Araştırma / Original Paper

The Effect of Chitosan Complexes on Biodistribution of siRNA

Emine Şalva[1,2], Naziye Özkan[2], Levent Kabasakal[3], Suna Özbaş Turan[1], Jülide Akbuğa[1]

[1]Marmara University, Faculty of Pharmacy, Department of Pharmaceutical Biotechnology, [2]Vocational Health School, Pathology Laboratory, [3]Faculty of Pharmacy, Department of Pharmacology, Istanbul-Turkey

Yazışma Adresi / Address reprint requests to: Jülide Akbuğa
Marmara Ünv. Eczacılık Fak. Farmasötik Biyoteknoloji ABD, İstanbul-Turkey
Telefon / Phone: +90-216-414-2962/1182 Faks / Fax: +90-216-345-2952 Elektronik posta adresi / E-mail address: jakbuga@marmara.edu.tr
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ABSTRACT:
The effect of chitosan complexes on biodistribution of siRNA

Objective: RNAi is a powerful tool for controlling cellular processes in the gene silencing and in the analysis of molecular mechanisms for many diseases including cancer. VEGF signaling is a potential therapeutic target for siRNA delivery in breast cancer. Although siRNA can be potential therapeutic agent for various diseases, intracellular delivery of siRNA is one of the major hurdles to turn siRNA into therapeutically active molecules. To date, numerous transfection methods or delivery systems have been developed. Among them, chitosan is potential gene carrier due to its characteristics such as biodegradability, biocompatibility, non immunogenic and toxicity. The purpose of this study was to investigate the biodistribution and tumor localization of chitosan/ VEGF siRNA complexes in breast cancer model of rat.
Method: In our study, we intravenously injected FITC labeled naked siRNA-VEGF (40 µg/rat) and chitosan/FITC labeled siRNA-VEGF complexes (40 µg/rat) to breast tumor-bearing rats.
Results: While the biodistribution of chitosan/siVEGF complexes to the brain and heart appeared almost similar to that observed for naked siVEGF, the accumulation was slightly lower in the spleen, liver, lungs, muscle and higher in the kidney. In the breast tumor tissue, chitosan/FITC-labeled VEGF siRNA complexes were localized in the tumor 15 min post-injection but naked FITC-siVEGF did not localize in tumor tissue.
Conclusion: In this preliminary study, we revealed the promising potential of chitosan as VEGF siRNA delivery system for biodistribution.
Key words: siRNA, VEGF, chitosan, biodistribution, breast cancer

ÖZET:
siRNA'nın biyodağılımına kitozan komplekslerinin etkisi

Amaç: RNAi kanser dahil olmak üzere birçok hastalığın moleküler mekanizmasının analizinde ve gen susturulmasında hücresel proseslerin kontrolü için önemli bir araçtır. VEGF sinyali meme kanserinde siRNA taşınmasında önemli bir hedeftir. siRNA farklı hastalıklar için potansiyel bir ajan olmasına rağmen, siRNA'nın intrasellüler taşınması, terapötik olarak aktif bir moleküle dönüşmesindeki önemli engellerden biridir. Bugüne kadar birçok transfeksiyon yöntemi ve taşıyıcı sistem geliştirilmiştir. Bunlar arasında kitozan, biyouyumlu, biyoparçalanabilir olması, toksik ve immunojenik olmaması gibi özellikleri nedeniyle önemli bir gen taşıyıcısıdır. Bu çalışmanın amacı, meme kanserinde kitozan/VEGF-siRNA komplekslerinin tümör lokalizasyonunu ve biyodağılımını araştırmaktır.
Yöntem: Çalışmamızda meme tümörü taşıyan sıçanlara serbest FITC-işaretli siVEGF (40 µg/sıçan) ve kitozan/ FITC-işaretli siVEGF (40 µg/sıçan) kompleksleri intravenöz olarak enjekte edildi.
Bulgular: Kitozan/siVEGF komplekslerinin beyin ve kalbe biyodağılımı, serbest siVEGF ile hemen hemen benzerken, dalak, karaciğer, akciğer ve kasta biraz daha düşük ve böbrekte ise biraz daha yüksektir. Meme tümör dokusunda, kompleksler enjeksiyon sonrası 15 dakikada tümörde lokalize iken, serbest FITC-siVEGF tümör dokusunda lokalize değildir.
Sonuç: Bu ön çalışmada, biz biyodağılım için VEGF siRNA taşıyıcı sistem olarak kitozanın umut verici olduğunu gösterdik.
Anahtar sözcükler: siRNA, VEGF, kitozan, biyodağılım, meme kanseri

INTRODUCTION

RNAi is a powerful tool for specific gene silencing and has considerably important potential in the new drug development to silence various disease genes. RNAi, induced by a double-stranded RNA, is a post-transcriptional gene silencing method. Small interfering RNA (siRNA) that can induce a gene-specific RNAi, is a small double stranded

RNA sequence composed of 21 to 25 base pairs (1). Vascular endothelial growth factor (VEGF) is a key stimulating factor on tumor angiogenesis. Inhibition of VEGF to interfere with tumor neovascularization is one of the most important strategies in antiangiogenesis therapy (2).

Although the use of siRNAs in clinical applications has attracted a lot of attention, there are major challenges such as 'off target' effect, immune stimulation and the delivery of siRNA to the desired cells, tissues or organs (3). Because of its highly negatively charged backbone containing phoshodiester linkages, naked siRNA does not freely cross the negatively charged cell membrane (4). Therefore, delivery systems are designed to overcome such major bottleneck in the development of siRNA therapies (3). An important challenge for delivering siRNA in vivo is the non-specific distribution of siRNA throughout the body when administered systemically. Non-formulated siRNA has an average size below 10 nm and is subject to high renal clearance, short half-life (~0.3 h) and consequently limited duration of therapeutic activity (3,5). Delivery systems can improve the pharmacokinetic, pharmacodynamic behavior, biodistribution and toxicological profiles of siRNAs by altered their physical size so as to reduce excretion by the kidneys and thereby prolong in vivo half-life (6,7).

Up till now, many gene delivery systems have been developed including viral and non-viral based vectors (8). Viral vectors are highly efficient delivery systems for nucleic acids, but their clinical application is hindered by their induction of toxic immune responses and inadvertent gene expression changes following random integration into the host genome (6). A number of non-viral vectors including polyethyleneimine (PEI), chitosan, poly-L-lysine (PLL), poly (lactide) (PLA), poly (D,L-lactide-co-glycolide) (PLGA), L-alpha-dioleoyl phosphatidylethanolamine (DOPE) and lipofectamine 2000 have been widely used for gene delivery (9). Among these, chitosan has been considered to be a good gene carrier since it is known as a biodegradable, biocompatible, non toxic and natural-derived biopolymer with high cationic potential. Chitosan is useful for alleviating poor cellular uptake and rapid degradation of naked siRNA both in vitro and in vivo via different administration routes (10).

Chitosan may modify the stability and pharmacokinetics of siRNA in vivo. Gao et al. (11) reported that chitosan altered the organ distribution pattern of siRNA and high siRNA accumulation of the chitosan (Mw 160 kDa, DD80%)/siRNA nanoparticles within the kidney was observed after intravenous injection to mice. At 30 min, the intensity of intact siRNA in the kidney was much stronger than in other organs, showing a 10-fold and 100-fold increase compared to lung and spleen, respectively. The level of siRNA in the kidney remained very high even after 24 h, the implying that chitosan/siRNA complexes preferentially accumulate in the kidneys while circumventing the rapid glomerular filtration observed for naked siRNA, showing the potential for development of clinically relevant RNAi therapeutics for renal diseases with targeting effect (10). Although many studies concerning siRNA delivering are present, the limited papers are available about the biodistribution of siRNA.

In our earlier study, we obtained good results after application of chitosan/siVEGF complexes to the breast cancer of rats (12,13). In this study, chitosan/siVEGF complexes were used to examine the biodistribution and tumor localization of siRNA complexes after i.v. injection in rats.

MATERIALS and METHODS

Materials

Low molecular weight chitosan (75 kDa, 75-85% deacetylation) from Sigma was used as gene carrier. The fluorescein isothiocyanate (FITC) labeled siVEGF oligonucleotides used in this study were purchased from Dharmacon (Germany). N-Nitroso-N-methylurea (NMU) was obtained from Sigma-Aldrich (USA). All other chemicals and reagents were obtained from Sigma-Aldrich, USA.

Preparation of Chitosan/siRNA Complexes

Complexes were prepared as reported earlier (14). Briefly, chitosan was dissolved in 0.1 M tris acetate/0.1 M acetate buffer (pH 5.4) to form a solution of 2.5 mg/ml (w/v). Lyophilized siRNAs were dissolved in 5xsiRNA buffer and final concentration was made 1µg/µl. Chitosan/siRNA complexes (15/1, +/-) were prepared by adding chitosan solution to siRNAs stock solutions during intense stirring on a vortex mixer for 15 s and left for 30 min for forming of the complexes completely. The formation of complexes were checked by agarose gel electrophoresis in PBS.

Characterization of Complexes

The diameter and zeta potential values of the complexes were determined using a Malvern Zetasizer (Malvern Instruments 3000 HS, UK). The instrument is equipped with both a particle sizer and a zetameter unit. The samples was measured in PBS in glass cuvettes at 25°C with a constant angle of 90°. Each measurement was done in triplicate.

Mammary Tumor Induction

Female Sprague-Dawley rats at 40 days old were supplied from Marmara University Experimental Animals Laboratory. Animal experiment was performed in accordance with the acceptance and guidance of Animal Ethic Committee of Marmara University (37.2006.mar). For tumor formation, rats in the experiment were injected intraperitonally 50 mg/kg NMU on the 45-50 day of age. After three or four months, tumors developed along the mammary lines from the axilla to inguinal areas of rats (15). Mammary tumor formation was controlled as macroscopically and microscopically using multiple frozen sections.

In Vivo Injection and Fluorescence Imaging

Naked FITC labeled siVEGF and chitosan/FITC-siVEGF complexes containing 40 µg of siRNA were injected to Sprague Dawley rats by intravenously. At 15 min of post-injection, rats were sacrificed and the various tissues, including liver, kidney and tumor were excised and snap frozen in liquid nitrogen. 5-6 µm sections were cut on a cryostat and examined by fluorescence microscopy (Olympus) (16).

RESULTS

Preparation and Control of Complexes

To identify the formation of chitosan/siRNA complexes, the gel retardation assay was performed. Full complexation was observed at chitosan/siRNA complexes in 15/1 ratio (Fig. 1). The size and surface charge of chitosan/siRNA nanoplexes (15/1) were measured around 307±12.1 nm and +20.2 ± 4.6 mV respectively.

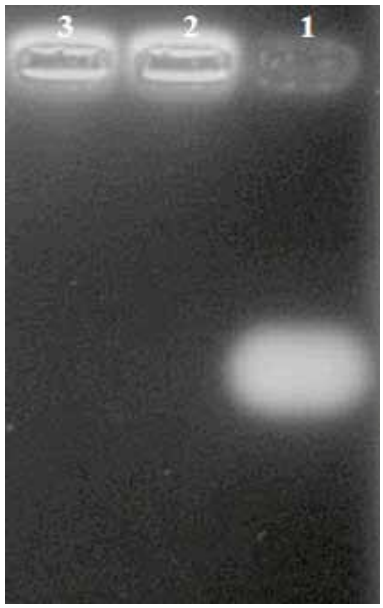
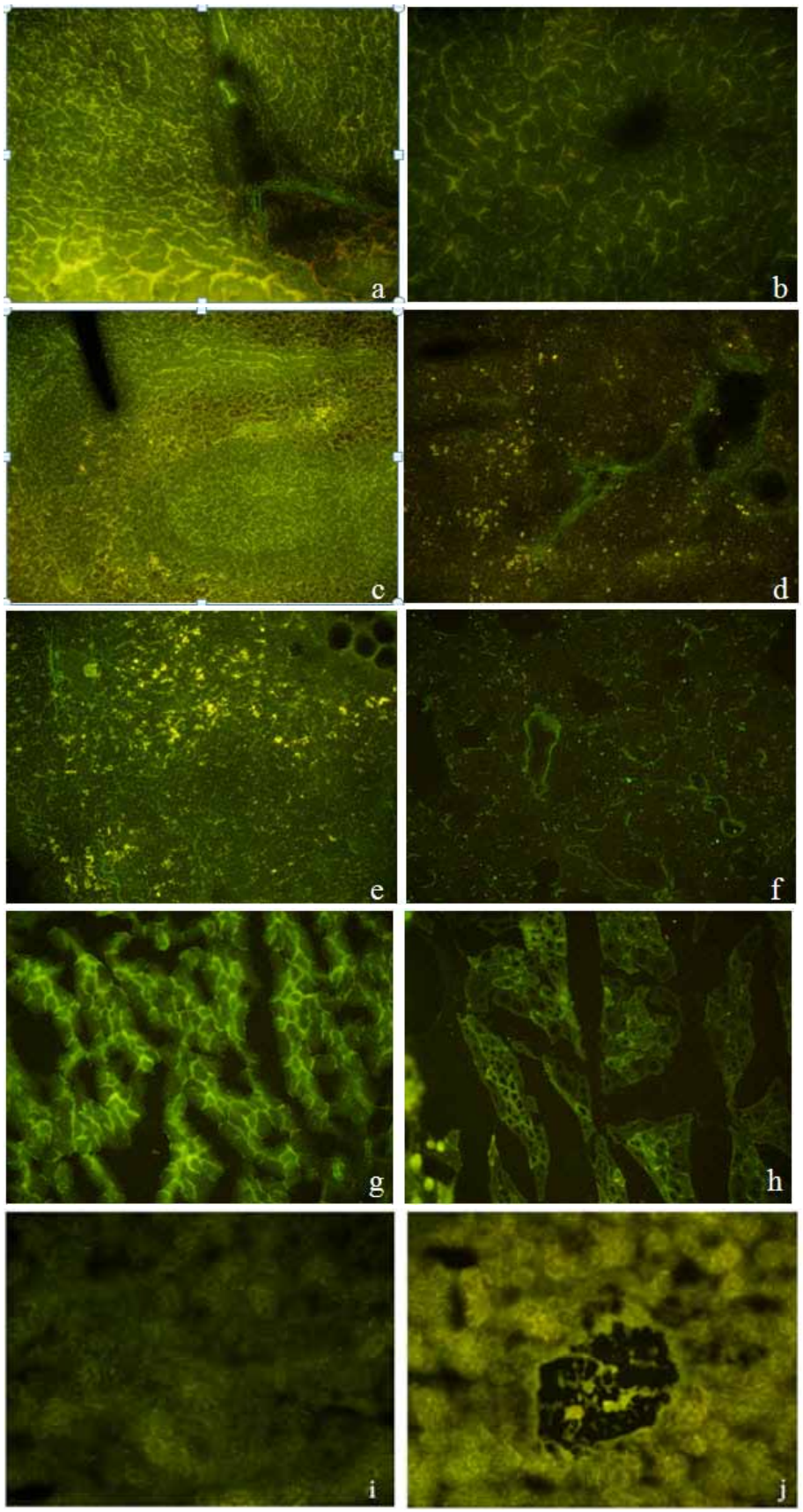


Figure 1: Agarose gel photograph of chitosan/siVEGF complexes 1.Free siVEGF, 2-3.Chitosan/siVEGF (+/-)complexes (10/1, 15/1).

Biodistribution

For the investigation of in vivo biodistribution and tumor localization of chitosan/siRNA complexes in tumor-bearing rats using fluorescence microscopy, we intravenously injected FITC labeled naked siRNA-VEGF (40 µg/rat) or chitosan/FITC labeled siRNA complexes (40 µg/rat) to invasive breast ductal carcinoma tumor-bearing rats. Tumor diameter is about 0,5-1 cm. We have showed the accumulation of fluorescently labeled siRNA in lung, liver, muscle, brain, heart, spleen, kidney and breast tumor of rats after intravenous injection (Fig. 2). While the biodistribution of chitosan/siVEGF complexes to the brain and heart of appeared almost similar to that observed for naked siVEGF (Fig.2.k-l, m-n), there was slightly lower spleen, liver, lungs and muscle (Fig.2.a-b, c-d, e-f, g-h) and there was slightly higher kidney (Fig.2.i-j). However, in the breast tumor tissue, chitosan/FITC-labeled VEGF siRNA complexes were localized in the tumor 15 min post-injection but no localization was observed in tumor tissue after naked FITC-siVEGF injection (Fig2.o-p). Naked siRNAs encounter difficulties in reaching the tumor site after intravenous administration. As a consequence, siRNAs show poor pharmacokinetics after intravenous administration together with a low level of accumulation at the distant tumor site, the short biological half-life (17).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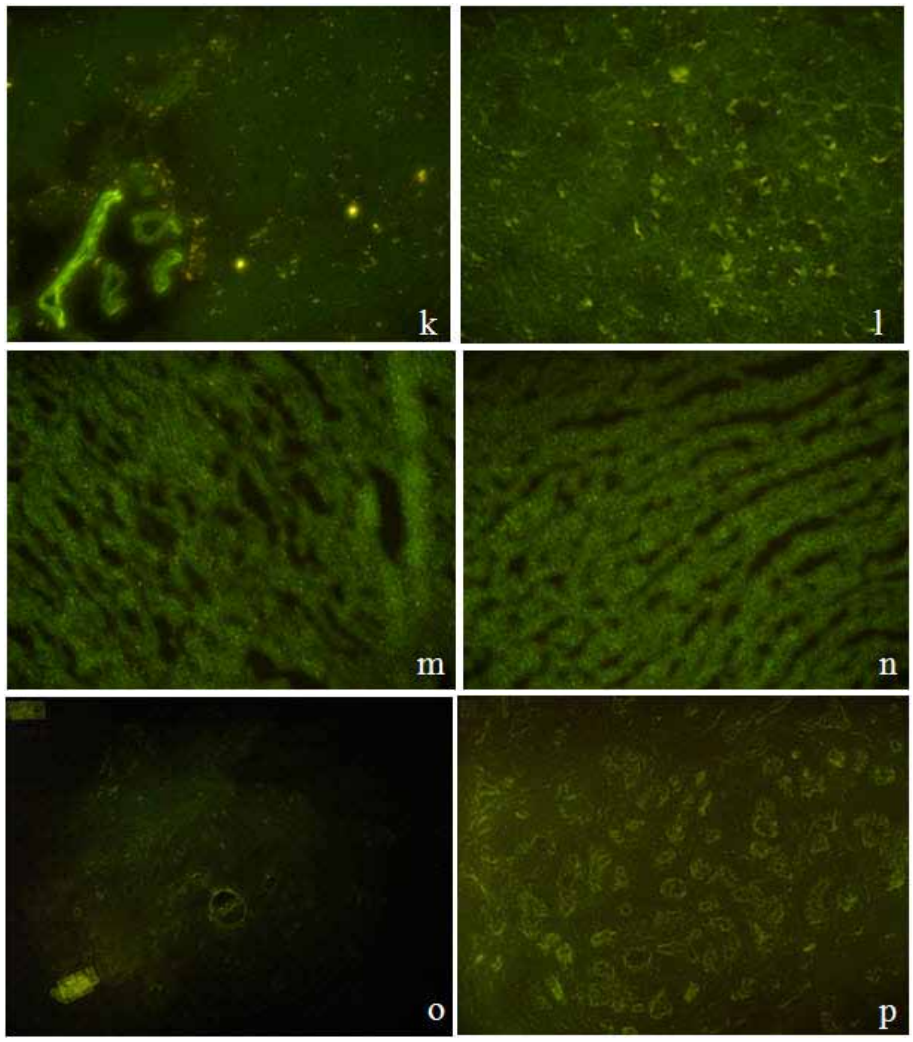

Figure 2: The accumulation of naked FITC-siVEGF and chitosan/FITC-siVEGF complexes in different tissues. FITC-siRNA was administered intravenously in the free form (FITC-siRNA) (left pictures; a,c,e,g,i,k,m and o) or chitosan/FITC-siVEGF complexes (right pictures; b,d,f,h,j,l,n and p) (a-b:liver, c-d:spleen, e-f:lung, g-h:muscle, i-j:kidney, k-l:brain, m-n:heart, o-p:breast tumor)

CONCLUSIONS

In this study, we have studied biodistribution of siRNA VEGF to the different tissues and the availability of chitosan for delivery of siRNA in vivo. Chitosan/siRNA complexes were prepared by self-assembled method as reported previously by us (14). The complex formation occurs due to the ionic interaction between the positively charged amino groups of chitosan and the negatively charged phosphate groups of siRNA. The N/P ratio is defined as the ratio between chitosan nitrogen (N) per DNA phosphate (P). The N/P ratio of complexes is an important parameter affected complex properties and transfection efficiency (18). In our study, full complex formation was observed at 15/1 (N/P, +/-) ratio (Fig. 1).

The surface charge of the complexes depends on the mixing molar stoichiometry of siRNA to chitosan (N/P ratio), which influences the efficiently condense ability of the particles and interact with cells whose membranes are negatively charged and therefore influences the transfection efficiency (19). In addition, increasing the charge ratio in the complex implies an increase in the chitosan concentration in the complex. There is an optimal range for the N/P ratio values specific to the chitosan used because using an N/P ratio that is too low will yield physically unstable complexes and poor transfection, while overly stable complexes prepared at an N/P ratio that is too high may also show reduced transfection (20). The particle size and zeta potential of complexes are important factors for the cellular uptake in

vivo studies (21). In this study, the size and surface charge of chitosan/siRNA complexes (15/1) were measured 307±12.1 nm and +20.2±4.6 mV respectively.

VEGF is one of the important angiogenic factors for tumor neovascularization. It is overexpressed in most tumors. Therefore, the intervention of VEGF has been considered as a potential strategy for tumor growth inhibition (22). The biodistribution experiments were performed on breast tumor-bearing rats after the intravenous injection of chitosan/FITC-siVEGF complexes. In our study, we prepared chitosan/siVEGF complexes and evaluated as an angiogenic endothelial polymeric gene carrier. These complexes containing siVEGF are also important in terms of tumor growth inhibition in vivo. However, tumor inhibition effect of siVEGF was not identified in this study. We previously reported that chitosan/siVEGF complexes could be effective for anti-angiogenic gene therapy (12,13).

Chitosan which a biopolymer is used in our study, is useful for increasing cellular uptake and preventing rapid degradation of naked siRNA in vivo. Chitosan may act to modify the stability and pharmacokinetics of siRNA in vivo (11). Gao et al (11) found that unmodified siRNA showed blood degradation in 1 minute after i.v. administration and siRNA has shown rapid clearance to kidney, but when siRNA was formulated with chitosan, chitosan prolonged the blood circulation time to 65% after 5 minutes but only ~5% remained at 30 minutes and ~1–2% at 24 hours (11).

In this study, to investigate the different tissues localization of complexed and non-complexed siRNAs, tissue sections were obtained and analyzed by fluorescence microscopy. The chitosan/FITC-siRNA complexes accumulated in the tumor region to greater extent than those obtained from naked siRNA. The systemic delivery of chitosan/siRNA complexes prevented loss of the injected dose. The chitosan complexes may be responsible for the decreased accumulation in the organs of the siRNAs (16). A delivery strategy for use of siRNA as a therapeutic agent could reduced glomerular filtration, firstly, and secondly optimize intracellular delivery to target cells, while also minimizing exposure to nuclease and distribution to non-target tissues (23). These observations are also consistent with those reported by de Wolf et al (17). They demonstrated that complexation of the siRNA with cationic liposome and cationic polymer had effect on the pharmacokinetics; both naked siRNA and siRNA packaged into the cationic carriers exhibited rapid blood clearance with tissue distribution within the first 15 min after injection. (17). Gao et al. (11) reported that kidney accumulation of the chitosan/siRNA nanoparticles within the kidney was observed after intravenous injection to mice. The level in the kidney remained very high even after 24 h, implying that chitosan/siRNA complexes preferentially accumulate in the kidneys while circumventing the rapid glomerular filtration observed for naked siRNA, showing the potential for development of clinically relevant RNAi therapeutics for renal diseases with targeting effect.

In conclusion, our study showed that chitosan is a promising tool for in vivo siRNA delivery in future therapeutic studies and further investigation is required for major determinants. In this preliminary study, we observed that accumulation of chitosan/FITC-siRNA complexes is higher than naked FITC-siRNA in the tumor region and the chitosan prevented loss of the injected dose.

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