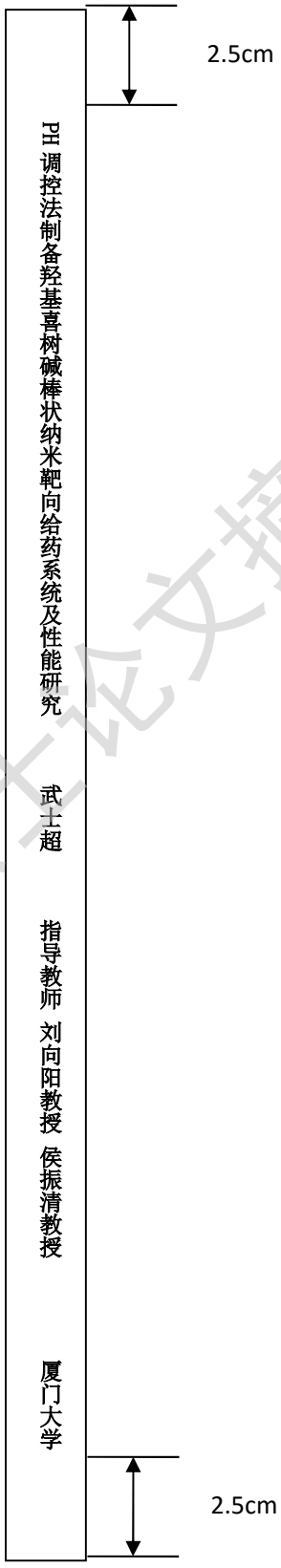

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摘 要

目前，在大部分给药纳米粒子制备方法中，疏水基团是必需的，因此这些制备方法都非常依赖于有机溶剂。而这些有机溶剂通过减压蒸馏或者冻干等传统方法是无法完全除去的，即使是透析也无法完全去除包裹在纳米粒子之内的残留溶剂，这样在纳米粒子内就会不可避免的存在残留有机溶剂。残留溶剂不仅对人体的健康有害，而且会加速药物制剂的变质失效。因此，如何减少或去除药物制剂中的残留溶剂一直是人们关注的问题。

羟基喜树碱(HCPT)和甲氨喋呤(MTX)都是临床常用的抗肿瘤药物，二者作用机制不同，羟基喜树碱是通过作用于 DNA 拓扑异构酶 I 来抑制 DNA 复制、转录和有丝分裂，而甲氨喋呤则是通过与二氢叶酸还原酶结合，干扰辅酶 F 的合成，从而抑制 DNA 的复制。二者联合用药能够产生非常好的协同作用，共同杀伤细胞。此外，甲氨喋呤还能够与癌细胞表面过度表达的叶酸受体相结合，介导纳米粒子的特异性细胞摄取，从而产生一定的靶向性。因此将二者共同负载到一个纳米粒上，能够使纳米粒既具有非常好的协同作用，又能有一定的靶向性。

非球形纳米粒子受到越来越多的关注，因其具有与球形粒子完全不同的体内和体外性质，尤其是高纵横比纳米粒子，实验证明，高纵横比纳米粒子的细胞摄取速率要大于球形的纳米粒子的细胞摄取速率，因此构建一种高纵横比的给药纳米粒子也许就能够提供一种新型的高效抗肿瘤制剂。

本文以壳聚糖为药物载体，以羟基喜树碱为模型药物，采用 pH 调控联合超声法制备出了叶酸修饰的和甲氨喋呤修饰的羟基喜树碱纳米棒，整个制备过程没有使用任何的有机溶剂，消除了纳米粒残留溶剂问题。并考察了制备条件对其两种粒子形貌的影响，并对两种纳米棒粒子的性质进行了表征，最后对它们的体内和体外抗肿瘤效果进行了系统评价，结果如下：

(1) 叶酸修饰的羟基喜树碱纳米棒（HFNDs）的制备。首先合成了壳聚糖-叶酸复合物（CS-FA），并进行了红外图谱的表征，证明的酰胺键的生成。将 CS-FA 溶于醋酸溶液，HCPT 溶于氢氧化钠溶液，在超声冰浴的条件下，将两溶液混合，制备出了叶酸修饰的羟基喜树碱纳米棒(HFNDs)。进行了制备条件的优化实验。优化条件投料比为 1：1，浓度为 10 μg/mL，温度为 4-6℃，反应最终溶液 pH=7，

超声功率为 200 W，超声时间为 3 s，间隙 3 s，冰浴条件下反复 150 次。根据优化实验结果，对优化条件下制备的 HFNDs 的粒径、Zeta 电位、载药量、包封率、形态、HCPT 的存在状态进行分析。结果表明：通过 pH 值调控成核法制备的 HFNDs 纳米粒子具有均一的形貌和大小。形状为棒，长度约为 800 nm，宽约为 80 nm，动态光散射分析其平均水化动力学粒径为 104.3 ± 5.7 nm，且粒径分布较窄，表面电势为+ (16.3 ± 1.9) mv。 HFNDs 的载药量为 70.2 ± 3.1%，包封率为 83.1%，且缓释效果良好，在 pH=7.4 的缓冲溶液能够在 48 h 内缓慢的释放药物。

(2) 体外及体内抗肿瘤效果评价。体外细胞摄取实验表明，HeLa 细胞对 HFNDs 的细胞摄取的速率大大快于未经叶酸修饰的羟基喜树碱-壳聚糖纳米棒(NDs)。活体荧光成像结果表明 HFNDs 在肿瘤部位的药物浓度要高于 NDs，以上两组实验说明，HFNDs 具有非常好的靶向性，能够很好的在肿瘤部位富集。体外细胞毒性实验表明，HFNDs 对 HeLa 的细胞毒性比 NDs 对 HeLa 的细胞毒性要明显大的多。小鼠抗肿瘤实验说明，HFNDs 的体内抗癌效果要远远好于相同药物剂量下的市售制剂以及 NDs，以上两组试验则表明，叶酸的引入大大提高了 HFNDs 的抗肿瘤效果。

(3) 双载药纳米棒粒子（MHNDs）的制备。首先合成了壳聚糖-甲氨喋呤复合物（CS-MTX），进行了红外图谱的表征，证明的酰胺键的生成，并通过紫外分光光度法，计算出 MTX 的百分含量为 28.6 ± 1.7%。将 CS-MTX 溶于醋酸溶液，HCPT 溶于氢氧化钠溶液，在超声冰浴的条件下，将两溶液混合，制备出了双载药纳米棒粒子(MHNDs)。并对制备的 MHNDs 的粒径、Zeta 电位、载药量、包封率、形态、HCPT 的存在状态进行分析。结果表明：通过 pH 值调控成核法制备的 MHNDs 纳米粒子形貌与 HFNDs 形貌非常类似，同样为棒状的纳米粒子，长度约为 600 nm，宽约为 80 nm，动态光散射分析其平均水化动力学粒径为 101.0 ± 5.8 nm，且粒径分布较窄，表面电势为+ (21.4 ± 2.1) mv。MHNDs 的 HCPT 的载药量为 68.8 ± 2.5%，包封率为 80.1%，MTX 的载药量为 8.9±0.3%。MHNDs 的缓释效果良好，能够在 48 h 内缓慢的释放药物。

(4)体外及体内抗肿瘤效果评价。体外细胞摄取实验表明，HeLa 细胞对 MHNDs 的细胞摄取的速率大大快于 NDs。叶酸竞争性抑制试验表明，在叶酸存在条件下，MHNDs 的细胞摄取明显下降，说明 MHNDs 是通过与叶酸受体的特

异性结合介导的特异性吞噬进入细胞的。活体荧光成像结果表明 MHNDs 在肿瘤部位的药物浓度要高于 NDs，以上实验说明，MHNDs 同样具有非常好的靶向性，能够很好的在肿瘤部位富集。体外细胞毒性实验说明 MHNDs 的细胞毒性不仅大于两种药物理混合物的细胞毒性，也强于 NDs 与甲氨喋呤的混合物的细胞毒性。小鼠抗肿瘤实验也说明，双载药纳米棒(MHNDs)的体内抗癌效果要远远好于相同药物剂量下的市售制剂以及单载药纳米棒(NDs)制剂，而且毒副作用也大大降低。以上两组实验说明双载药纳米棒(MHNDs)不仅具有很好的协同作用，也具有一定的靶向性。

关键词：羟基喜树碱 壳聚糖 甲氨喋呤 pH 调控法 纳米棒 超声

Abstract

Since the hydrophobic group is essential to the synthesis of the drug-loaded nanoparticles, a majority of the methods rely heavily on organic solvents. These organic solvents might be residual within the particles and could not be completely removed by conventional methods, such as reduced pressure distillation or freeze drying. As a result, trace of organic solvents would remain in the medicine, which are called residual solvents. Although residual solvents are extremely little and always meet the special directions published in pharmacopeias that have been strictly controlling the maximum allowable amounts of the residual solvents in pharmaceutical products, the residual solvents would be accumulating in the body, and might accentuate the disease or cause other serious issues to the patients. Hence,all the manufacturers have been aspiring to minimize the amount of the organic solvents used in drug production process. Therefore, it will be a pretty major leap for the human health to use “green” chemistry into the pharmaceutical industry, although facing amounts of difficulties.

10-hydroxycamptothecin (HCPT) is a promising broad-spectrum antitumor agent which targets nuclear enzyme topoisomerase I and has achieved remarkable success in the early clinical trials. MTX is also a commonly used clinical antitumor drug, which works by affecting folate metabolism. The function mechanism of the two drugs is different, which will lead to good synergistic effect when combined the two drugs. Moreover, MTX can function as a tumor-targeting agent as well. Promisingly, interfacial integration of MTX with another anticancer drug in the nanohybrid could result in the dual-drug delivery system with improved therapeutic efficiency, compared with that of traditional formulations.

In the current study, an aqueous drug nanoformulation, composed of 10-hydroxycamptothecine (HCPT) and the MTX-chitosan/FA-chitosan conjugate, was fabricated in a green coprecipitation process driven by the abrupt pH switch in the aqueous mixture. The hybrid nanorods are characteristic of bearing a nanocrystalline HCPT core integrated with a MTX-chitosan/FA-chitosan conjugated shell.

Nanohybrids with high HCPT loading showed the prolonged and sustained release property due to the presence of the conjugated protection layer. *In vitro* and *in vivo s*tudies will be then systematically carried out to examine the effect of the nanorods against cancer cells.

The contents are summarized as follows:

(1) Preparation and characterization of Folate-modified, HCPT-loaded nanorods (HFNDs). Firstly, we conjugate folate to chitosan via an amido bond. The FTIR spectrum illustrated that the amido bond was exist and the conjugate CS-FA was successfully synthesized. Then, the HCPT and the CS-FA were dissolved in alkali and acids, respectively. When the two solutions were mixed, neutralization reaction would happen. The produced mixture was controlled to be neutral, which would be a poor solvent for both HCPT and CS-FA. The decrease of the solubility triggered by pH changes provided an opportunity for the nucleation of HCPT nanorods and the accompanying coprecipitation of CS-FA onto the growing HCPT nanorods. To optimize the formulation conditions, a condition experiment was designed to study the effect of the ratio of HCPT to CS-FA, ultrasonic power, pH of the produced mixture, and concentration of the produced mixture on the the morphology of HFNDs. The results were as follows: the ratio of HCPT to CS-FA was 1:1; the pH of the produced mixture was 7.0; the power of the sonication was 200 W; the sonication time was sonication for 3 s, interval for 3 s and repeated for 150 times in ice-bath. The obtained nanorods were characterized by scanning electron microscopy (SEM)/transmission electron microscopy (TEM), dynamic light scattering (DLS), and X-ray diffraction (XRD). Based on these experimental results, it was proposed that HFNDs exhibited well dispersed, fairly uniform size and a pointed-end, needle-like shape with a length of about 800 nm and a width of about 80 nm. The DLS analysis illustrated that the size of HFNDs was 104.3± 5.7 nm, the zeta potential was + 16.3 ± 1.9 V. The drug-loading and entrapment efficiency of HFNDs were 70.2 ± 3.1% and 83.1%, respectively. The release behavior in vitro showed that HFNDs could slowly release the drug in 48 h in PBS (pH=7.4).

(2) The in vitro and in vivo studies of HFNDs. The in vitro and in vivo studies

were then systematically carried out to examine the effect of HFNDs against cancer cells. The cellular uptake test was carried out with HeLa cells and used HCPT-chitosan nanorods (NDs) with the same size as control. The cellular uptake of HFNDs was much faster and higher than that of the NDs. The in-vivo fluorescence test also stated the targeting property of HFNDs was better than that of NDs. These tests stated that enhanced cellular uptake of HFNDs was caused by the modification of folate, which would lead to the receptor-mediated endocytosis. The cytotoxicity test indicated that the killing ability of HFNDs was stronger than that of the individual drugs, or NDs. The in-vivo antitumor experiment showed the excellent anticancer property and the slight side effect. These two tests illustrated the good anticancer effect of HFNDs.

(3) Preparation and characterization of MTX-modified, HCPT-loaded nanorods (MHNDs). Firstly, we conjugate MTX to chitosan via an amido bond. The FTIR spectrum illustrated that the amido bond was exist and the conjugate CS-MTX was successfully synthesized. We calculated the percentage of the MTX in the conjugation via ultraviolet spectroscopy. Then, the fast mixing of an alkaline HCPT solution (pH = 13) with an acidic CS-MTX one (pH = 2.0) in the presence of ultrasound led to coprecipitation of both ingredients. Thus, the dual-drug loaded nanorods was successfully prepared. The obtained nanorods were characterized by scanning electron microscopy (SEM), dynamic light scattering (DLS), and X-ray diffraction (XRD). Based on these experimental results, it was proposed that MHNDs exhibited well dispersed, fairly uniform size and a pointed-end, needle-like shape with a length of about 600 nm and a width of about 80 nm. The DLS analysis illustrated that the size of HFNDs was 101.0 ± 5.8 nm, the zeta potential was +21.4 ± 2.1 mV. The drug-loading and entrapment efficiency of HCPT in MHNDs were 68.8 ± 2.5% and 80.1%, respectively. The drug-loading of MTX was 8.9±0.3%. The release behavior in vitro showed that MHNDs could slowly release both drugs in 48 h in PBS (pH=7.4).

(4) The in vitro and in vivo studies of MHNDs. The in vitro and in vivo studies were then systematically carried out to examine the effect of MHNDs against cancer cells. The cellular uptake test was carried out with HeLa cells and used NDs as control. The cellular uptake of MHNDs was much faster and higher than that of the NDs. And

this enhancement would disappear, when the folate receptors on the cell membranes were blocked with folate. This stated that the enhanced cellular uptake of MHNDs was caused by the affinity between MTX and the folate receptors in target cells, which would also lead to the receptor-mediated endocytosis. The cytotoxicity test indicated that the killing ability of the dual drug loaded nanorods was stronger than that of the individual drugs, the mixture of individual drugs, or the mixture of MTX and the NDs. This illustrated the good synergistic effect and targeting property of MHNDs. The in-vivo fluorescence test also stated the good targeting property of MHNDs. The in-vivo antitumor experiment showed the excellent anticancer property and the slight side effect.

Keywords： HCPT · MTX · Chitosan · Nanorods · pH switch · Sonication

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