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Specific Renal Uptake of Randomly 50% *N*-Acetylated Low Molecular Weight Chitosan

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Abstract: In our previous studies, randomly 50% *N*-acetylated low molecular weight chitosan (LMWC) has been confirmed as a potential carrier for the site-specific delivery of prednisolone to kidney, suggesting specific uptake of LMWC in kidney. Interestingly, aminoglycoside, a well-known ligand of megalin receptor, shares a similar glucosamine unit level with LMWC. Based on these, we proposed that the specific renal uptake of LMWC might also be mediated by the megalin receptor. To test this hypothesis, we performed the present study to further investigate the renal uptake process of LMWC and its possible mechanism as well. First, LMWC was found by fluorescent microscopy to selectively accumulate in the kidneys, especially in the renal proximal tubules after iv injection in mice. Then, our research also revealed that LMWC was internalized into renal tubular cells (RTCs) through endocytic pathway, with a concentration-dependent and saturable pattern. The uptake of LMWC could be competitively inhibited in the presence of gentamycin, a kind of aminoglycosides. In addition, cytotoxicity assay showed that there were no obvious effects of LMWC on the viability of L929 and RTCs lines. Finally, megalin-shedding mouse models were established and the distribution of LMWC in tissues of normal and megalin-shedding mice was evaluated. Consistent with gentamycin inhibition assay, *in vivo* results also suggested the role of megalin in the uptake of LMWC in kidney. In conclusion, LMWC could be specifically taken up by RTCs, where the megalin receptor would likely mediate its binding and uptake.

Keywords: Low molecular weight chitosan; renal uptake; megalin receptor; renal targeting carrier; drug delivery

Introduction

The renal uptake or reabsorption of drugs[1−3] and low molecular weight proteins[4−6] mainly occurs in renal proximal tubule cells, known as the most active cells in kidney. Indeed, the internalization of these compounds is mediated by a multiligand endocytic receptor, named megalin. It is a 600 kDa glycoprotein, highly expressed in the endocytic pathway of renal proximal tubule.[7,8] The cytoplasmic tail of megalin contains a NPXY sequence, which is necessary for coated pit-mediated internalization.[9] Mediating endocytosis of a

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large variety of ligands, such as aminoglycoside and low molecular weight proteins, megalin functions as one of the most important receptors in the proximal tubule.

Chitosan is a copolymer of glucosamine and *N*-acetylglucosamine derived from chitin, the second most abundant polymer in nature. Due to excellent biocompatibility and biodegradability, chitosan has been widely used in the biomedical field including drug delivery systems and wound dressings. Over the last decades, chitosan has played a significant role in the development of drug delivery systems, such as specific site delivery,[10−12] controlled drug release[13] and delivery of gene or protein drug.[14,15]

In our previous study, we have explored the potential of randomly 50% *N*-acetylated low molecular weight chitosan (LMWC) as a novel renal targeting carrier for renal specific delivery of prednisolone.[10] Specifically, when prednisolone was conjugated to LMWC with molecular weight of 31 kDa and 19 kDa, it presented a relatively higher renal accumulation rate than other candidates. However, before further applications of LMWC, some key aspects should be addressed, such as the distribution site of LMWC, the internalization process and possible uptake mechanism.

It is worth mentioning that aminoglycoside, a well-known ligand of megalin receptor,[1−3,8] shares a similar glucosamine unit level with LMWC. This leads us to investigate whether LMWC can bind to megalin, which accounts for its specific renal uptake. To test the hypothesis, a megalin-shedding animal model was established and competitive inhibition assay was performed *in vitro*.

In this paper, we mainly focused on the specific uptake of LMWC in kidney. First, we found LMWC could selectively accumulate in the kidneys, especially in the renal proximal tubules. Then an uptake study was carried out, indicating that the renal uptake of LMWC might be a concentration-dependent and saturable endocytic pathway. In addition, *in vivo* study using megalin-shedding mouse models demonstrated that the megalin receptor was probably involved in the uptake process of LMWC in kidney. Based on these, we come to the conclusion that LMWC is specifically taken up by renal tubular cells (RTCs), where the megalin receptor would likely mediate its binding and uptake. Therefore, our research may shed light on the mechanism of the accumulation of LMWC in kidney, which will pave the way for the development of LMWC as a promising renal targeting carrier.

Experimental Section

Materials and Animals. Chitosan (deactylation degree = 90%, Mw = 6.5×10^5) was purchased from Bo'ao biochemical Co. Ltd. (Shanghai, China). Fluorescent probe fluorescein isothiocyanate (FITC), propidium iodide (PI) and Triton x-100 were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO). Disodium maleate was obtained from Kasel Kogyo Co. (Tokyo, Japan). The rabbit polyclonal antimegalin antibody was provided by Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Micro BCA protein assay kit was bought from Pierce Chemical Co. (Rockford, IL). All other chemicals used were of reagent grade.

Kunming mice, 22−25 g, were provided by West China Experimental Animal Center of Sichuan University (China). All *in vivo* protocols were approved by the Institutional Animal Care and Use Committee of Sichuan University and Project of Sichuan Animal Experiment Committee, license 045, China.

FITC Labeled LMWC. LMWC with molecular weight of 31 kDa and 19 kDa (LMWC-31k, -19k) was prepared according to previous methods.[10] FITC labeled LMWC was prepared according to published protocols[16] and used throughout the study. FITC labeled ratio of LMWC-31k and -19k was 28.08 ± 0.19 and 29.95 ± 0.24 μg/mg, respectively.

Fluorescence Imaging of LMWC. Taking LMWC-31k as an example, 100 mg/kg of FITC labeled LMWC-31k and the equivalent mixture of FITC and LMWC-31k were given to mice intravenously respectively and then the mice were sacrificed 60 min after injection. Before imaging, the mice were shaved to expose the tissues to a 470 nm light source of the LT-99D2 Illumatool Dual Light System (Lightools Research, Encinitas, CA). The images were recorded by a

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built-in CCD camera. Then, the tissues were collected and observed by the Dual Light System. Kidneys were frozen at −25 °C for sectioning (Leica, Nussloch, Germany), observed under fluorescent microscope (Carl Zeiss, Oberkochen, Germany).

Cell Culture. Human RTCs (HK-2 cells) and L929 cells were obtained from ATCC (Manassas, VA). HK-2 cells were cultured in DMEM (Gibco, Grand Island, NY) containing 5% new born calf serum, 100 U/mL penicillin. L929 cells were cultured in RPMI1640 (Gibco) containing 10% newborn calf serum, 100 U/mL penicillin and 100 U/mL streptomycin. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C.

Cytotoxicity. HK-2 and L929 cells were used to evaluate cytotoxicity of LMWC. Dextran (50 kDa) served as a negative control and chitosan hydrochloride (640 kDa) as a positive control. Cells were seeded into 96-well plates at a density of 1×10^4 cells/well. The plates were left in the incubator overnight. The following day, the LMWC were added to the wells at increasing concentrations (0.4−5 mg/mL) in a final volume of 200 μL and the plates were then returned to the incubator for 24 h. After 24 h, the viability of cells was determined by MTT assay.[17]

Uptake of LMWC by HK-2 Cells. HK-2 cells were seeded on 24-mm diameter polyester filters (1×10^5 cells/well) with a pore size of 0.4 μm (Transwell clears, Corning-Costar, Cambridge, MA). The cells were cultured for 5 days when the cell monolayer was polarized, as demonstrated by transmembrane electrical resistance (>700 $\Omega \cdot cm^2$). Then, FITC labeled LMWC was added to the apical (in the presence or absence of 6 mg/mL unlabeled LMWC) or basolateral compartments at final concentration of 1 mg/mL to incubate with cells at 37 °C for 2 h. The experiment was terminated by washing the cell monolayer three times with ice-cold phosphate buffer (PBS: 8 g/L NaCl, 0.2 g/L KCl, 1.56 g/L Na_2HPO_4 and 0.2 g/L KH_2PO_4 in water at pH 7.2) and lysing the cells with 0.2 mL of 1% Triton X-100 in 50 mM Tris-HCl buffer (pH 8.0). The obtained cell lysate solutions were determined by RF-5301PC spectrofluorophotometer (Shimadzu, Japan, λ ex 492 nm, λ em 519 nm) to quantify cell-associated LMWC. Uptake was expressed as the amount (μg) of LMWC associated with a unit weight (1 mg) of cellular protein. The protein content of the cell lysate was measured using the Micro BCA protein assay kit.

HK-2 cell monolayer in 12-well plate was preincubated for 1 h in 1 mL of serum-free DMEM at 37 °C. LMWC was dissolved into prewarmed serum-free DMEM solution to obtain equivalent concentrations from 0.15 to 2.0 mg/mL. Uptake was initiated by exchanging the DMEM solution with 1 mL of specified dosing LMWC solution and incubating the cells at 37 °C for 4 h. The experiment was terminated with ice-cold PBS. The same procedure was carried out following the steps mentioned above.

Laser Scanning Confocal Microscopy. HK-2 cells were incubated with prewarmed LMWC (1 mg/mL) at 37 °C. After 4 h of incubation, LMWC was removed and the cells were washed in triplicate with ice-cold PBS. Then, the cells were fixed in 4% paraformaldehyde for 10 min and stained with 10 μg/mL PI (containing 25 μg/mL RNase A) at 37 °C for 30 min. Finally, the fixed cells were washed with PBS for three times and observed under confocal microscope (Leica SP5, Mannheim, Germany).

Competitive Inhibition Assay. HK-2 cells were incubated with 1 mg/mL LMWC diluted into serum-free DMEM in the absence or presence of 1 mg/mL gentamycin or 1 mM EDTA. After incubation for 4 h at 37 °C, cells were rinsed three times with ice-cold PBS and observed under confocal microscope.

Animal Studies. According to the previous studies,[1,18] disodium maleate (400 mg/kg) was injected into the Kunming mice by intraperitoneal injection. Subsequently, the mice were sacrificed at prearranged time between 45 min and 6 h 45 min and the kidneys and urine samples were collected. The renal cortex was separated from the kidneys and put into liquid nitrogen. After pulverization of the cortex in liquid nitrogen, total protein was extracted by RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X100, 10 g/L sodium deoxycholate, 1 g/L SDS, 1 mM PMSF, 1 mM EDTA, pH 7.4) for Western blot analysis. The urine was concentrated by freeze-drying. By immunohistochemistry and Western blot analysis, the shedding of megalin was confirmed.

The normal mice were divided into two groups and injected intravenously with 100 mg/kg FITC labeled LMWC (31k and 19k), respectively. The megalin-shedding mice after maleate treated for 45 min were also divided into two groups and injected with an equivalent dose of FITC labeled LMWC (31k and 19k), respectively. The mice were sacrificed 3, 5, 10, 15, 30 min, 1, 2, 6, 11 and 24 h after injection. Then, the tissues were collected and treated according to Onishi's method.[16] The yielded samples were assessed by spectrofluorophotometer. The area under the concentration−time curve (AUC_{normal} and $AUC_{megalin\text{-}shedding}$) and mean residence time (MRT) were calculated with Drug and Statistics software (DAS, 2.0 version, China). Finally, shedding rate (R) was estimated with the following equation:

$$R = 1 - \frac{AUC_{megalin\text{-}shedding}}{AUC_{normal}} \times 100\% \quad (1)$$

Results

Renal Targeting of LMWC. To examine the tissue-specific localization of LMWC *in vivo*, fluorescence imaging was used to visualize the accumulation of FITC labeled LMWC in the kidneys in mice (Figure 1A−E). The images showed that FITC labeled LMWC selectively accumulated in the kidneys within 60 min while it was scarcely observed

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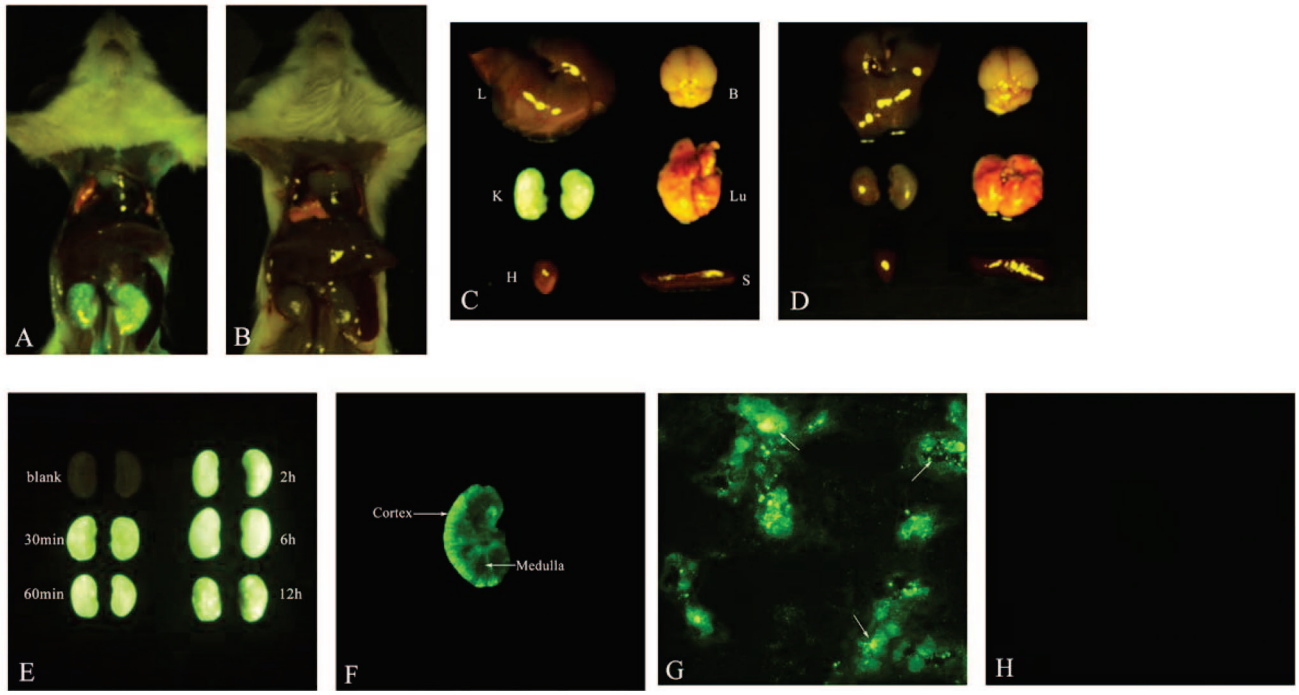

Figure 1. (A−E) Fluorescence imaging of kidneys in mice after injection with LMWC. The mouse was injected intravenously with FITC labeled LMWC-31k (100 mg/kg) and the equivalent mixture of FITC and LMWC-31k, respectively. All tissues were visualized by whole body imaging using a 470 nm light source. Representative whole body distribution of FITC labeled LMWC-31k (A) and the mixture (B) 60 min after injection indicated renal specificity of LMWC. Then, the tissues were collected and exposed to the same light source. The fluorescence images were recorded. Sectioned tissues of mice showed FITC labeled LMWC (C) selectively accumulated in the kidneys. Conversely, no fluorescent signal was observed in any tissues in the mixture group (D). Mice received FITC labeled LMWC were sacrificed 30 min, 60 min, 2 h, 6 and 12 h after intravenous administration. Then, the kidneys were collected (E). The kidneys of mice sacrificed at various time points showed that LMWC specifically accumulated in the kidneys and exhibited high renal retention. L, liver; K, kidney; H, heart; B, brain; S, spleen; Lu, lung. (F−H) Specific distribution site of LMWC. A longitudinal section of the kidney from a mouse sacrificed 60 min after injection with FITC labeled LMWC-31k. Most FITC labeled LMWC accumulated in the renal cortex, rather than the medulla (F). Then, histological sections were prepared to show the distribution site of FITC labeled LMWC. Compared with glomeruli, stronger fluorescent intensity was observed in the proximal tubules (arrow), suggesting specific location of LMWC in proximal tubules (G). However, no signal was observed in the section after injection with the equivalent mixture of FITC and LMWC-31k for 60 min (H). Magnification (G, H), ×400.

in other tissues (Figure 1A,C). Besides, no fluorescent signal was detected in the kidneys of mice received mixture of FITC and LMWC (Figure 1B,D). Even 12 h after injection, the fluorescence intensity of FITC labeled LMWC was still strong (Figure 1E). A longitudinal section of the kidney of mice 60 min after injection with FITC labeled LMWC showed that most LMWC accumulated in the renal cortex, rather than the medulla (Figure 1F). Then, histological sections indicated that LMWC specifically distributed in the proximal tubules (Figure 1G). However, no signal was detected in the section after injection with the equivalent mixture of FITC and LMWC for 60 min (Figure 1H), which was consistent with the result of Figure 1B. Therefore, the visual images revealed LMWC specifically accumulated in the kidney, especially in the renal proximal tubules and exhibited high renal retention.

Cytotoxicity. First, we evaluated the cytotoxicity of LMWC to L929 and HK-2 cell lines. The results indicated that LMWC-19k and LMWC-31k had no obvious effect on the viability of L929 and HK-2 cell lines (Figure 2), while cationic chitosan presented apparent cytotoxicity to both types of cells, in accordance with Begona's study.[19] In addition, dextran showed a certain effect on the viability of both cell lines at a high concentration.

Cellular Uptake of LMWC. To pursue the major route of cellular uptake of LMWC, Transwell plates were employed to examine the uptake of LMWC in HK-2 cells from apical or basolateral side. As shown in Figure 3A, the uptake of LMWC from apical side was much higher than that from basolateral side ($p < 0.001$) and was reduced in the presence of excess unlabeled LMWC, which suggested that LMWC was dominantly taken up at apical surface of HK-2 cells and the binding sites for LMWC should be localized on the apical side.

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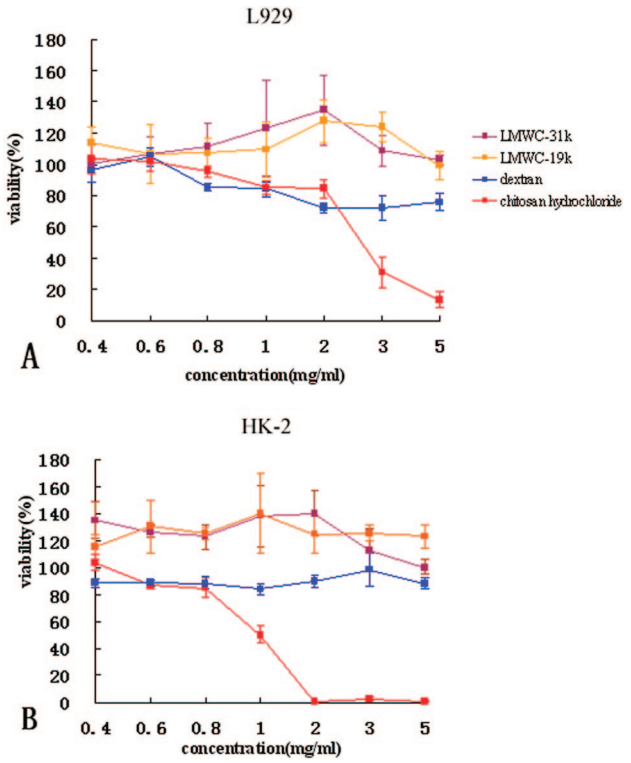

Figure 2. *In vitro* cytotoxicity assay of LMWC on L929 cells (A) and HK-2 cells (B) as measured by MTT assay. Cell viability was calculated as the percentage of mean absorbance of treated cells over the mean absorbance of untreated cells. LMWC-19k and LMWC-31k had no obvious effect on the viability of both types of cells. Dextran (50 kDa) as negative control; chitosan hydrochloride (640 kDa) as positive control. Error bars represent standard deviation of the mean ($n = 5$).

For *in vitro* study, the concentration of LMWC is a feature of great interest in renal uptake, considering its significant influence on the uptake process of LMWC. As shown in Figure 3B, the uptake rate of LMWC-31k increased by 4-fold as its concentration was raised from 0.15 to 2.0 mg/mL. The same tendency was also observed in the uptake of LMWC-19k, with a higher increase (4.4-fold) over the same concentration range (Figure 3C). The process seemed saturable at the concentration of 1 mg/mL, and there was a good fit when the data were transformed to the Michaelis−Menten equation ($R = 0.991$ and 0.987). The K_m values of LMWC-31k and -19k were 0.546 and 0.705 mg/mL, and the V_{max} values were 0.924 and 1.01 μg/mg/h, respectively.

To confirm whether LMWC was internalized into the cytoplasma or not, laser scanning confocal microscopy was performed. Figure 4A,B shows a montage of images of a single HK-2 cell incubated with FITC labeled LMWC-31k for 4 h. Strong fluorescence was observed on the surface of the HK-2 cell. Intense fluorescence signals were concentrated between 3 and 9 μm from the apical surface of the cell (Figure 4A3−9). In addition, there was a fair amount of fluorescence in the cytoplasma (Figure 4A3−9,B). These provided evidence that LMWC was partly adsorbed on the surfaces of RTCs and the remaining part was internalized into the cytoplasma.

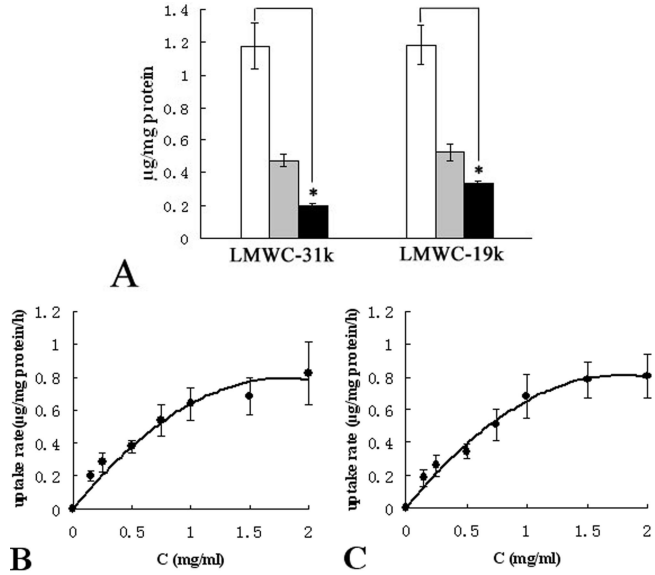

Figure 3. (A) The major route of cellular uptake of LMWC. HK-2 cells were seeded on 24-mm diameter polyester filters with a pore size of 0.4 μm and cultured for 5 days. FITC labeled LMWC was then added to the apical or basolateral medium compartments at final concentration of 1 mg/mL at 37 °C for 2 h. The uptake of LMWC from either the apical (white bars) or basolateral (black bars) side of the renal tubular epithelial cells was assessed. The uptake from apical side was significantly greater than that from basolateral side (*$p < 0.001$) and was reduced in the presence of excess unlabeled LMWC (6 mg/mL) (gray bars), suggesting that LMWC is dominantly taken up at apical surface of HK-2 cells and the binding sites for LMWC should be localized on the apical side. (B,C) Uptake rate curve of LMWC-31k (B) and LMWC-19k (C) by HK-2 cells. Five-day-old confluent cells were incubated with LMWC in equivalent concentrations from 0.15 to 2.0 mg/mL for 4 h at 37 °C. The uptake rate was calculated. Uptake of LMWC-31k and LMWC-19k increased accordingly with the raising dose. When the concentration raised up to 1 mg/mL, the uptake process was saturated. Error bars represent standard deviation of the mean ($n = 3$).

Megalin Mediating the Renal Uptake of LMWC. Having established the specific uptake of LMWC in kidney, we attempted to inhibit the uptake of LMWC by HK-2 cells with gentamycin and EDTA (Figure 4C−E). HK-2 cells, expressing megalin receptors, are commonly used in the related study of megalin.[20,21] The result showed that in the absence of competitors, LMWC was readily endocytosed. By contrast, in the presence of competitors, gentamycin and EDTA, the uptake of LMWC (Figure 4D,E) was completely inhibited. Previous studies have shown that the ligand binding process to megalin is calcium-dependent[22,23] and could be inhibited by coincubation with EDTA. Therefore, the results

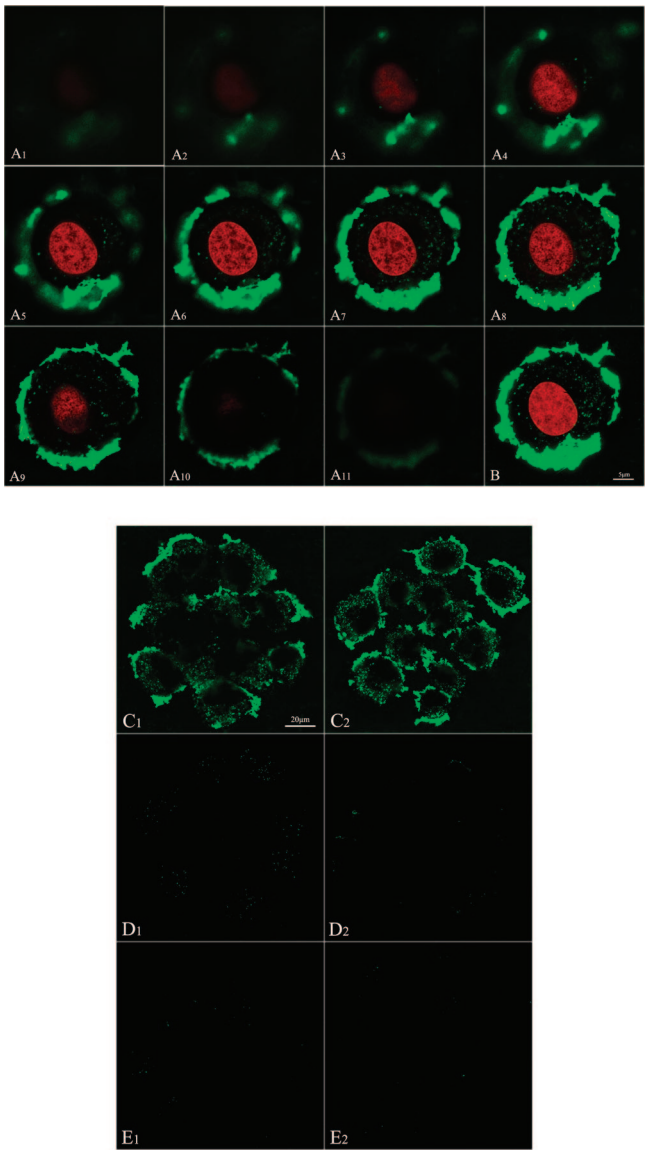

Figure 4. (A,B) A montage of images of a single HK-2 cell incubated with FITC labeled LMWC (A1−11). The cell was fixed in 4% paraformaldehyde, and nuclei were counterstained with propidium iodide (red signal, containing RNase A). A three-dimensional reconstruction of fluorescence signals was performed (B). LMWC was probably endocytosed into the endosomes (arrow). The images indicated that LMWC was partly adsorbed on the surfaces of cells and the remaining part was internalized into the cytoplasma. "Z series" images were recorded with 1 μm increments. (C−E) Inhibitory effect of gentamycin and EDTA upon the uptake of LMWC in HK-2 cells. HK-2 cells were incubated at 37 °C for 4 h with 1 mg/mL FITC labeled LMWC-31k (C1-E1) or FITC labeled LMWC-19k (C2-E2) in the absence or presence of 1 mg/mL gentamycin or 1 mM EDTA. LMWC (C1, C2) was internalized by HK-2 cells. More than 95% of cells were labeled with LMWC. Gentamycin (D1, D2) and EDTA (E1, E2) completely inhibited the uptake of LMWC, suggesting that the internalization of LMWC is probably mediated by megalin. Magnification, ×630.

supported the hypothesis that megalin probably mediated the internalization of LMWC.

To further verify the role of megalin in renal uptake of LMWC, megalin-shedding mouse models were established using disodium maleate. In this study, megalin levels in the renal cortex and in the urine from maleate-treated mice were examined by immunohistochemistry and Western blot analysis. As shown in Figure 5A−D, the amount of megalin in the kidneys suffered an enormous decrease after administration of maleate. Similar results were observed in Figure 5E: the megalin level decreased in the renal cortex after maleate injection for 45 min. In addition, urinary megalin was detected in maleate-treated mice, but could hardly be examined in the normal one under the same concentration. These results indicated that megalin-shedding mouse models have been established successfully.

Our previous study mainly focused on LMWC as a carrier to deliver prednisolone to mouse kidneys.[10] However, the behavior of LMWC *in vivo* was not clarified. To further verify renal targeting and possible accumulation mechanism, LMWC was injected intravenously in mice and the biodistributions of LMWC in various tissues were determined by spectrofluorophotometer. The AUC of LMWC in the plasma and tissues were presented in Figure 6A,B. The AUC of LMWC-31k (A) and -19k (B) in the kidneys was 325.94 ± 21.62 and 150.00 ± 9.16 mg·min/g respectively, with a significant increase in the uptake of LMWC ($p < 0.01$) compared with other tissues. Furthermore, LMWC exhibited higher retention in the kidneys, whereas they could hardly be detected in the other tissues 6 h after iv injection (data not shown). As shown in Table 1, the MRT of LMWC in the kidneys also underwent a significant increase ($p < 0.01$). In agreement with results indicated by fluorescence imaging (Figure 1), these data suggested the potential of LMWC as a renal targeting carrier. In megalin-shedding mice, the accumulation of LMWC-31k and -19k in the kidneys was dramatically reduced by ∼70% and ∼60% as compared with normal animals, respectively (Figure 6C,D). Moreover, concentration of LMWC in the kidneys at various time points was significantly decreased (Figure 6 C,D inset). Calculated from eq 1, shedding rates (R) were close (76.90% ± 0.57% and 66.97% ± 4.59%), which suggested the renal uptake of

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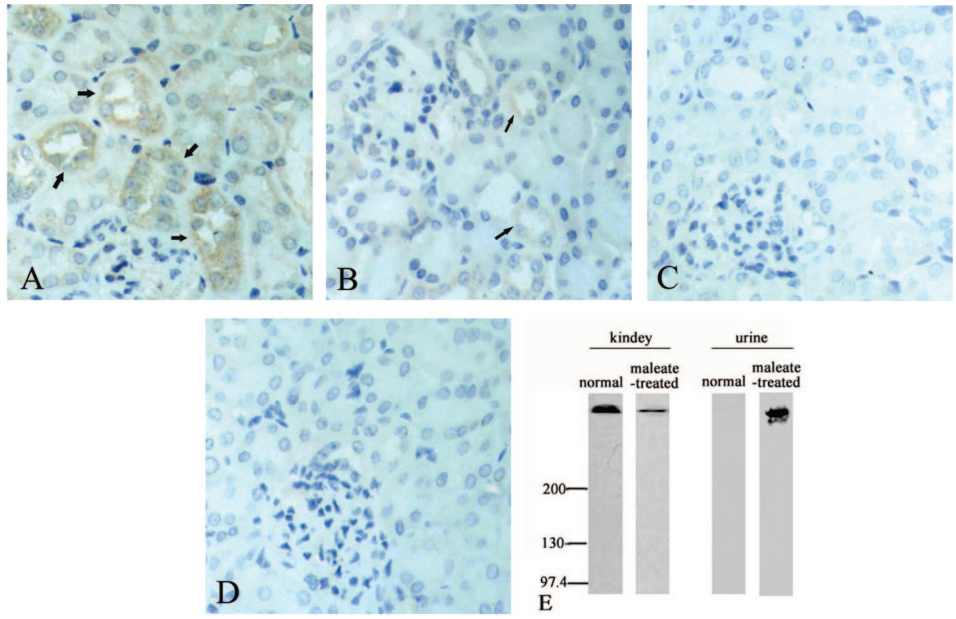

Figure 5. (A−D) Immunohistochemical detection of megalin in the paraffin sections of normal mouse kidneys and maleate-treated mouse kidneys. In contrast to normal kidneys (A), only a little megalin was detected at the luminal plasma membrane of the affected kidneys after maleate injection for 45 min (B) and even no megalin could be identified after maleate injection for 3 h 45 min (C) and 6 h 45 min (D), resulting in a functional megalin deficiency. The results are consistent with the previous studies.[1] The arrow indicates the megalin at the luminal plasma membrane. The rabbit polyclonal antimegalin antibody was used at 1:200. An HRP conjugated goat antirabbit antibody (1:500 dilution) was used as the secondary and counterstained with hematoxylin. Magnification, ×400. (E) Western blot analysis for megalin in renal cortex samples and urine samples of normal mice and maleate-treated mice after injection for 45 min. The decrease in megalin level was found in the renal cortex after maleate injection. In addition, urinary megalin was detected in maleate-treated mice, but it could hardly be examined in the normal one under the same concentration. Samples (renal cortex: 30 μg/lane, urine: 50 μg/lane) were subjected to 4% SDS−PAGE under reducing conditions, and the proteins were transferred to PVDF membranes. Immunoreactive sites on PVDF membranes were revealed by enhanced chemiluminescence by exposure to SuperSignal West Pico chemiluminescent substrate (Pierce-Perbio Science, Northumberland, U.K.). Western blots were then exposed to Kodak Biomax film and developed using a Kodak autodeveloper (Kodak, Hemel Hempstead, U.K.). Primary antibody: rabbit polyclonal antimegalin antibody (1:300 dilution). Secondary antibody: HRP conjugated goat antirabbit antibody (1:5000 dilution).

LMWC-31k and LMWC-19k was affected to the same extent by the shedding of the megalin receptor. Therefore, the results confirmed that megalin receptor mediated process was responsible for the specific renal uptake of LMWC.

Discussion

In the present study, we have identified the selective distribution of LMWC in the kidneys, specifically in the renal proximal tubules, using fluorescent imaging. Then, we have further demonstrated that HK-2 cells expressing megalin receptors[20,21] are able to internalize LMWC, the uptake of which can be completely inhibited by gentamycin and EDTA, known as the competitive inhibitors of megalin receptor. In addition, it was shown *in vivo* study that renal targeting can be achieved through iv injection of LMWC in normal mice. However, in megalin-shedding mice models, we found the renal accumulation of LMWC decreased significantly.

In recent years, optical imaging has been developed as a simple yet powerful technique for the trace of drug and the carrier as well, therefore enabling the evaluation of whether successful delivery to the desired organ or tissue has been achieved.[24,25] In particular, in current studies on chitosan, fluorescence labeling is widely used[16,26] to track the both *in vitro* and *in vivo* behavior of chitosan due to its easy conjugation with FITC. This strategy is also applied in our experiments, which allows visualization of the distribution of LMWC in tissues, as well as the internalization of LMWC in cells by fluorescence microscopy and confocal microscopy.

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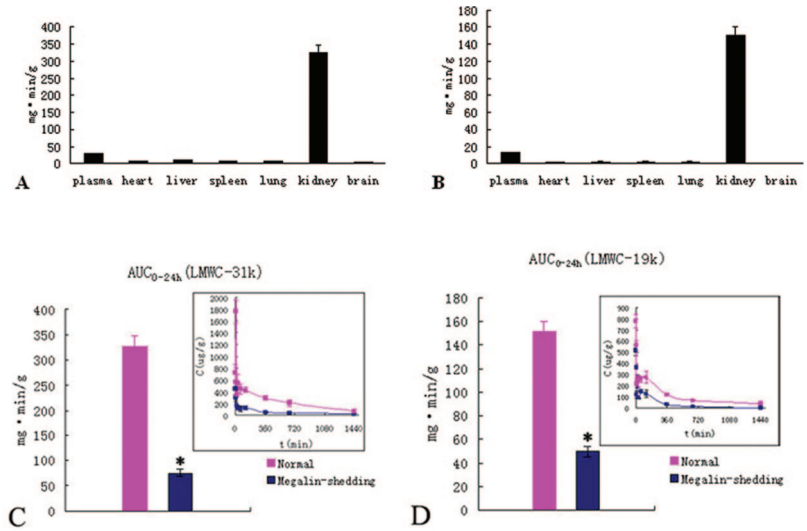

Figure 6. (A,B) Accumulation of LMWC in plasma and tissues of normal mice for 24 h. Normal mice were given an intravenous dose of FITC labeled LMWC (100 mg/kg). Mice were killed at prearranged time points, and plasma and tissues were collected. Then, the amount of LMWC was quantified. The 24-h area under the curve (AUC) is shown for LMWC-31k (A) and LMWC-19k (B). Analysis of variance showed a significantly greater amount of LMWC in the kidneys ($p < 0.01$) than that in other tissues. The results are in line with fluorescence imaging that is presented in Figure 1. (C,D) Renal accumulation of LMWC in normal and megalin-shedding mice for 24 h. Normal mice or megalin-shedding mice were given an intravenous dose of FITC labeled LMWC (100 mg/kg; C, LMWC-31k; D, LMWC-19k). Mice were killed at prearranged time points, and kidneys were collected. The amount of LMWC in the kidneys was quantified. Then, AUC in the kidneys was calculated. There was a significant reduction in uptake of LMWC in megalin-shedding kidneys compared with that in normal kidney (*$p < 0.001$). The inset depicts the concentration−time curve of LMWC in the kidneys of normal mice and megalin-shedding mice. Error bars represent standard deviation of the mean ($n = 3$).

Table 1. The Mean Residence Time (MRT) of LMWC-31k and LMWC-19k in the Plasma and Tissues[a]

samples	plasma	heart	liver	spleen	lung	kidney	brain
LMWC-31k	87.04 ± 21.70	72.05 ± 1.80	98.74 ± 3.38	110.71 ± 8.60	68.73 ± 1.39	445.03 ± 55.54*	114.17 ± 7.89
LMWC-19k	49.27 ± 8.39	28.88 ± 0.96	63.12 ± 8.41	115.21 ± 12.19	23.70 ± 4.30	426.51 ± 17.07**	12.73 ± 0.98

[a] Each value is the mean ± SD ($n = 3$). MRT in the kidney exhibited a significant increase compared with that in other tissues, (*) $p < 0.01$, (**) $p < 0.005$.

It was clear in fluorescent images that LMWC accumulated with high specificity in the kidneys, especially in the renal proximal tubules, while there was merely no LMWC in glomeruli (Figure 1). Indeed, functioning as an extensive apical endocytic apparatus, the renal proximal tubule reabsorbs a large variety of molecules filtered through glomeruli. Furthermore, acting in both conservation of protein and reabsorption of aminoglycoside, several key receptors existing in renal proximal tubule cells have been reported to be involved in the process. It is worth mentioning that in recent research, megalin was convinced as the most important receptor in the endocytic pathway.[8,27,28] Taken together, these findings suggested that some receptors on the membrane of RTCs, such as megalin, might participate in the specific renal uptake of LMWC.

To investigate the possible uptake mechanism of LMWC in kidney, further *in vitro* and *in vivo* experiments were carried out. However, before uptake assay *in vitro*, it is necessary to evaluate the cytotoxicity of LMWC. According to data obtained from the cytotoxicity assessment, LMWC had no obvious effect on the viability of L929 and HK-2 cell lines (Figure 2) even with a dose up to 5 mg/mL.

According to intracellular fluorescent images (Figure 4A,B), the uptake of LMWC was an endocytic process. This was also supported by the uptake study, which indicated that the uptake of LMWC by RTCs followed a concentration-dependent and saturable manner (Figure 3B,C). Nevertheless, this internalization could not be attributed to a disruption of the cellular plasma membrane because LMWC did not bring in any significant changes in cell viability as assessed by MTT method (Figure 2).

Analysis by fluorometry could not differentiate between intracellular and surface located LMWC in a direct-view way. Therefore, laser scanning confocal microscopy was applied in order to confirm whether LMWC was internalized into

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the cytoplasma, as shown in Figure 4B. Confocal imaging is able to make "z series" images, which are essentially serial sections through the same specimen, allowing for three-dimensional analysis of a tissue or cell sections.[29,30]

Chitosan salts have been reported to interact with Caco-2 epithelial cell membrane through their positive charges,[31] but the zeta potential of LMWC in a base form[32,33] in our experiments was below zero (∼−1.5mV pH 7.0). Thus, it seemed that the uptake of LMWC by RTCs was unlikely mediated by a charge-dependent mechanism. In addition, aminoglycoside has been identified as one of the ligands[1−3,8] of megalin receptor, which serves as a scavenger mediating the reabsorption of aminoglycoside and proteins in the glomerular filtrate by binding a number of ligands in a Ca^{2+} dependent manner.[34−36] The involvement of Ca^{2+} in the binding of ligands to megalin may be explained by two possible models: Ca^{2+} may either directly bridge the interaction between the ligand and megalin, or maintain megalin in a right conformation that is receptive for ligand binding.[21] Therefore, if megalin acts in the renal uptake of LMWC as described above, gentamycin and EDTA will be capable of decreasing or inhibiting the uptake of LMWC in RTCs due to competitive inhibition of the binding to megalin or lack of dissociated Ca^{2+}. To test the possibility of a role of megalin in the uptake of LMWC, experiments on the effects of gentamycin and EDTA were performed, the results of which confirmed our hypothesis and suggested that LMWC may share part or all of binding sites on the megalin with gentamycin.

In the present research, the pharmacokinetic properties of LMWC were first investigated. The AUC and MRT of various tissues in normal mice were calculated, and the data showed that the accumulation rate of LMWC in the kidneys was considerably higher than that in other tissues, which demonstrated the renal targeting of LMWC. Besides, previous reports have confirmed that the reabsorption of megalin ligands—gentamycin,[1] aprotinin[37] and lysozyme[38]—in kidney can be decreased in megalin-shedding animal models. The administration of disodium maleate reduced the renal accumulation of LMWC, following the decrease in megalin level in the kidney (Figure 6C,D). These provide evidence that the renal accumulation of LMWC is dependent on the megalin level in kidney. In addition, the uptake of LMWC in RTCs could be completely inhibited by gentamycin (Figure 4D). Inspired by these observations, we proposed a possible interaction between LMWC and megalin. Gentamycin, aprotinin and lysozyme are recognized as the ligands of megalin. Based on these, we draw a conclusion that megalin plays an important role in the uptake of LMWC.

Dextran with the structure of glycoside unit is also a polysaccharide carrier. Unlike LMWC, dextran was found highly accumulated in the liver and spleen.[39,40] Thus, the structure of glucosamine unit in LMWC seems essential for renal accumulation. However, the exact mechanism by which megalin binds such a structurally diverse group of LMWC remains unclear. Fortunately, information on the structural and chemical properties of LMWC can be found in previous kinetic studies on the renal accumulation [41−43] and nephrotoxicity[44−46] of aminoglycoside. The reabsorption of aminoglycoside by proximal tubular cells was shown to be a saturable and highly selective process.[1,2,47] Although the binding site was not identified in these studies, the negative charge of aminoglycoside with polybase was proved to be a major determinant for the binding to megalin on the luminal surface.[3,48] Thus, clusters of

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acidic amino acids in megalin, such as the highly conserved Ser-Asp-Glu sequence contained in each ligand binding repeat and/or anionic carbohydrates, may participate in binding aminoglycoside. Besides, LMWC and aminoglycoside share the similar structure. According to the result of competitive inhibition assay (Figure 4D), gentamycin and LMWC probably share the same ligand-binding repeats in megalin as a site for receptor−ligand interaction. Megalin is therefore an important receptor for us to explore a series of chitosan derivatives as novel renal targeting carriers. Future studies will optimize the design of the molecular structure of chitosan, with the aim to enhancing affinity to megalin and avoiding nonspecific binding.

In conclusion, we have revealed that LMWC can be specifically taken up in the kidney and megalin may play a primary role in its internalization process. In view of the severe extrarenal side effects of most drugs used for renal diseases, in particular immunosuppressive agents, LMWC might attract more and more interest as a drug delivery carrier for selective accumulation in kidney, with minimized side effects.

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