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Preparation, property of the complex of carboxymethyl chitosan grafted copolymer with iodine and application of it in cervical antibacterial biomembrane

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ABSTRACT

Cervical erosion is one of the common diseases of women. The loop electrosurgical excisional procedure (LEEP) has been used widely in the treatment of the cervical diseases. However, there are no effective wound dressings for the postoperative care to protect the wound area from further infection, leading to increased secretion and longer healing time. Iodine is a widely used inorganic antibacterial agent with many advantages. However, the carrier for stable iodine complex antibacterial agents is lack. In the present study, a novel iodine carrier, Carboxymethyl chitosan-g-(poly(sodium acrylate)-*co*-polyvinylpyrrolidone) (CMCTS-g-(PAANa-*co*-PVP), was prepared by graft copolymerization of sodium acrylate (AANa) and *N*-vinylpyrrolidone (NVP) to a carboxymethyl chitosan (CMCTS) skeleton. The obtained structure could combine prominent property of poly(sodium acrylate) (PAANa) anionic polyelectrolyte segment and good complex property of polyvinylpyrrolidone (PVP) segment to iodine. The bioactivity of CMCTS could also be kept. The properties of the complex, CMCTS-g-(PAANa-*co*-PVP)-I_2, were studied. The *in vitro* experiment shows that it has broad-spectrum bactericidal effects to virus, fungus, gram-positive bacteria and gram-negative bacteria. A CMCTS-g-(PAANa-*co*-PVP)-I_2 complex contained cervical antibacterial biomembrane (CABM) was prepared. The iodine release from the CABM is pH-dependent. The clinic trial results indicate that CABM has better treatment effectiveness than the conventional treatment in the postoperative care of the LEEP operation.

1. Introduction

Cervical erosion is one of the common diseases of women. It is considered as an immediate cause for cervical carcinoma [1]. The resistance of the endocervical columnar epithelium on the top of the ectocervical erosion area is very low. Therefore, the cervix is easy to be invaded by the pathogen when the cervical erosion occurs, inducing inflammations. Due to the numerous mucosal folds in the cervical canal, the pathogen is hard to be removed once the inflammation occurred. The inflammation is deferred and becomes worse, eventually inducing chronic cervicitis [2]. Various treatments for the cervical erosion have been used in clinic. However, the recurrence rate after the traditional physicotherapeutics and medication is very high. In 1989, Prendiville et al. proposed a loop electrosurgical excisional procedure (LEEP) to treat the dysplasia of the cervical epithelial cells [3]. Since then, this LEEP has been used worldwide in the treatment of the cervical diseases [4]. However, there are no effective wound dressings for the postoperative care to protect the wound area from further infection. Then colporrhagia can be induced by scabs, vascular rupture and wound infection, leading to increased secretion and longer healing time. The probability of secretion increasing is enhanced and the duration was longer. The above problems are the puzzle in the clinic of this operation method [5].

Iodine is a widely used inorganic antibacterial agent [6]. The complex formed between iodine and the carrier is a broad-spectrum bactericide with high antibacterial effect, good stability and low toxicity [7]. Iodine is usually combined with various small molecular surfactants, such as lecithin [8], and polymers, such as polyvinylpyrrolidone [9] and polyoxyethylene ether [10], and so on. Recently, Ding et al. used amphiphilic derivates of chitosan to complex with the iodine [11]. Polyvinylpyrrolidone-iodine (Trade name, Povidone-Iodine) is the only iodine antibacterial agent widely used in clinic [12]. Povidone-Iodine has low toxicity and excellent treatment effect. It can be used to kill both bacteria and virus [13]. However, Povidone-Iodine is an external use medicine and thus its antibacterial persistence and efficiency are weak. It cannot be used *in vivo* and orifice. Although the hydrophility of polyvinylpyrrolidone is good, the surface of the solidified Povidone-Iodine is rigid, the swelling rate of it is slow, and the adhesion to mucosa

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is very poor. All these confine the application of solid Povidone-Iodine complex antibacterial agent. Efforts have been made to improve the antimicrobial effects of PVP-I_2 by constructing its hydrogel forms [14,15].

In the present work, carboxymethyl chitosan (CMCTS), an important derivate of marine polymers, was used as a skeleton to graft copolymerize with the fully neutralized acrylic acid and vinylpyrrolidone. The above polymer possesses the properties of both poly(sodium acrylate) (PAANa) and PVP, including the high hydrophilicity, good tissue adhesion and pH sensitivity of PAANa and the excellent complex ability of PVP to iodine. The prepared copolymer CMCTS-g-(PAANa-*co*-PVP) was characterized and used as a carrier to prepare a CMCTS-g-(PAANa-*co*-PVP)-I_2 antibacterial agent. Then a CMCTS-g-(PAANa-*co*-PVP)-I_2 contained antibacterial biomembrane (CABM) was prepared. After being LEEP treated for the patients suffered from cervical erosion, the prepared CABM containing CMCTS-g-(PAANa-*co*-PVP)-I_2 was used on the surface of a wound to study the postoperative care effect of it.

2. Materials and methods

2.1. Materials

N,*O*-carboxymethyl chitosan (CMCTS) was synthesized according to a method reported in the literature [16]. The substitution degree of the carboxymethyl was determined as 0.75 by elemental analysis. Acrylic acid (AA, A.R.) and *N*-vinylpyrrolidone (NVP, A.R.) were purchased from Tianjin Chemical Reagent Institute (Tianjin, China) and MERCK-Schuchardt Co. (Germany), respectively. Both were purified by vacuum distillation before use. Azodiisobutyronitrile (AIBN, A.R.) was purchased from Shanghai Tianlian Fine Chemical Co., Ltd. (Shanghai, China) and used as the initiator for the polymerization. Iodine, *n*-heptane, alcohol and sodium hydroxide were analytical grade and used as received.

Type II herpes simplex virus (HSV-II strain) was supplied by the Key Lab of Pathogen Associated Molecular Biology at Zhejiang Academy of Medical Sciences. Vero cell used as the experimental cell strain was supplied by Shanghai Institute of Cell Biology. DMEM cell culture medium, trypsin (GIBCO) and fetal bovine serum was purchased from Hangzhou Sijiqing Biomaterial Co., Ltd. (Hangzhou, China).

Seven species of bacteria with 10 strains for each, including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Entercoccus faecalis*, *Klebsiella pneumonia*, *Candida albicans* and *Na. gonococcus*, were separated from patients by the clinical laboratory at Hangzhou Hospital of Traditional Chinese Medicine. Four species of standard bacteria including *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923), *Pseudomonas aeruginosa* (ATCC27853) and *Candida albicans* (AATCC10231) were provided by National Key Laboratory of Safety Evaluation for New Drug at Zhejiang Academy of Medical Sciences. MH agar, Paul's agar, bouillon culture medium and *Neisseria gonorrhoeae* culture medium were purchased from Hangzhou Tianhe Microbial Reagents Co., Ltd. (Hangzhou, China).

2.2. Synthesis of CMCTS-g-(PAANa-co-PVP)

First, 1.5 g CMCTS was dissolved in 120 mL water and the solution was transferred to a three-necked flask. The CMCTS solution was stirred for 30 min under the protection of nitrogen and refluxed at 60 °C in a water bath. 0.35 g AIBN was slowly added into the flask to initiate the graft polymerization and refluxed at 60 °C for 30 min. NVP and sodium acrylate fully neutralized by NaOH with a total amount of 15 g were then added to reaction mixtures and refluxed at 60 °C for 6 h under the protection of nitrogen. The final volume of the reaction mixture was controlled to 200 mL. The reaction mixture was then precipitated by alcohol and filtered. The residue was thoroughly washed with the alcohol/water (4:1, v/v) for several times under high-speed stirring, soaked in the alcohol/water (4:1, v/v) for 24 h and filtrated. The residue was collected, dried in vacuum and shivered to powder.

2.3. Preparation of CMCTS-g-(PAANa-co-PVP)-I_2

CMCTS-g-(PAANa-*co*-PVP) powder was dissolved in a water/alcohol solution. The solution was neutralized to desired pH by 5% acetic acid solution and heated to 50 °C. An iodine solution in alcohol was slowly added into the CMCTS-g-(PAANa-*co*-PVP) solution and stirred for a certain period of time as shown in Fig. 2d. The reaction mixture was concentrated by evaporation under reduced pressure and then soaked in *n*-heptane for 5 h. The suspension solution was filtrated and the residue was washed with ether twice and dried in vacuum to achieve CMCTS-g-(PAANa-*co*-PVP)-I_2. The detailed reaction conditions are showed Fig. 2.

2.4. Characterization of CMCTS-g-(PAANa-co-PVP) and CMCTS-g-(PAANa-co-PVP)-I_2

FTIR spectra were recorded on a NEXUS-470 FTIR spectrometer (Nicolet Co., USA) over 200 scans at a resolution of 3 cm^{-1} with KBr pellet samples.

The UV–Vis absorption spectra of CMCTS-g-(PAANa-*co*-PVP)-I_2 in water at room temperature was recorded on a 2102 PC UV–Vis spectrophotometer (Unico Co., USA) with deionized water as a reference.

Wide angle X-ray diffraction (XRD) spectra were measured with an X'pert Pro MPD type X-ray diffractometer (PaNalystical Co., Holland) in the scanning range of 12–60°.

Thermogravimetric analysis (TGA) was conducted on a TA 2000 thermogravimetric analyzer (Dupont Co., USA) at a heating rate of 15 °C/min with nitrogen as the purge gas.

XPS measurements were performed on an Φ 5300 X-ray photoelectron spectroscopy (Perkin-Elmer Physical Electronics Co., USA) under a vacuum $<10^{-7}$ Pa (7.5×10^{-10} Torr). MgKa was used as the target (1253.6 eV) and the power was set to 250 W (12.5 kV at 20 mA).

The activated iodine content in CMCTS-g-(PAANa-*co*-PVP)-I_2 was determined by a titration method as described in the literature [17]. Briefly, 1 g precisely weighted CMCTS-g-(PAANa-*co*-PVP)-I_2 was dissolved in 200 mL of deionized water and titrated with 0.1 mol/L $Na_2S_2O_3$ aqueous solution. One milliliter $Na_2S_2O_3$ titrant is equal to 12.69 mg of the activated iodine.

2.5. Inactivation of CMCTS-g-(PAANa-co-PVP)-I_2 to herpes simplex virus

Frozen Vero cells were rapidly thawed in a 37 °C water bath, washed with cell culture medium and transferred to cell culture bottles contained 10 mL of cell culture medium. The cell culture bottles were put in a 37 °C CO_2 incubator with 5% CO_2. After a monolayer of cell was grown in the bottle, 0.25% trypsin was added to the cell culture medium to release the cells. The released cells were diluted to 2×10^4/mL and 0.1 mL cell suspension was seeded to the wells of a 96-well culture plate. The culture plate was put in the CO_2 incubator until a monolayer of cells was grown in each well. The culture plate was then kept in low temperature before use.

The frozen herpes simplex virus was quickly thawed in a 37 °C water bath, diluted 10 times with the cell culture medium, transferred to the culture bottle with a monolayer of Vero cell and incubated in the CO_2 incubator until ¾ cytopathic effects on the cells observed. The cultured virus was frozen, thawed and centrifuged. The supernatant was aliquoted and cryo-stored at −80 °C for further use.

CMCTS-g-(PAANa-*co*-PVP)-I_2 solutions with the activated iodine contents of 16×10^{-3} mg/mL, 24×10^{-3} mg/mL and 32×10^{-3} mg/mL were diluted with DMEM cell culture medium. Each solution (1.8 mL) was added into a sterile test tube and then 0.2 mL of virus supernatant was added into each tube and mixed well. At the time intervals of 5 min, 15 min, 30 min, 24 h, 48 h and 72 h, 0.1 mL of the mixture was taken and neutralized with 0.9 mL neutralizer (0.1% $Na_2S_2O_3$ in DMEM culture medium) for 10 min. The titer of the virus was tested by a dilution method. Briefly, the neutralized solution was diluted 10 times with DMEM cell culture medium and 0.1 mL of the solution was