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Article

Zn^{2+}-Loaded Cellulose Beads Stabilized by Chitosan and Prepared via Freeze-Drying for Removing Human Testosterone in Plasma

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Abstract: Immobilized metal ion affinity adsorbents have been widely used in separation technique to purify proteins. Due to the leakage of metal ion from the adsorbents, there is no metal ion affinity adsorbent for hemoperfusion has been applied to clinical trial. In this study, in order to prevent the leakage of Zn^{2+} loaded from cellulose beads based adsorbent, improve its stability and adsorption capacity for testosterone, Freeze-drying method was used to enhance the porosity of cellulose beads, improve the surface area of the cellulose beads and adsorption capacity for testosterone. Chitosan was used to coat the adsorbents for preventing the leakage of Zn^{2+} loaded and improve the adsorbent's stability. Moreover, the factors affecting adsorption ability and some components in plasma were also investigated. The results indicate the adsorption ability of the adsorbent can be significantly improved by freeze-drying. After the adsorbent was coated with 0.02% chitosan solution, the highest adsorption percentage reached 48%. During adsorption, the Zn^{2+} concentration in plasma did not rise. In addition, the adsorption percentage for total proteins was below 15%. The results may be caused by the pore size and surface area of the adsorbent enlarged via freeze-drying, and the chitosan solution went into the pores and coated the outer and inner surface of the adsorbent. The adsorbent has a potential clinical application to remove testosterone in patients with recurrent and metastatic prostate cancer.

Keywords: testosterone; cellulose bead; chitosan; coating; zinc ion; freeze drying; adsorbent

1. Introduction

Prostate cancer is one of the most common malignant tumor threatening old age man worldwide [1-8]. Research has demonstrated that high concentration of testosterone in human plasma is the most important factors causing prostate cancer. Decline the testosterone level in human blood can effectively alleviate the syndrome of patients, therefore, Androgen-Deprivation Therapy (ADT) has been regarded as a routine therapy method [4]. But it can cause serious side effects, such as reduced or absent libido (sexual desire); erectile dysfunction; hot flashes; gynaecomastia; osteoporosis; anaemia; decreased mental acuity; loss of muscle mass; weight gain; fatigue; depression [1-3,6]. In addition, recent research indicated that ADT can increase risk of cardiovascular disease and diabetes. Due to intermittent ADT (IADT) can improve the tolerability of treatment [4], The European Association of Urology (EAU) recently acknowledged that this new

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therapy method should no longer be considered an experimental therapy [6]. Adsorbent for hemoperfusion has been widely applied to treat autoimmune diseases due to it can specifically remove the pathogens existing in human blood without serious side effects. Furthermore, compared with medical or surgical ADT treatment, the treatment of hemoperfusion based adsorbent can remove testosterone secreted not only by the testes but also by other tissues [9,10]. The development of adsorbent is important for the treatment of hemoperfusion to remove human testosterone in plasma. There are binding sites for Zn^{2+} in the surface of SHBG and albumin [11,12]. Our preliminary investigation indicates the immobilized metal ion affinity adsorbent can adsorb human testosterone in plasma. It is known that immobilized metal ion affinity adsorbents are widely used in separation technique to purify proteins. Some researchers attempt to prepare the absorbent, such as Cu^{2+} loaded porous chitosan (CS) particles [13], to adsorb the protein in plasma. Unfortunately, the leakage of metal ion from the adsorbents hindered its further application in clinical trials [14,15]. In order to resolve this problem and improve adsorption capacity for testosterone, freeze-drying method was used to enhance the porosity of cellulose (CA) beads; chitosan was used to coat the adsorbents for preventing the leakage of Zn^{2+} loaded and improve the adsorbent's stability. Experimental results demonstrated that the adsorption ability for testosterone can be significantly improved via freeze-drying and chitosan coating membrane can prevent the leakage of Zn^{2+} loaded. The adsorbent coated with 0.02% chitosan solution has the highest adsorption percentage reached 48%. Moreover, all the factors affecting the stability and adsorption capacity for testosterone were also investigated in detail, experimental results show that the adsorbent we synthesized is potential to be applied to clinical trial.

2. Materials and Methods

2.1. Materials

Chitosan (89.4% deacetylated, Mw: 702 kDa) was purchased from Aladdin Industrial Corporation (Shanghai, China). Diethylenediamine and zinc sulfate heptahydrate were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Epichlorohydrin was purchased from Lingfeng Chemical Reagent Co. Ltd (Shanghai, China). All other reagents are analytical grade.

In the study, ethical approval was given by the Medical Ethics Committee of Shiyan Renmin Hospital (reference number 2017-005). Written consent for participation was obtained from volunteers before participation. Plasma was collected from several male volunteers aged between 18 and 60 years old in Shiyan Renmin Hospital, whose routine blood tests were not seen significantly abnormal. Then the plasma samples were mixed, sub-packed in EP tubes, and stored at -20 ℃ until being used.

2.2. Preparation of cellulose beads based Zn^{2+}loaded adsorbent with chitosan membrane coating (Zn^{2+}-CA^{cs}_{fd} beads)

In this paper, Zn^{2+} is selected as ligand. Cellulose bead is used as carrier. Between Cellulose bead and Zn^{2+}, diethylenediamine is introduced as spacer. The freeze-drying is used to enhance the adsorption capacity of the adsorbent. Chitosan is used to coat the adsorbent and improve the stability of Zn^{2+}, the schematic diagram of synthesis route is shown in Figure 1.

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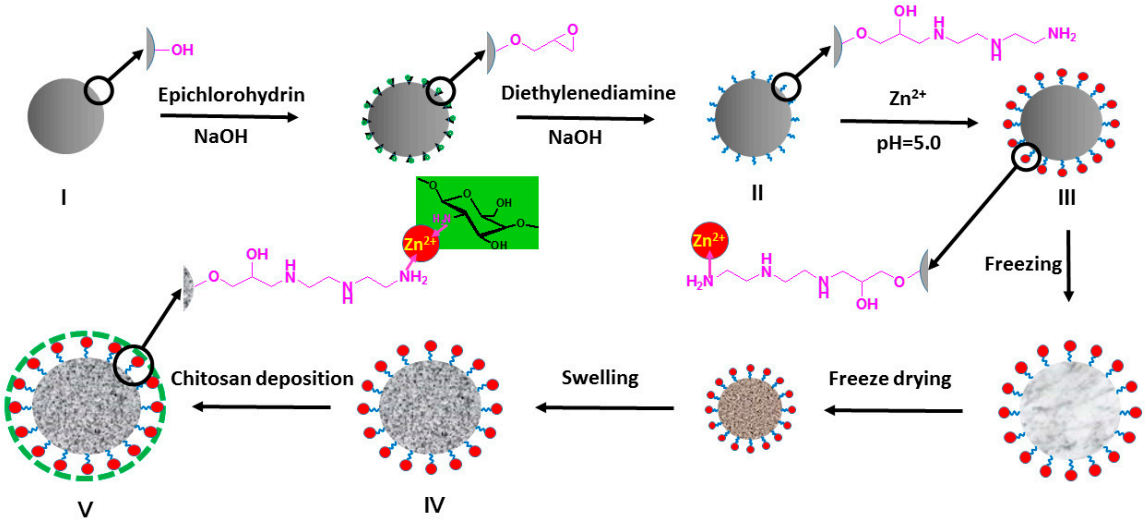

Figure 1. Schematic diagram for preparation process of the adsorbent. Ⅰ: cellulose bead; Ⅱ: diethylenediamine-cellulose bead; Ⅲ: Zn^{2+}-cellulose bead (Zn^{2+}-CA bead); Ⅳ: Freeze-dried Zn^{2+}-cellulose bead (Zn^{2+}-CA_{fd} bead); Ⅴ: Zn^{2+}-CA_{fd} bead coated with chitosan (Zn^{2+}-CA_{fd}^{cs} bead).

2.2.1. Preparation of Zn^{2+} loaded cellulose adsorbent and frozen-dried Zn^{2+} loaded cellulose adsorbent

The epoxidized cellulose beads were prepared by reaction with epichlorohydrin according to a previously published procedure [16]. 1.0 mL epoxidized cellulose bead was suspended in admixture solution with 3 mL of diethylenediamine and 18 mL of NaOH (0.125 M) and shaken for 4 h at 65 ℃. Afterwards, the beads were collected, washed with deionized water until neutrality, and the diethylenediamine-cellulose beads were obtained. Exactly 1 g diethylenediamine-cellulose bead was incubated with 24 mL of 10 mg·mL^{-1} Zn^{2+} solution using zinc sulphate as the source of Zn^{2+} for 1.5 h at 40 ℃. After washed with deionized water till no Zn^{2+} in eluent, which means Zn^{2+} has been loaded on the cellulose beads successfully, (abbreviated Zn^{2+}-CA beads). After isolated, Zn^{2+}-CA beads was frozen at -20 ℃ to form ice crystals, and transferred to freeze-dryer to remove the water in the beads at -50 ℃ over night. The freeze-dried beads (abbreviated Zn^{2+}-CA_{fd} bead) were swelled to normal size by immersing them into normal saline solution for 15 min. Finally, the Zn^{2+}-CA beads and Zn^{2+}-CA_{fd} beads were collected and stored at 4 ℃ until being used.

2.2.2. Coating Zn^{2+}-CA_{fd} beads with chitosan

In order to improve the stability and adsorption capacity of Zn^{2+}-CA_{fd} beads, chitosan was coated on the surface of Zn^{2+}-CA_{fd} beads by phase inversion technique referring to previously reported researches [17,18]. The product is abbreviated as Zn^{2+}-CA_{fd}^{cs} beads. Briefly, chitosan powder was added into acetic acid solution (2%, v/v), and stirred for 30 min at room temperature. Zn^{2+}-CA_{fd} beads (1.5 g) were mixed with 4.5 mL the obtained solution and stirred for 12 h. Thereafter, the beads were isolated by the sieve (40 mesh) to remove soluble substances and added into a flask with 15 ml deionized water. The mixed suspension was regulated to pH 8.0 with NaOH solution (5%, w/v) and stirred for 2 h. The Zn^{2+}-CA_{fd}^{cs} beads were collected and washed with deionized water until neutrality, respectively.

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2.3. Characterization

2.3.1. FTIR-ATR spectra

A series of freeze-dried beads were measured by FTIR-ATR. FTIR-ATR spectra were recorded with spectrometer (VERTEX 70, Bruker, Germany) scanning from 4000 cm^{-1} to 450 cm^{-1} at room temperature.

2.3.2. Thermogravimetric analysis-differential scanning calorimetry (TG-DSC)

TG-DSC was used to determine the content of water and polymer and conducted by STA449F3 Jupiter (Netzsch, Germany) in a nitrogen atmosphere at a heating rate of 10 ℃·min^{-1} from 40 ℃ to 800 ℃. The wet-state beads were used during TG-DSC.

2.3.3. Field emission- Scanning electron microscopy (FE-SEM) with energy dispersive spectroscopy (EDS) analysis

The freeze-dried beads were coated with a thin layer of gold powder. The morphologies of beads were observed by FE-SEM (FEI Nova NanoSEM 450, Netherlands). The energy profiles of carbon, oxygen, nitrogen, and zinc distributed in the beads were analysed by EDS attached to the FE-SEM.

2.3.4. Confocal microscopy

Fluorescein thiocyanate(FITC)-labelled chitosan was synthesized according to the previous method [19]. The FITC-labelled chitosan was used to prepare Zn^{2+}-CA_{fd}^{cs} beads by the same method as described above. The distribution of FITC-labelled chitosan on Zn^{2+}-CA_{fd}^{cs} beads was observed by confocal laser scanning inverted microscope (CLSM) (Zeiss Axio Observer; Zeiss, Göttingen, Germany).

2.3.5. Elements analysis

Elemental analysis (Vario Micro cube, Elementar, Germany) was performed to determine the content of carbon, hydrogen and nitrogen in the synthetic process of Zn^{2+}-CA_{fd}^{cs} beads. Freeze-dried beads were used during elemental analysis.

2.3.6. Determination of ligand contents

The amino group content of diethylenediamine-cellulose beads was determined by acid-base titration [20]. Spectrophotometric determination (LabRAM HR800, Horiba JobinYvon, France) was used with zincon for determination of Zn^{2+} in the solution [21]. The amount of loaded Zn^{2+} was calculated from the amount change before and after incubation and the weight of beads.

2.3.7. Adsorption experiments

As described in a previous article [22], 1 g wet-state bead was incubated with 3 mL plasma and stirred for 2 h at 37 ℃. Plasma samples were collected at 0, 15, 30, 45, 60, 90, and 120 min intervals, respectively. The total testosterone concentration was determined by an ELISA KIT (Human T ELISA KIT, MLBIO, China). The Zn^{2+} concentration was determined by a Zinc assay (Nanjing Jiancheng Bioengineering Institute, China). The total proteins concentration was determined by a Total protein quantitative assay kit (Nanjing Jiancheng Bioengineering Institute, China). Adsorption capacity and adsorption percentage were calculated according to the following equations:

$$AC = [([C]_B - [C]_A)V_P]/M \quad (1)$$

$$AP = ([C]_B - [C]_A)/[C]_B \times 100\% \quad (2)$$

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where AC and AP stand for adsorption capacity and adsorption percentage, respectively; $[C]_B$ is testosterone concentration before adsorption, $[C]_A$ is testosterone concentration after adsorption, V_P is volume of incubated plasma, M is mass of wet-state adsorbent.

2.4. Statistical analysis

All data were expressed as mean ± SD and analysed for statistical significance using Student's *t*-test. *P*-values less than 0.05 were considered to be significant.

3. Results and Discussion

3.1. Synthesis and characterization

The Zn^{2+}-CA^{cs}_{fd} beads were synthesized according to the route shown in Figure 1. Cellulose is a natural polymer, which shows good mechanical strength, blood compatibility, and cytotoxicity [16]. Diethylenediamine has been used to adsorb low density lipoprotein as spacer [23]. Zn^{2+} is an indispensable nutrition microelement for human body. Therefore, these materials were used to synthesize the adsorbent. Freeze-drying was used to increase the adsorption percentage of the adsorbent due to it can enhance pore size. In order to improve the adsorbent stability, the adsorbent was coated with a chitosan membrane. The chitosan has good haemocompatibility and amino groups that could chelate directly with Zn^{2+}. During the synthetic process, a series of beads with different compositions were characterized by the technique described above, respectively. Results indicate that the amino group content of wet-state diethylenediamine-cellulose bead was 228.9 μmol·g^{-1}. The amount of chelated Zn^{2+} on wet-state diethylenediamine-cellulose bead was 124.5 μmol·g^{-1}.

FTIR-ATR spectra of cellulose beads, diethylenediamine-cellulose beads, Zn^{2+}-CA beads, and Zn^{2+}-CA^{cs}_{fd} beads are shown in Figure 2. The absorption band at approximately 1560 cm^{-1} is assigned to the bending vibration of amino group. In comparison with the spectrum of cellulose beads, the band at 1560 cm^{-1} for diethylenediamine-cellulose beads is relatively stronger, which indicates that diethylenediamine reacted with epoxidized cellulose beads and gave rise to abundant amino groups (see Figure 2a and b). The FTIR spectrum of Zn^{2+}-CA beads shows a decrease in intensity of the amino group at 1560 cm^{-1}, indicating that a complex band of amino group and Zn^{2+} is formed (Figure 2c). In comparison with the spectra of Zn^{2+}-CA beads, the band at 1560 cm^{-1} for Zn^{2+}-CA^{cs}_{fd} beads was stronger. The slight change suggests that chitosan has been coated on Zn^{2+}-CA_{fd} beads, which strengthens amino group peak. All the changes show that Zn^{2+}-CA^{cs}_{fd} beads have been prepared successfully.

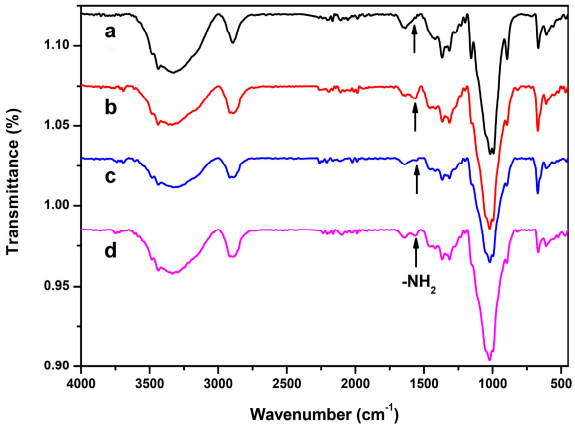

Figure 2. FTIR-ATR spectra of (a) cellulose beads, (b) diethylenediamine-cellulose beads, (c) Zn^{2+}-CA beads, and (d) Zn^{2+}-CA^{cs}_{fd} beads.

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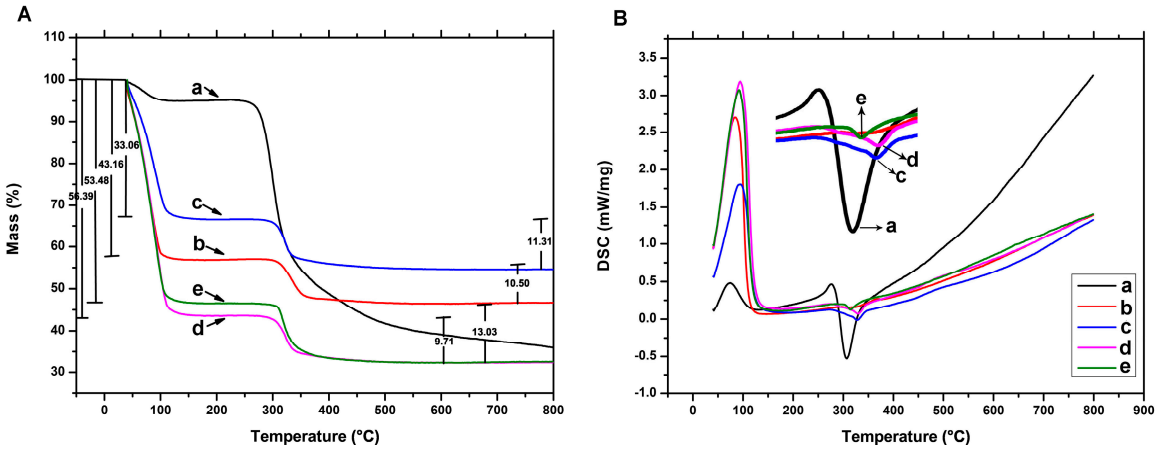

Figure 3. TGA curves (A) and DCS thermograms (B) of (a) chitosan powder, (b) cellulose beads, (c) Zn^{2+}-CA beads, (d) Zn^{2+}-CA$_{fd}$ beads and (e) Zn^{2+}-CA$_{fd}^{cs}$ beads.

TGA curves and DCS thermograms are shown in Figure 3. The TGA curves of Zn^{2+}-CA$_{fd}$ beads and Zn^{2+}-CA$_{fd}^{cs}$ beads show the weight loss is 9.71% and 13.03% during 300-350 ℃, respectively. The results indicate that the polymers content of the beads is increased by 3.32 %, which may be caused by the chitosan membrane.

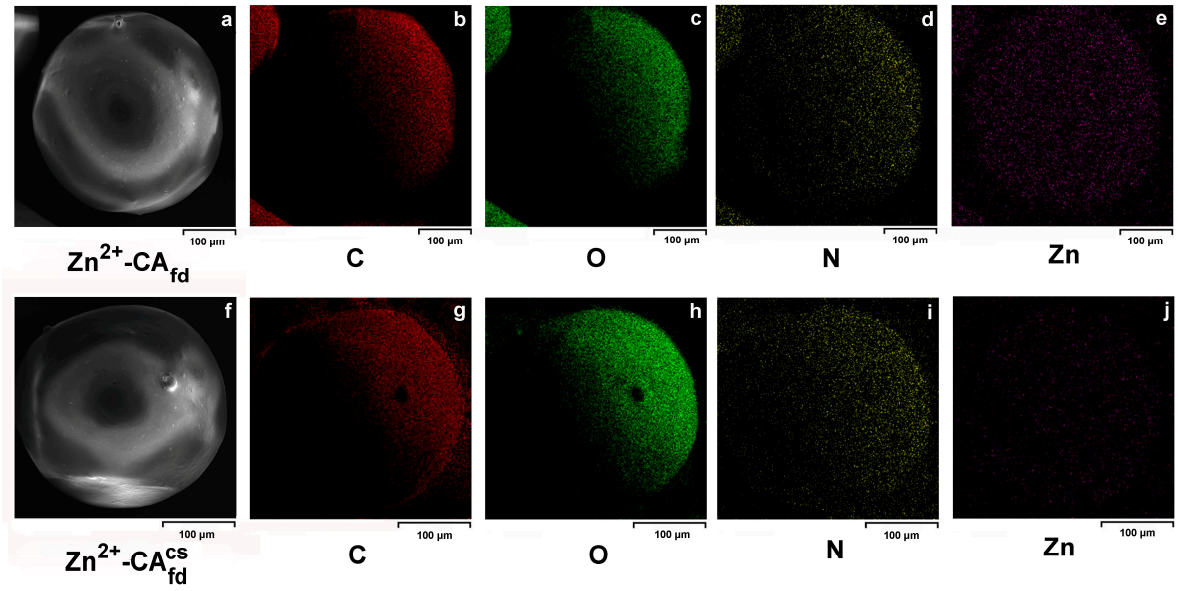

Figure 4. FE-SEM and EDS images of Zn^{2+}-CA$_{fd}$ bead and Zn^{2+}-CA$_{fd}^{cs}$ bead.

Figure 4 shows the FE-SEM and EDS images of Zn^{2+}-CA$_{fd}$ bead and Zn^{2+}-CA$_{fd}^{cs}$ bead. The FE-SEM images confirm that the Zn^{2+}-CA$_{fd}$ bead was shrunk in shape after freeze-drying, which makes the size of bead smaller. The EDS images demonstrate the uniform distribution of carbon, oxygen, nitrogen, and zinc on the surface of the Zn^{2+}-CA$_{fd}$ bead and Zn^{2+}-CA$_{fd}^{cs}$ bead, respectively.

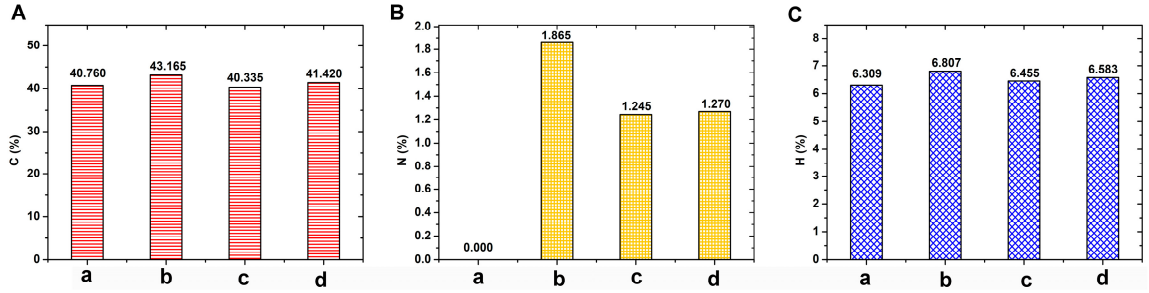

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Figure 5. Elemental analysis of (A) C, (B) N, and (C) H for (a) cellulose beads, (b) diethylenediamine-cellulose beads, (c) Zn^{2+}-CA_{fd} beads and (d) Zn^{2+}-CA_{fd}^{cs} beads, respectively.

Figure 5 shows the contents of C, N, and H for cellulose beads, diethylenediamine-cellulose beads, Zn^{2+}-CA_{fd} beads and Zn^{2+}-CA_{fd}^{cs} beads, respectively. Compared diethylenediamine-cellulose beads with cellulose beads, the contents of C, H and N increase in diethylenediamine-cellulose beads, which indicate that diethylenediamine has been coupled with the cellulose beads. Compared Zn^{2+}-CA_{fd} beads with diethylenediamine-cellulose beads, the contents of C, H and N decrease in Zn^{2+}-CA_{fd} beads. The reason is that the increment of Zn^{2+} content lead to the decrease of C, H and N percentage content relatively. Compared Zn^{2+}-CA_{fd} beads with Zn^{2+}-CA_{fd}^{cs} beads, the contents of C, H and N in Zn^{2+}-CA_{fd}^{cs} beads increase relatively again. The result demonstrate Zn^{2+}-CA_{fd}^{cs} beads are coated with chitosan successfully.

3.2. Effect of freeze-drying and membrane coating on adsorption capacity for testosterone

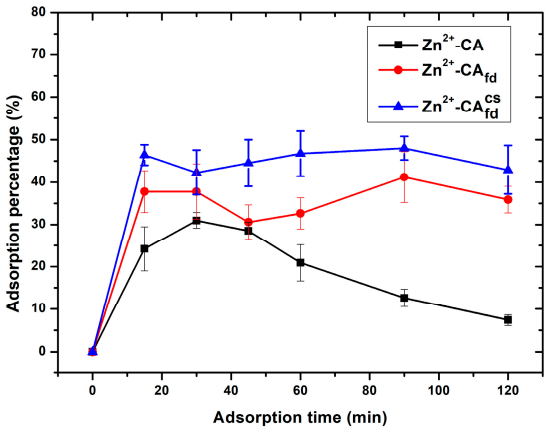

Figure 6. Effect of freeze-drying and membrane coating on adsorption percentage for testosterone in plasma (n=3).

The absorption results for testosterone are shown in Figure 6. The adsorption percentage for testosterone by wet-state Zn^{2+}-CA_{fd} beads is more than that by Zn^{2+}-CA beads. The maximum adsorption percentage of wet-state Zn^{2+}-CA_{fd} beads increase about 11%. The results suggest that after freeze-dried and swelled, the adsorption ability of the adsorbent can be significantly improved. The adsorption percentage of Zn^{2+}-CA_{fd}^{cs} beads (0.02% chitosan solution) for testosterone is higher than that of wet-state Zn^{2+}-CA_{fd} beads.The results can be ascribed that chitosan membrane improve Zn^{2+} ligands stability and its ability for chelating with SHBG. After 120min adsorption, the Zn^{2+} contents in plasma using Zn^{2+}-CA beads and wet-state Zn^{2+}-CA_{fd} beads as adsorbents are shown in Figure 7, respectively. The Zn^{2+} concentrations in plasma increase more than two times compared to Zn^{2+}-CA_{fd}^{cs} beads as adsorbent.

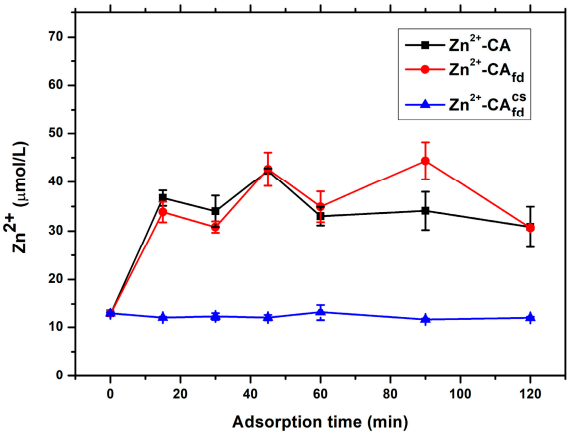

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Figure 7. Effect of Zn^{2+}-CA beads, wet-state Zn^{2+}-CA_{fd} beads, and Zn^{2+}-CA_{fd}^{cs} beads (0.02% chitosan) on Zn^{2+} concentration in plasma (n=3).

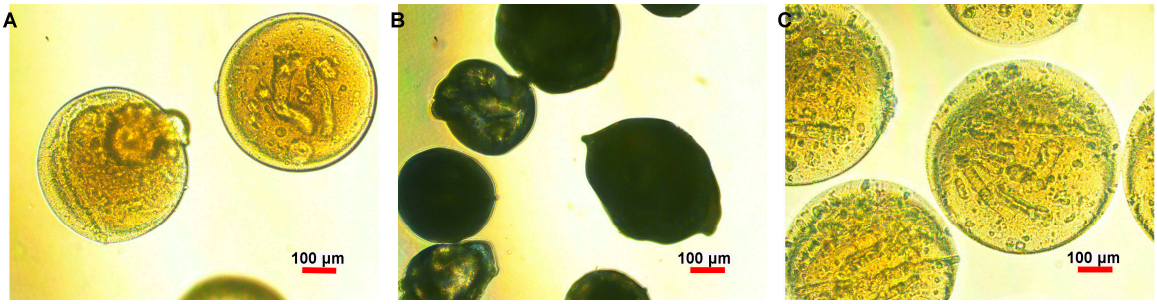

Figure 8. Microscope images of (A) Zn^{2+}-CA beads, (B) Zn^{2+}-CA_{fd} beads, (C) wet-state Zn^{2+}-CA_{fd}^{cs} beads.

Freeze-drying is a representative technique to prepare porous material[24]. Although it can destroy the structure of bead, freeze-drying has some advantages, such as fast and cost-effective[25]. Considering cellulose beads have very good swelling behavior [26] and freeze-dried beads at wet state are easy to be coated by chitosan. After freeze-dried, the beads can shrink in shape (Figure 8B). When it was added into normal saline solution, the freeze-dried beads soon restore their original shape, as shown in Figure 8A and Figure 8C. From Figure 3A, in comparison with Zn^{2+}-CA beads, the water content of wet-state Zn^{2+}-CA_{fd} beads increase by 23.33%. The result suggests pore size of the adsorbent become bigger after freeze-drying and more water was adsorbed. It can cause increment of the adsorption percentage for testosterone.

3.3. Effect of different concentration of chitosan solution on the adsorption capacity of wet-state Zn^{2+}-CA_{fd}^{cs} beads for testosterone

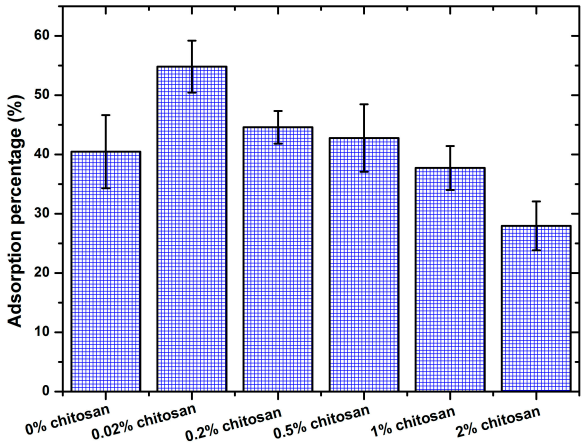

Figure 9. Adsorption percentage of wet-state Zn^{2+}-CA_{fd} beads coated by different concentration of chitosan at 90 min (n=3).

A series of Zn^{2+}-CA_{fd}^{cs} beads were prepared with 2%, 1%, 0.5%, 0.2%, 0.02% and 0% (w/v) chitosan solution by the method described above. The adsorption percentage for testosterone at 90 min are presented in Figure 9. The results show Zn^{2+}-CA_{fd}^{cs} beads (0.02% chitosan solution) had best adsorption capacity for testosterone. The optimal adsorption percentage for testosterone is about 48% at 90 min and the adsorption capacity for testosterone is 0.0176 nmol·g^{-1}.

In order to investigate the reasons behind the above interesting phenomenon, FITC-labelled chitosan was synthesized to prepare Zn^{2+}-CA_{fd}^{cs} beads with 2%, 1%, 0.5%, 0.2%, and 0.02% (w/v) chitosan solution. In Figure 10a-e, a layer with bright green fluorescence appeared on outer surface of Zn^{2+}-CA_{fd}^{cs} beads and gradually became weaker with the decrease of chitosan concentration.

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Simultaneously, FITC-labelled chitosan diffused from outer to inner. When 0.02% (w/v) chitosan solution was used to coat Zn^{2+}-CA_{fd} beads, the layer with bright green fluorescence disappeared and FITC-labelled chitosan uniformly distributed on outer and inner surface.

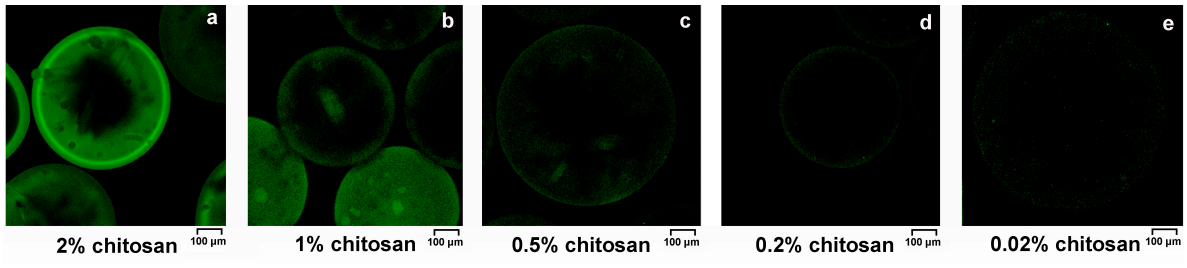

Figure 10. CLSM images of distribution of FITC-labelled chitosan on wet-state Zn^{2+}-CA_{fd} beads.

Note: (a-e) CLSM images in the cross-section of wet-state Zn^{2+}-CA_{fd}^{cs} beads

These images suggest that wet-state Zn^{2+}-CA_{fd}^{cs} beads were coated with chitosan solution (0.02%, w/v) without impact on the porosity. The pores on the surface still existed (as shown in Figure 11A). The chitosan solution can coat the inner surface from the pores. Uniform chitosan layer was formed on the outer and inner surface, which may cause the fact that Zn^{2+}-CA_{fd}^{cs} beads (0.02% chitosan solution) had best adsorption capacity for testosterone (as shown in Figure 11B).

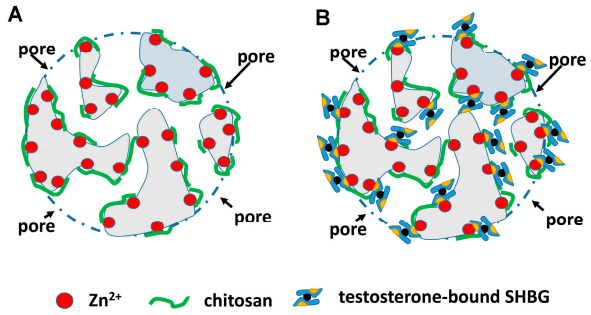

Figure 11. Schematic diagram of Zn^{2+}-CA_{fd}^{cs} beads (0.02% chitosan). (A) before adsorption; (B) after adsorption.

3.4. Effect of the adsorbent on plasma total proteins adsorption

The optimal Zn^{2+}-CA_{fd}^{cs} beads (0.02% chitosan solution) were used to test the non-specific adsorption, results show that adsorption percentage for plasma total proteins was 12.74% at 90 min (P=0.021) (as shown in Figure 12), which indicates the adsorbent has better stability and selectivity [22].

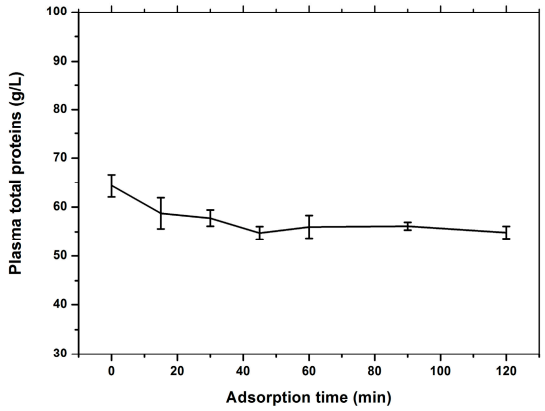

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Figure 12. Effect of optimal Zn^{2+}-CA_{fd}^{cs} beads on total proteins concentration in plasma (n=3).

3.5. Storage stability

As shown in Figure 7, the Zn^{2+} concentration in plasma decreased from 12.88 μM to 11.62 μM, about 9.78 % (P=0.027) at 90 min. The optimal Zn^{2+}-CA_{fd}^{cs} beads (0.02% chitosan solution) were stored at 4 ℃ in refrigerator, the adsorption capacity for testosterone at 0, 2, 4, 8, 12 weeks were measured. No significant decrease in adsorption capacity was observed after 12 weeks of storage at 4 ℃.

4. Conclusions

In this paper, loaded Zn^{2+} cellulose beads with a chitosan layer were prepared for the removal of human testosterone in plasma. The factors affecting adsorption ability and some components in plasma were investigated. The adsorption ability of the adsorbent can be significantly improved by freeze-drying and coating. During adsorption, the Zn^{2+} concentration in plasma doesn't rise. In addition, the adsorption percentage for total proteins is below 15%. The adsorbent has a potential clinical application for androgen deprivation therapy to remove testosterone in patients with recurrent and metastatic prostate cancer. In addition, the method originated from this work may be useful for other similar materials to improve their adsorption ability and stability.

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Author Contributions: Huibin Yu, Shenqi Wang and Hongqin Ke conceived and designed the experiments; Huibin Yu, Xing Li and Shenglong Tan performed the experiments; Huibin Yu analyzed the data; Tu Chen and Hongqin Ke had significant contribution in collection for plasma; Huibin Yu, Shenqi Wang and Lei Zhou wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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