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Influence of the Deacetylation Degree on the *In Vivo* Distribution of Chitosan in Mice

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SUMMARY. **Deacetylation degree (DD) is one of the most important characteristics of chitosan and has great impact on its physicochemical and biological properties. In this study, three kinds of chitosan with different DD were prepared. By means of living imaging technique and fluorescent quantitative technique, the influences of different DD on the distribution of chitosan *in vivo* were investigated. The results showed that chitosan with molecular weight between 20,000 and 26,000 mainly distributed in kidney after intravenous injection. The influences of DD on the distribution of chitosan in kidney was obvious. The renal distribution of chitosan with 38, 51, and 61% DD achieved the maximum concentration in 60, 30, and 60 min, respectively. The concentration of chitosan with 51% DD was much higher than that of chitosan with 38% and 61% DD, which suggested that the chitosan with 51% DD might obtain better kidney targeting capacities and be considered as a better kidney targeting drug delivery carrier.**

INTRODUCTION

Low-molecular-weight drugs play an important role in disease prevention, but great side effects may be accompanied with some of them. The plasma concentration of them is usually higher than their therapeutic concentration in a short time after administration,which may cause many side effects. Meanwhile, by virtue of the rapid biological metabolism rate, short half-life time and being easily excreted, the drug concentration reduces much faster and ultimately affects the drug efficacy. On the other hand, low-molecular-weight drugs cannot be selectively targeted to the specific tissue, which leads to high dosage, low efficacy and severe side effects. For this reason, advanced drug delivery systems to improve drug efficacy has attracted pharmacy scientist's attention [1]. One particular approach towards an improved use of drugs for therapeutic applications is the design of polymeric prodrugs or polymer-drug conjugates. Researches and applications of polymeric prodrugs have been gradually concerned by researchers. Polymeric prodrugs possess two extremely outstanding characteristics: prolongation of action of the drug and targeting [2]. A suitable polymeric carrier is essential for the designation of polymeric prodrugs, which should be required with the following basic properties: biodegradability, low toxicity, non-immunogenicity, non-accumulation, obtaining appropriate functional groups to link small molecular drugs, being able to protect the original properties of drugs and release the active compounds to the target sites [2-4]. Therefore, the characteristics of polymer have great influences on the prodrugs.

As the unique natural alkalescence amino glucidamin, chitosan's superordinary physicochemical and biological properties make it to be widely concerned [5]. Chitosan is biological degradable and non-toxicity [6], consequently, it is also widely used in pharmaceutical areas [7-9]. Chitosans are the fully or partially N-deacetylated derivatives of the chitin polymers, and the

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chemical and biochemical properties of chitosan depend heavily on the degree of deacetylation (DD). *i.e.* the average number of D-glucosamine units per 100 monomers expressed as a percentage [10]. The DD is one of the most important chemical characteristics as it influences the performance of chitosan in many of its applications [11]. Recently, microparticle drug delivery system and macromolecular prodrugs using chitosan as a drug carrier have received quick growth [12-14]. It had been found that the conjugation of methotrexate and chitosan with the partially deacetylated demonstrated very slow and incomplete degradation *in vivo* [15]. This proposed that chitosan might accumulate in some special organs of the body. The *in vivo* distribution of chitosan is of significant importance to chitosan as a targeting carrier. Recently, some scientists had in succession studied the *in vivo* distribution of chitosan after peritoneal injection, subcutaneous injection, and even oral administration [16-19]. To further demonstrate the *in vivo* distribution of chitosan and the influences of DD on the distribution, in this work chitosan with different DD were prepared, then its *in vivo* distribution was studied after intravenous administration in mice according to Onishi & Mashida [16] and Kamiyama *et al.* [20]. Meanwhile, effects of different DD on the distribution of chitosan in mice were also investigated in depth. We hope it can be used for a helpful reference to the research of chitosan as a targeting carrier.

MATERIALS AND METHODS

Materials

RF-5301 fluorospectrophotometer (Shimadzu, Japan), BP211D electronic analytical balance (Sartorius Stedim Biotech Company Ltd., Germany), magnetic stirrer (Sile instrument Company Ltd., Shanghai, China), Allegen 22R high speed refrigeration centrifuge (Beckman, USA), Axiovert 40 CFC fluorescent microscope (Caisi optical instrument Company Ltd., Shanghai, China), whole-body imaging system (Lightools Research, USA).

Fluorescinisothiocyate (FITC) was purchased from Sigma Chemical Co. (USA). Chitosan (base form, deactylation degree = 95%, Mw = 6.5 × 10^5) was purchased from Shanghai Bo'ao Biochemical Co. Ltd. (China), Buffer bicarbonate was self-prepared and other reagents was all of analytical grade. Chitosan (Mw = 20~26 kDa, 95% deacetylation degree) was obtained from oxidative degradation of chitosan (Mw = 6.5 × 10^5) by H_2O_2. The intrinsic viscosity of the chitosan solutions in aqueous 0.5 M CH_3COOH/0.5 M CH_3COONa at 25 °C was measured using a dilution-type Ubbelohde viscometer [21].

Healthy Kunming mice (6 weeks old), 25-30 g, were provided by West China Experimental Animal Center of Sichuan University (China).

Preparation of chitosan with different deacetylation degrees

Chitosan was dissolved in the aqueous acetic acid, then the mixture of ethanol, pyridine and acetic anhydride was added in the chitosan solution. Acetylation of chitosan with acetic anhydride was carried out in the homogeneous aqueous solution. The water-soluble chitosan with different deacetylation degrees were prepared by controlling the amount of acetic anhydride [22-24]. In brief, 0.5 g low molecular weight chitosan (20~26 kDa) was dissolved in 2.8% acetic acid (12.5 mL). Then 12.5 mL ethanol, 4 mL pyridine and a amount of acetic anhydride were added into the reaction system under a continuous stirring condition.After stirring at room temperature for several hours, a amount of ethanol (chitosan: ethanol = 1:200, w/v) was added into the reaction system to precipitate the low molecular weight chitosan. By centrifugation, the supernatant was discarded and the precipitation was dissolved in deionized water; after centrifuging again, the sediments were discarded. Some quantity of ethanol was added in the supernatant again, and the centrifuging operation was replicated. The precipitate (chitosan) was washed with ethanol, acetone and diethyl ether in succession until neutral. Finally, the product was obtained by rotary evaporation (60 °C, 0.09 MPa) for 3 h and stored in the desiccators for the next applications.

Synthesis of FITC labeled chitosan (FITC-chitosan)

Low molecular weight chitosan (100.25 mg, M_w = 20~26 kDa) was dissolved in $NaHCO_3$ solution (10 mL), pH 8.31, after magnetic stirring for 3 h. Meanwhhile, FITC (5 mg) was also dissolved in NaHCO3 solution, pH 8.31.Afterwards, FITC solution was added into the chitosan solution, and the mixture was protected from light and stirred under the condition of icebath for 24 h. The reaction product was dispersed with ethanol (30 mL) and successively stirred. Much floccular precipitate was separated and more precipitate was obtained if in refrigerator.The mixture was centrifuged at 8000 rpm for 15 min and the supernatant was discarded. The precipitate was washed with ethanol (15 mL) and centrifuged twice until FITC was not detected in the

supernatant. Finally, the precipitate was collected, dissolved with distilled water and then freeze-dried. The final product FITC-Chitosan (106 mg) was put into a glass bottle for protecting from wet and stored in a refrigerator at -20 °C.

The content of the fluoresceinthiocarbamyl moiety (FTC) of FITC-Chitosan was determined at 520 nm with an excitation wavelength at 485 nm using an RF-5301 fluorospectrophotometer (Shimadzu, Japan) [16].

Fluorescence imaging of FITC-Chitosan in mice

Two mg of FITC-Chitosan was precisely weighted and dissolved in physiological saline water 1 mL). FITC-Chitosan solution (0.2 mL) was administered intravenously to Kunming mice (n = 3) at a dosage of 20 mg/kg, and free FITC solution (120 μg/mL) was administered to the control mice (n = 3). Thirty min after administration the mice were sacrificed and the abdominal cavity were unfolded to expose the kidney, then whole body fluorescence imaging of all mice was recorded. Afterwards, the tissue samples including hearts, livers, spleens, lungs and kidneys were collected and the fluorescence imaging of these organs were also recorded. Fluorescence imaging were all performed in macro imaging system LT-9 equipped with illumatool dual light system LT-99D2 (Lightools Research, Encinitas, CA, USA).

In vivo distribution of FITC-Chitosan in mice

Determination of extraction recovery ratios

The extraction recovery ratios of FITC-Chitosan from various organs (heart, liver, spleen, lung and kidney) of mice were first analyzed. Healthy Kunning mice were killed by cervical dislocation. The heart, liver, spleen, lung and kidney were removed, blotted by filter paper and weighted. FITC-Chitosan solutions with concentrations of 1 mg/mL were prepared using saline, and FITC-Chitosan solution was added to the tissue with the same weight. The mixture of the tissue and FITC-Chitosan was homogenized using a glass homogenizer and 1M HCl aqueous solution was added to the obtained homogenate by 9-fold volume, then the samples were centrifuged at 3000 rpm for 5 min after sufficient mixing. The obtained supernatant was used in the final preparation for fluorescence measurement.

The obtained supernatant (0.5 mL) was mixed with 1M NaOH aqueous solution (0.4 mL) and 0.2 M phosphate buffer, pH 6.15, (9.1 mL) using a vortex shaker. The resulting solution was measured at 516 nm with an excitation at 492 nm using the RF-5301 fluorospectrophotometer (Shimadzu, Japan) to determine the fluorescence intensity of FITC-Chitosan in blank tissue (Intsample). For the determination of fluorescence intensity of blank tissue (Int_{blank}) and FITC-Chitosan solution $(Int_{standard})$, The blank tissue samples were made by the addition of physiological saline instead of FITC-Chitosan solution. In the preparation of the standard FITC-Chitosan solution, physiological saline was used instead of the tissue and the other procedures remained the same. Finally the extraction recovery ratios of FITC-Chitosan in various tissues was calculated according to the following equation:

$$Extraction\ recovery\ (\%) = \frac{Int_{sample} - Int_{blank}}{Int_{standard}} \times 100$$

In vivo distribution of FITC-Chitosan after i.v in mice

FITC-Chitosan was dissolved in physiological saline to obtain a concentration of 1mg/mL solution for intravenous (i.v.) administration. Twelve Kunming mice weighing 18 to 24 g were randomly assigned to 4 groups (3 mice per group) and administered the prepared FITC-Chitosan solution with a single dose of 0.1 mL/10 g via tail vein. At each time point (0.25, 0.5, 1, and 2 h), the mice were sacrificed and the heart, liver, spleen, lung and kidney were separated and weighted. The fluorescence intensity of FITC-Chitosan in different organs were determined as described before.

RESULTS AND DISCUSSION

Preparation of chitosan with different DD

The DD of acetylated chitosan was closely interrelated to the dosage of acetic anhydride [25]. The results showed that the DD of chitosan derivative reduced remarkably along with the increase of acetic anhydride amount and an almost good linear relationship also could be found (Fig. 1). However, there were no remarkable influences of the amount of acetic anhydride on the DD of chitosan when a certain amount had reached, which might be relative to the polymer molecular structure of chitosan molecule.When the amount of acetic anhydride was 0.88 and 1.00 mL, DD of two chitosan derivative was almost equal. Along with the continuous acetylation reaction, the insert of acetyl group made the steric hindrance of chitosan increase gradually and finally blocked the

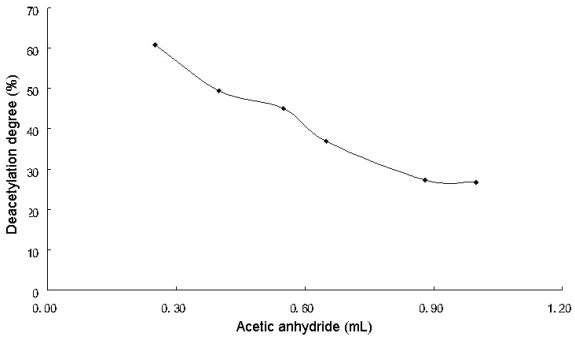

Figure 1. Influences of the acetic anhydride on the deacetylation degree of chitosan.

acetylated reaction. In this experiment, the acetylation of chitosan was controlled by means of the different amount of acetic anhydride. Classic acid-base titration method and ultraviolet spectroscopy were utilized to determine the DD of chitosan [26,27] and the results were in good agreement with each other.

Fluorescence imaging of FITC-Chitosan in mice

Fluorescent whole-body imaging technique was applied to investigate the qualitative distribution of FITC-Chitosan in mice. Figs. 2 and 3 had shown that the kidneys of FITC-Chitosan group exhibited strong fluorescence both *in vivo* and *in vitro,* but the other organs didn't emit obvious fluorescence. By contrast, the organs of FITC group didn't emit significant fluorescence *in vivo* or *in vitro,* which indicated that FITC gathered more into kidney after coupling to chitosan, and chitosan might assemble into kidney after intravenous injection. The results further indicated the possibility of chitosan to be a kidney targeting carrier. Moreover, it was observed that the urine of dead mice in FITC group emitted strong fluorescence, but none was found within the kidney. By virtue of the fact, it could be predicted that the FITC was still excreted through urine after intravenous injection, but the renal tubules showed poor re-absorption to FITC. Therefore, the FITC was observed in urine but not in kidney. However, the FITC could be observed in kidney in FITC-Chitosan group, which might be caused by the re-absorption of renal tubules. As a result, it could be concluded that the mechanism of kidney distribution of FITC-Chitosan might result from the renal tubule re-absorption.

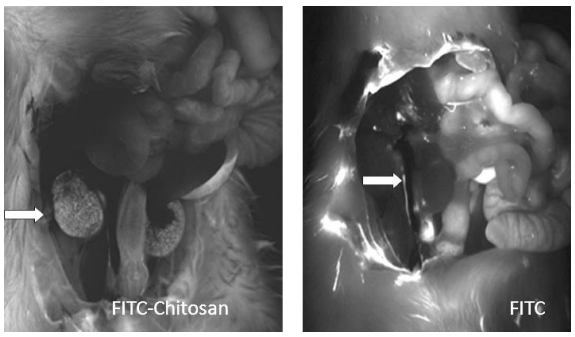

Figure 2. *In vivo* fluorescence imaging of FITC-Chitosan and FITC in mice.

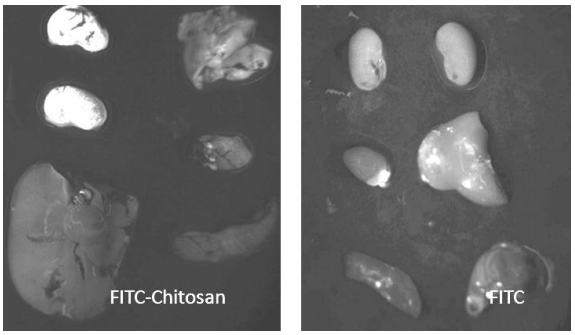

Figure 3. *In vitro* fluorescence imaging of FITC-Chitosan and FITC in mice.

In vivo distribution of chitosan with different deacetylation degrees in mice

The *in vivo* distribution of chitosan with 38, 51, and 61% DD labeled by FITC had been experimented in mice by means of fluorescent quantitation method, to explore the influences of different DD on the distribution of chitosan *in vivo*. In our previous studies, some endogenous substances in the organs could emit fluorescent. Therefore, the extraction recoveries of abodominal organs were studied in order to avoid the influences of organs themselves on the fluorescent quantitation. Results were shown in Table 1. The extraction recoveries of each organ was various, which can be explored that the species and quantity of endogenous substances in different organs were not the same.

The fluorescence intensities of organs after i.v FITC-Chitosan were detected by fluorospec-

Organs	Extraction recovery (%)		
	Chitosan (38% DD)	Chitosan (51% DD)	Chitosan (61% DD)
Heart	82.36	81.52	83.09
Liver	73.68	84.29	72.36
Spleen	92.33	104.03	81.63
Lung	98.83	100.15	95.05
Kidney	90.14	90.56	88.31

Table 1. Extraction recovery of FITC-Chitosan with different DD in different organs.

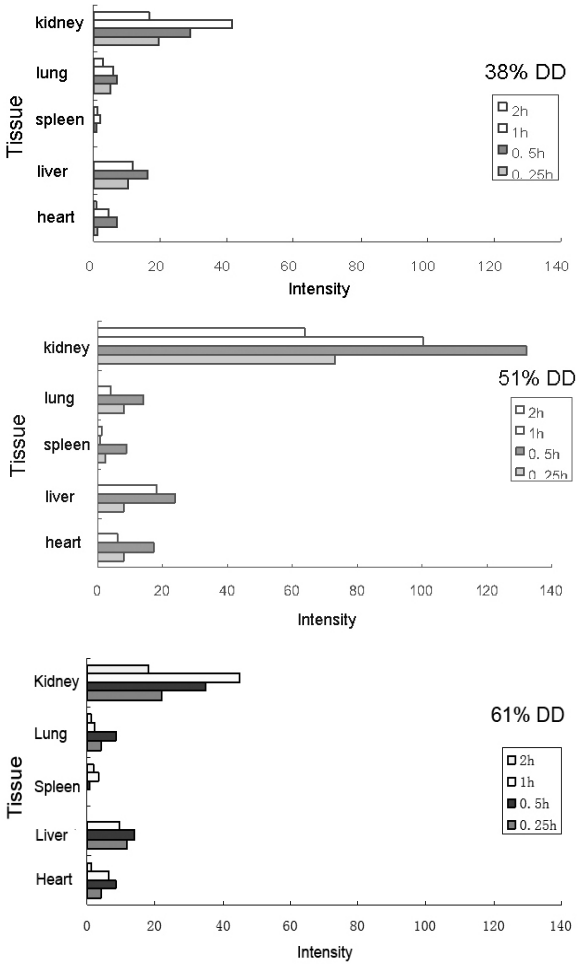

Figure 4. Comparison of fluorescence intensity after i.v. FITC-Chitosan with 38, 51, and 61% DD.

trophotometer. On the base of extraction recovery, the actual fluorescence intensity of each organ was calculated and compared (Fig. 4).

It could be seen from Fig. 4 that obvious fluorescence had been detected in some organs at 0.25 h after intravenous administration of chitosan with 38 ,51, and 61% DD labeled by FITC, which illustrated that FITC-Chitosan conjugates distributed quickly from blood to organs. The maximal fluorescence intensity of the experimental organs reached at 0.5h after intravenous administration of FITC-Chitosan with 51% DD. Meanwhile, after i.v. FITC-Chitosan with 38 and 61% DD, the fluorescence intensity of heart, liver, spleen and lung also reached the maximal value at 0.5 h and the maximal fluorescence intensity happened to kidney at 1 h. From 0.5 to 2 h after i.v. three FITC-Chitosan solution, the fluorescence intensities of kidney were all significantly higher than the other organs, indicating that the content of FITC-chitosan in kidney were much more than the other organs, and the next was in liver. The relative higher fluorescence still could be detected in kidney after 2 h while almost no fluorescence could be observed in other organs except kidney.All these showed that FITC-Chitosan could distribute into kidney quickly after i.v. As time passed, the fluorescence intensity of organs decreased gradually and there was no any fluorescence signal at 2 h after injection except kidney. Moreover, by comparison the fluorescence intensities in kidney, it could be concluded that chitosan with 51% DD distributed into kidney fastest and the fluorescence intensity in kidney was the most among the three chitosans with different DD. These results indicated that the kidney targeting of chitosan with 51% DD could be superior to that of chitosan with 38 and 61% DD (Fig. 5).

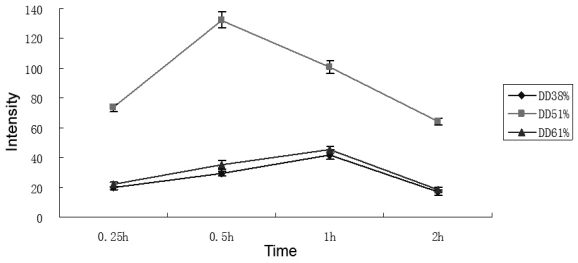

Figure 5. Changes of fluorescence intensities with time in kidney after iv different DD chitosan.

It was assumed that the kidney targeting degree of chitosan was involved with its solubility in water and the quantity of FITC conjugated with chitosan. The correlation of chitosan's solubility in water and its deacetylation degree have been confirmed.When the DD was about 50%, chitosan's solubility in water was the maximal.It was explored that the acetamido (CH_3CONH) group and the amino (NH_2) groups in the structure of chitosan with 50% DD was almost equal,which caused the minimal degree of crystallinity in the chitosan molecule. As a result, Van der Waals force among the chitosan molecule with 50% DD was minimal and this chitosan was easier dissolved in water.So a certain molecular weight of chitosan with 50% DD were easier filtrated through the glomerular and retained in the kidney through the renal tubule re-absorption.

On the other side, in our experiments the kidney targeting of chitosan was studied by means of fluorescent quantitation method. The coupling reaction between FITC and chitosan principally took place in the amorphous region of chitosan, then proceeded from the edge to

the inside of the crystalline region [28]. When the DD was about 50%, the degree of crystallinity in the chitosan was minimal and more FITC were conjugated with chitosan molecule.While the chitosan with 50% DD was targeted in the kidney, it was taken for granted that the fluorescence intensities were higher than the other chitosan with less and more DD.Provided that the FITC molecule were changed to low molecule drug, the drug molecules were probably targeted in the kidney. It can be concluded that the chitosan with 51% DD might obtain better kidney targeting capacities and be considered as a better kidney targeting drug delivery carrier.

According to the results consigned above, FITC-Chitosan distributed into kidney rapidly after i.v, and chitosan with 51% DD could obtain better kidney targeting. Some low molecular agents could be conjugated with chitosan and produce good accumulation and retention in the kidneys after intravenous administration. However, the deacetylation degree of chitosan played an important role in kidney targeting of chitosan. The targeting effect of chitosan with 51% DD was better than that of chitosan with 38 and 61% DD, which was coincided with the result reported by Hiraku Onishi that chitosan with about 50% deacetylation degree obtained good kidney targeting [16]. The mechanism of the influences of deacetylaton degree on kidney targeting still needed to be studied in depth.

CONCLUSION

In this study, the influences of different deacetylation degree on the distribution of chitosan in mice were investigated. Three kinds of chitosan with different DD were prepared by acylation of chitosan, and then *in vivo* distribution of chitosan was studied by living imaging technology and fluorescent quantitative technique. The results showed that three kinds of chitosan (Mw = 20~26 kDa, 38, 51, and 61% DD) mainly distributed in kidney after intravenous injection. The maximum distribution time is 60, 30, and 60 min, respectively. The fluorescence intensity of FITC-chitosan with 51% DD was higher than that of chitosan with 38 and 61% DD, This means that chitosan with 51% DD obtained better kidney targeting capacities it may be a better kidney targeting drug delivery carrier.

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