Bioorganic & Medicinal Chemistry Letters 20 (2010) 4840–4844

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Fluorine-18 labeled galactosylated chitosan for asialoglycoprotein-receptor-mediated hepatocyte imaging

Wenjiang Yang [a], Tiantian Mou [a], Wenyan Guo [a], Huihui Jing [a], Cheng Peng [b], Xianzhong Zhang [a,*], Yunchuan Ma [b], Boli Liu [a,*]

[a] Key Laboratory of Radiopharmaceuticals (Beijing Normal University), Ministry of Education, College of Chemistry, Beijing Normal University, 19 Xinjiekou Outer St., Beijing 100 875, China
[b] PET Center of Xuanwu Hospital, Capital Medical University, Beijing 100 053, China

ARTICLE INFO

Article history:
Received 18 April 2010
Revised 8 June 2010
Accepted 19 June 2010
Available online 25 June 2010

Keywords:
^{18}F
Galactosylated chitosan
[^{18}F]FB-GC
Asialoglycoprotein receptor

ABSTRACT

Galactosylated chitosan (GC) was prepared by reacting lactobionic acid with water-soluble chitosan. GC was labeled with fluorine-18 by conjugation with *N*-succinimidyl-4-^{18}F-fluorobenzoate ([^{18}F]SFB) under a slightly basic condition. After rapid purification with HiTrap desalting column, [^{18}F]FB-GC was obtained with high radiochemical purity (>97%) determined by radio-HPLC. The total reaction time for [^{18}F]FB-GC was about 150 min. Typical decay-corrected radiochemical yield was about 4–8%. Ex vivo biodistribution in normal mice showed that [^{18}F]FB-GC had moderate activity accumulation in liver with very good retention (11.13 ± 1.63, 10.97 ± 1.90 and 10.77 ± 0.95% ID/g at 10, 60, 120 min after injection, respectively). The other tissues except kidney showed relative low radioactivity accumulation. The high liver/background ratio affords promising biological properties to get clear images. The specific binding of this radiotracer to the ASGP receptor was confirmed by blocking experiment in mice. Compared with the non-blocking group the hepatic uptake of [^{18}F]FB-GC significantly declined in all selected time points. The better liver retention properties of [^{18}F]FB-GC than that of albumin based imaging agents may improve imaging quality and simplify pharmacokinetic model of liver function in the future application with PET imaging.

In 1980s, neogalactosylalbumin (NGA) had been developed by attaching the galactosyl unit to human serum albumin (HSA), and then labeled with ^{99m}Tc for imaging the liver.[1] Later, to simplify the labeling procedure, diethylenetriaminepentaacetic acid galactosyl human serum albumin (GSA) was obtained and developed as an instant kit.[2,3] These radiopharmaceuticals are known to bind asialoglycoprotein receptors (ASGP-R) that specifically exist on the mammalian liver and situate on the surface of hepatocyte membrane.[4] ASGP receptors participate in the hepatic metabolism of serum proteins and can recognize a glycoprotein with galactose residues on the terminal position of the saccharide chain, such as asialoglycoprotein. Quantitative imaging of ASGP receptors could estimate the function of the liver. The derivates of glycoprotein radio-labeled with technetium-99m[5–7], iodine-125/131[8], and indium-111[9] have been reported for single photon emission computed tomography (SPECT) imaging applications. ^{68}Ga-DF-NGA[10] and [^{18}F]FNGA[11] were introduced as positron emission tomography (PET) imaging agents. The degree of hepatic uptake of these radiopharmaceuticals correlates well with hepatic function. These agents are similar in that each employs a protein as the backbone for galactose or lactose. Chitosan have been tried out as drug carriers.[12] Water-soluble chitosan has useful characteristics, such as biocompatible, biodegradable, nonimmunogenic, relatively nontoxic, and cleared very rapidly from the bloodstream. Recently, several researchers have reported that galactosylated chitosan (GC) was able to bind to hepatocytes due to the galactose residue positioned on chitosan's exterior.[13] ^{99m}Tc labeled galactosyl-methylated chitosan (GMC) and hydrazinonicotinamide-galactosylated chitosan (HGC) were reported by Kim et al.[14,15] Both ^{99m}Tc-GMC and ^{99m}Tc-HGC showed specific and rapid targeting of hepatocytes.

We previously labeled the NGA with ^{18}F through *N*-succinimidyl-4-^{18}F-fluorobenzoate ([^{18}F]SFB) to get a novel PET tracer [^{18}F]FNGA.[11] Here we report the radiolabeling galactosylated chitosan with ^{18}F for hepatic asialoglycoprotein receptor imaging with PET.

The galactosylated chitosan was prepared by reacting the carboxylic group of lactobionic acid (Acros Organics) with the amino group of chitosan (molecule weight <10 kDa, degree of deacetylation is about 88%, kindly donated by Dr. Yong He, Beijing University of Chemical Technology) according to the procedure of Kim et al.[14]

* Corresponding authors. Tel.: +86 10 58802038; fax: +86 10 62208126 (X.Z.); tel.: +86 10 58808891 (B.L.).
E-mail addresses: zhangxzh@gmail.com, zhangxzh@bnu.edu.cn (X. Zhang), liuboli@bnu.edu.cn (B. Liu).

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.06.106

Briefly, chitosan (0.005 mmol) and lactobionic acid (0.1 mmol) were dissolved in 6 mL MES buffer (2-(*N*-morpholino)ethanesulfonic acid, 0.1 mol/L, pH 6.0). Then 0.2 mmol 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, J&K Chemical LTD) and 0.8 mmol *N*-hydroxysuccinimide (NHS, Alfa Aesar) were added to this solution and stirred for 48 h at room temperature. The final GC solution was dialyzed against distilled water for 3 d and lyophilized (molecular weight cutoff = 3000–5000). The reaction is schematically shown in Figure 1.

The GC was analyzed by ^{1}HNMR spectra in deuterated water, using a 400 MHz spectrometer (Bruker). Figure 2 shows the ^{1}HNMR spectra of GC. The substitution value of galactose in GC was calculated by comparing the characteristic peak areas of the galactose group (4.0 ppm) with that of the peak attributed to the original acetamide group of chitosan (1.9 ppm). The substitution value of lactobionic acid coupled with chitosan in GC was estimated to be 20.6%.

Starting with [^{18}F]F$^-$, the [^{18}F]SFB was synthesized by two-step reaction which was described before.[11] The methylene chloride solution of [^{18}F]SFB was evaporated to dryness with a stream of nitrogen. The residue was dissolved in 50 μL dimethyl sulfoxide and then 200 μL of a solution of GC (1 mg) in 0.1 mol/L borate buffer (pH 8.6) was added and incubated for 30 min at room temperature. After reaction the crude product was passed through a 0.22 μm millipore filter and loaded onto a HiTrap desalting column, eluted with 0.05 mol/L phosphate buffer, pH 7.5, to give the final complex [^{18}F]FB-GC. Starting with [^{18}F]F$^-$ in Kryptofix 2.2.2./K_2CO_3 solution, the total reaction time was about 150 ± 20 min. The typical overall radiochemical yield with decay correction was about 4–8%, including final purification. The radiochemical yield was lower than that of [^{18}F]FNGA, which was reported before[11], may due to the residual amino in GC had larger steric hindrance compared to the ε-amino of lysine in NGA. Starting with 185–555 MBq [^{18}F]fluoride, the specific activity of [^{18}F]FB-GC ranged from 24 to 290 GBq/mmol.

After purification the radiochemical purity was evaluated by ITLC chromatography and radio-HPLC. By ITLC, in ACD buffer, [^{18}F]FB-GC was remaining at the point of spotting (R_f = 0–0.1), while other radioactive impurities moved with the solvent front (R_f = 0.8–1.0). Radio-HPLC was performed on a SHIMADZU system with reversed-phase Kromasil C-4 column (4.6 × 250 mm, 5 μm, Eka Chemicals, Sweden). The mobile phase was changed from 70% solvent A (0.1% trifluoroacetic acid [TFA] in water) and 30% solvent B (0.1% TFA in acetonitrile [ACN]) to 30% solvent A and 70% solvent B at 30 min. The HPLC pattern of [^{18}F]FB-GC is shown in Figure 3. The retention time of [^{18}F]FB, [^{18}F]SFB and [^{18}F]FB-GC were 7.2, 10.2 and 3.1 min in our gradient, respectively. After rapid purified with HiTrap desalting column, the radiochemical purity of [^{18}F]FB-GC determined by both ITLC and radio-HPLC was above 97%.

To evaluate the biological properties of [^{18}F]FB-GC, the ex vivo biodistribution studies were performed in normal Kunming mice. [^{18}F]FB-GC (about 0.037 MBq in 100 μL solution contained about 20 μg GC) was injected through the tail vein. At selected time points (10, 60 and 120 min), mice (n = 5 at each time point) were sacrificed, and the tissues and organs of interest were collected, wet weighed and counted in a γ-counter. The percentage of injected dose per gram (% ID/g) for each sample was calculated by comparing its activity with appropriate standard of injected dose (ID), the values are expressed as mean ± SD. All biodistribution studies were carried out in compliance with the national laws related to the conduct of animal experimentation.

Chitosan is relatively nontoxic. But it may cause some problems when used in a blood contacting situation, especially by the intravenous route.[16] Intravenous injection of chitosan to rabbits at 4.5 mg/kg/day for 10 days produced no abnormal changes, a higher dose of 50 mg/kg/day likely caused blood cell aggregation.[17] Galactosylated chitosan (GC) has higher hydrophilicity than that of chitosan. It can dissolve in water in neutral or even basic

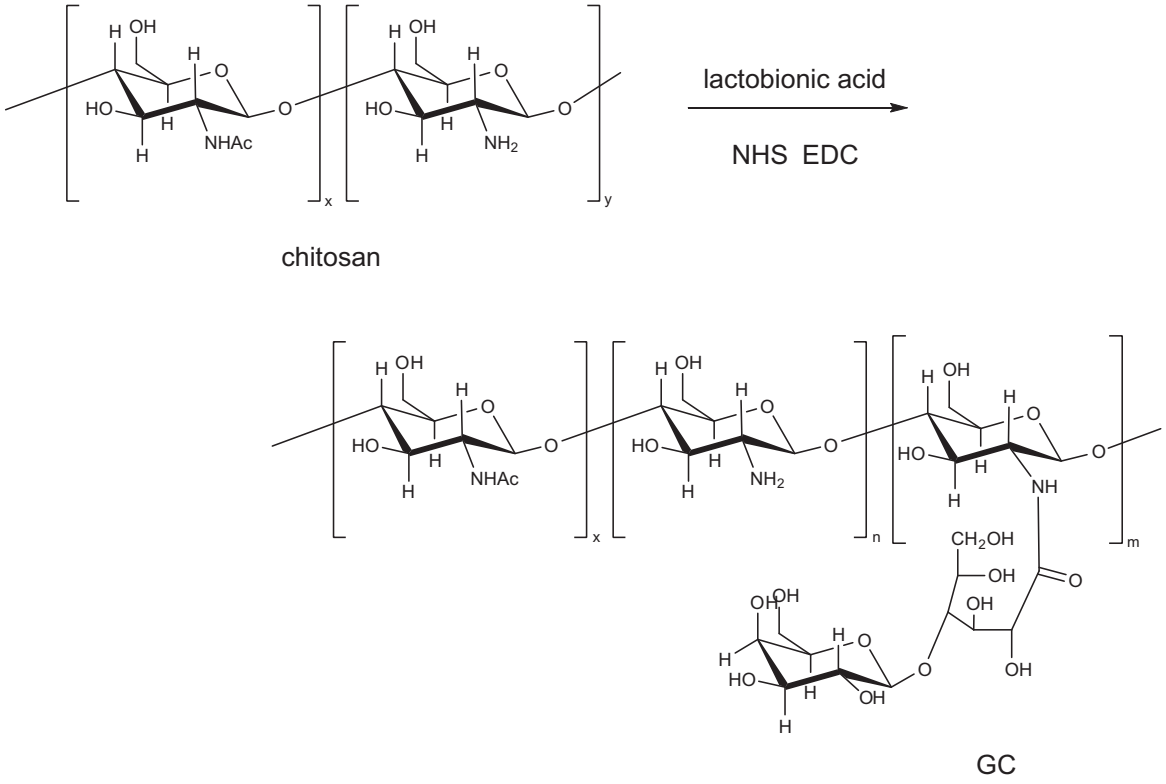

Figure 1. The synthetic scheme of GC.

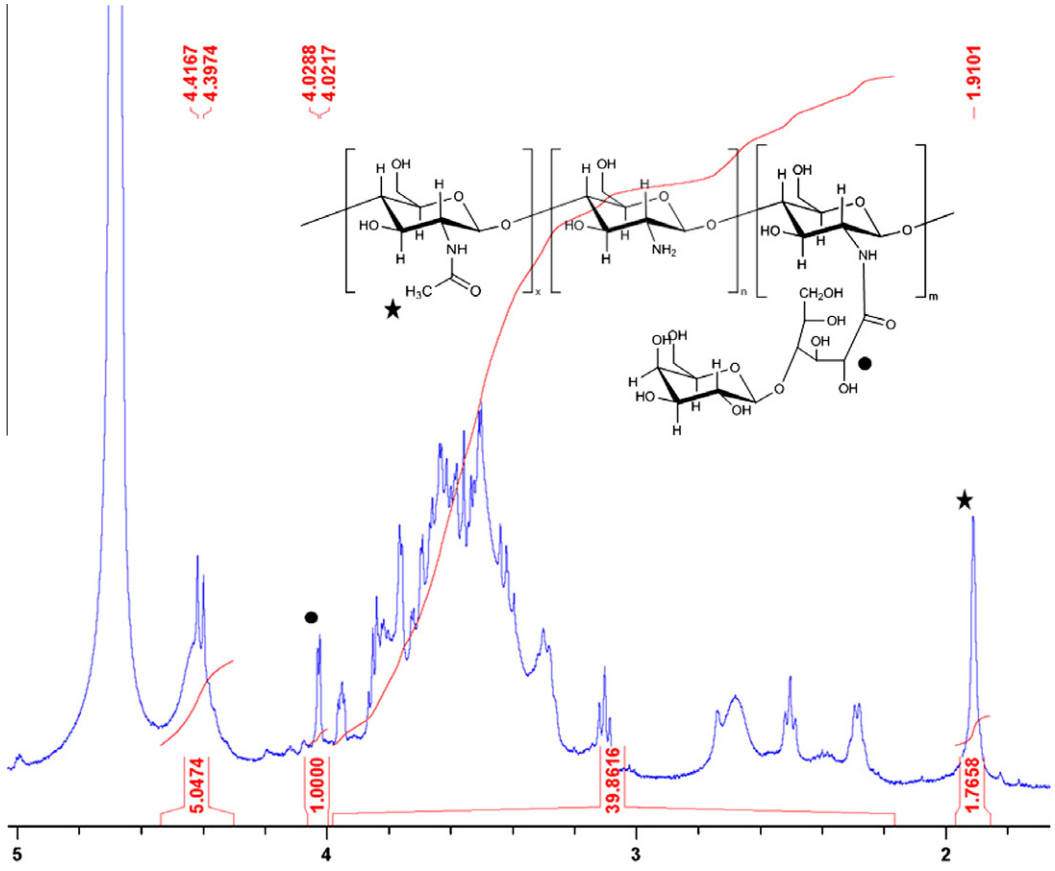

Figure 2. The ^{1}HNMR spectra of GC. Star and circle correspond to proton of acetamide group of chitosan (1.9 ppm) and proton in opened pyranose ring linked between grafted galactose group and chitosan main chain (4.0 ppm), respectively.

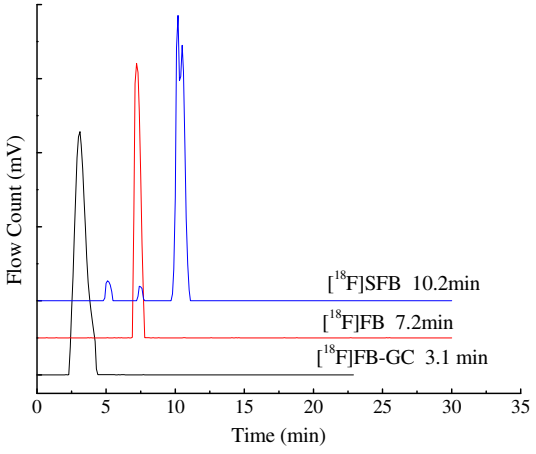

Figure 3. The radio-HPLC radiochromatograms of the [^{18}F]FB, [^{18}F]SFB, and [^{18}F]FB-GC. The retention time was 7.2, 10.2 and 3.1 min, respectively in our gradient system.

conditions. Therefore, the GC is not easy to form colloid over the physiological pH regions. Acute toxicity studies in mice were carried out according to the Chinese Pharmacopoeia (2010 Edition). No abnormal reaction was observed during the 72 h after intravenous injection of GC to mice (18–20 g) at 50 mg/kg. Furthermore, in our biological experiments, the animals were not observed any adverse reactions. The injected dose of [^{18}F]FB-GC as an imaging probe is much lower than 50 mg/kg. Therefore, we think the [^{18}F]FB-GC is safe for intravenous administration as a PET tracer. The specific activity of [^{18}F]FB-GC was relatively low. We inject as low as possible amount of tracer on the premise that the results can be accurately measured according to the detection limit. The specific activity of [^{18}F]FB-GC could be improved through increase the activity of initial reactant [^{18}F]F^{-} for the potential clinical use in the future.

The biodistribution results of [^{18}F]FB-GC are shown in Figure 4A. [^{18}F]FB-GC showed moderate liver accumulation with very good retention. The liver uptakes were 11.13 ± 1.63, 10.97 ± 1.90 and 10.77 ± 0.95% ID/g at 10, 60 and 120 min post-injection time, respectively. Very high accumulation of [^{18}F]FB-GC was observed in kidneys. Localization to the kidney is consistent with the known characterization of water-soluble chitosan.[12] The blood showed relatively low activity uptake and fast clearance. At 60 min after injection, the radioactivity concentration in blood was only 0.13% ID/g. The liver/background ratios of [^{18}F]FB-GC and [^{18}F]FB-NGA at different time points were compared in Table 1. The liver/background ratios of [^{18}F]FB-GC were higher than [^{18}F]FB-NGA after a period of time due to the good retention of [^{18}F]FB-GC in liver and fast clearance in other tissues. Compared with the report of Kim's, the liver accumulation of [^{18}F]FB-GC is similar to that of ^{99m}Tc labeled GC, while the uptake in other organs except kidney, such as spleen and blood are much lower.[14,15] The differences in kidney uptake of [^{18}F]FB-GC with ^{99m}Tc labeled GC may due to the different nature of our selected chitosan material. The molecular weight of our chitosan is near 10,000 (88% deacetylated), while the chitosan Kim et al. used is about 5000 (97% deacetylated). On the other hand, the degree of substitution of LA moieties in our GC is about 20.6%, which is higher than Kim's reports (7.42%[14] and 7%[15]). These differences

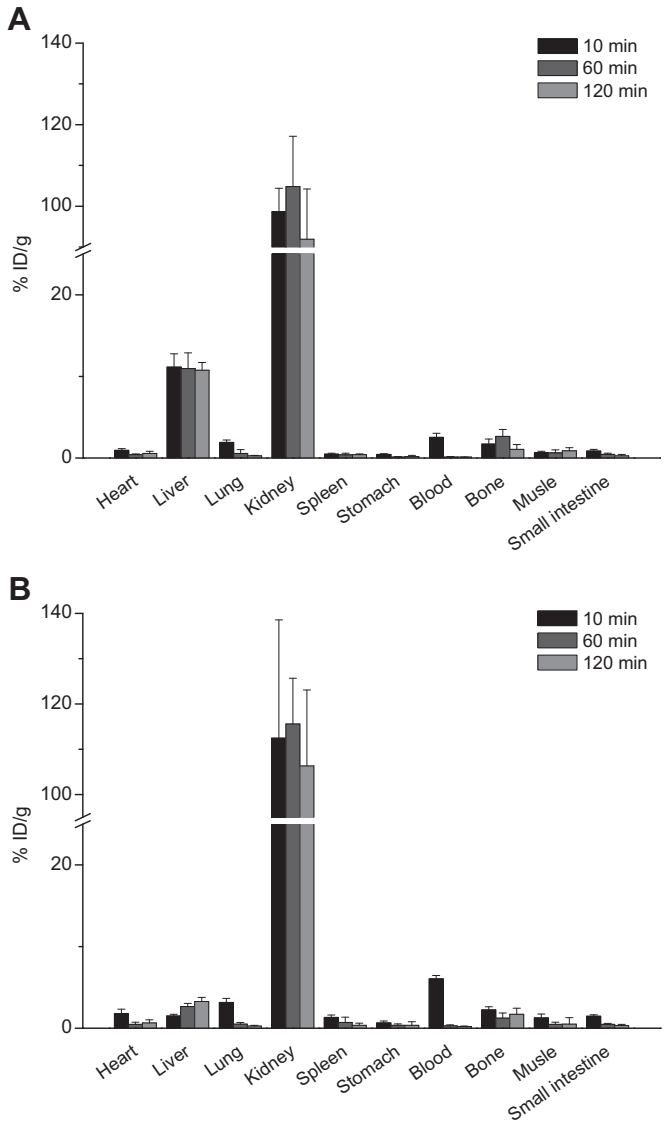

Figure 4. The biodistribution of the [^{18}F]FB-GC in normal mice: (A) The biodistribution after 10, 60 and 120 min following intravenous injection of [^{18}F]FB-GC into normal mice. (B) The results of blocking experiment with the preinjecting of free NGA at 10 mg/kg mice body weight. Data are expressed as mean% ID/g ± SD, n = 5.

Table 1
The liver/background ratios at different time points after injection of the [^{18}F]FB-GC and [^{18}F]FB-NGA in normal mice

Liver/background	[^{18}F]FB-GC			[^{18}F]FB-NGA	
	10 min	60 min	120 min	5 min	30 min
Liver/blood	4.40	84.55	91.13	23.23	5.80
Liver/kidney	0.11	0.11	0.12	28.37	0.69
Liver/heart	11.74	26.01	19.46	76.99	13.39
Liver/muscle	17.99	17.38	12.04	213.53	14.74
Liver/lung	5.84	19.58	39.95	40.64	7.95

of GC may affect the tracers' water solubility, receptor affinity, and metabolic rate, which could be the main reasons for the difference of in vivo distribution after intravenous injection of the GC.

We had reported the radiolabeling of NGA with ^{18}F for hepatic imaging with PET. The protein backbone of [^{18}F]FNGA is degraded after lysosomal proteolysis in liver. The radioactivity metabolite is quickly cleared from liver and eliminated from the body. When we use chitosan as backbone, the radioactivity shows slow elimination rate from the liver. The liver uptake at 120 min post-injection shows no significant different with that of at 10 min, this may due to the high in vivo stability of [^{18}F]FB-GC. The chitosan backbone may not be metabolized in the liver lysosome, the intact radiotracer remains in the liver for a long period. In the future, we will test and verify this assumption through experiments.

In order to further confirm that [^{18}F]FB-GC had specific receptor binding, blocking study was performed by conducting the biodistribution experiment in the presence of free NGA (10 mg/kg body weight) as blocking agent. Five minutes after the first injection of free NGA, [^{18}F]FB-GC was intravenously injected (about 0.037 MBq in 100 μL solution). Mice were sacrificed at selected time points (10, 60 and 120 min, n = 5). Results were expressed as the percentage of the injected dose per gram tissue (% ID/g). Averages and standard deviations were calculated.

The results are shown in Figure 4B. NGA (neoglycoalbumin) is known to have high affinity with the ASGP receptors and was selected as inhibitor in our study. The liver uptake was decreased significantly (P <0.01, unpaired two-tail t-test) in the blocking group at all selected time points (1.51 ± 0.20% ID/g, 2.65 ± 0.39% ID/g and 3.29 ± 0.47% ID/g at 10, 60 and 120 min post-injection, respectively) when compared with control group (without blocking). After blocking, the radioactivity in the blood only increased slightly at 5 min and there have no difference in blood uptake between blocking and control group after 60 min. The kidney uptake increased a little, while other organs had no significant changes. The results of ex vivo biodistribution showed that the [^{18}F]FB-GC has high affinity with the ASGP receptor, and its uptake in the liver via receptor-mediated.

The results of blocking assay were different from ^{99m}Tc-GSA. When excess inhibitor was preinjected, most receptors which situated on the surface of hepatocyte membrane were occupied. The following radiotracers would not be accumulated by the liver through receptor mediated, as result they circulated in the blood. After 30 min injection or longer, with the ASGP receptors re-circulating to the cell surface, ^{99m}Tc-GSA in blood would be re-accumulated by the hepatocyte. Because of recycling of receptors and high blood stability, the liver uptake of ^{99m}Tc-GSA between blocking and non-blocking was gradually converged after a period of time. Therefore, in the clinical use of ^{99m}Tc-GSA, the initial imaging of liver is very vague especially for the patient who has severe cirrhosis or cancer. The high radioactivity in hepatic blood pool will affect the quality of the liver imaging. On the other hand, the radioactivity accumulation in liver will increase gradually with the time, which makes it difficult to tell the imaging of a minor damaged liver from a normal one at 30 min or longer after injection. And due to the re-uptake of the radiotracer, the pharmacokinetic model may be more complicated. Facts mentioned above may have negative effects on accurate assessment of liver function. We expect the [^{18}F]FNGA may encounter the same problems with the ^{99m}Tc-GSA because of the natural character of albumin which they both use as backbone. Water-soluble chitosan will be cleared very rapidly from the bloodstream and excrete through kidney that shows different features from albumin. After the injection of [^{18}F]FB-GC, one part of the radiotracers would rapidly entry hepatocyte through ASGP receptor mediated endocytosis, while the others would be metabolized and excreted by kidney due to the natural characterization of water-soluble chitosan. After inhibition the radioactivity remaining in the blood could be cleared by kidney rapidly. The re-uptake phenomenon induced by the receptor re-circulated was not obvious. The features are different from those radiotracers using albumin as backbone, such as ^{99m}Tc-GSA and [^{18}F]FNGA. The [^{18}F]FB-GC might have an unique performance in liver imaging in the future application. It may not only provide clear imaging pictures and a distinct liver outline but also predict the liver function more accurate and simple.

In summary, galactosylated chitosan was successfully labeled with fluorine-18 through the prosthetic labeling group [^{18}F]SFB. We got [^{18}F]FB-GC with high radiochemical purity (>97%). [^{18}F]FB-GC had moderate activity accumulation in liver via ASGP receptor-mediated endocytosis. The other tissues except kidney showed relative low radioactivity uptake. The high liver/background ratio affords promising biological properties to get clear images. The specific binding of this radiotracer to the ASGP receptor was also confirmed by blocking experiment in mice. The blocking experiment of [^{18}F]FB-GC showed different features with ^{99m}Tc-GSA and [^{18}F]FNGA. [^{18}F]FB-GC might become a novel ASGP receptor imaging agent, which could improve imaging quality and simplify pharmacokinetic model of liver function.

Acknowledgments

The Project was sponsored by the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry, and partly by the National Natural Science Foundation of China (20871020), Beijing Natural Science Foundation (2092018) and the Scientific Research Foundation of Beijing Normal University. Dr. Yong He is acknowledged for his kindly donation of chitosan.

References and notes

1. Vera, D. R.; Stadalnik, R. C.; Krohn, K. A. *J. Nucl. Med.* **1985**, *26*, 1157.
2. Kubota, Y.; Kojima, M.; Hazama, H.; Kawa, S.; Nakazawa, M.; Nishiyama, Y.; Nakagawa, S.; Murase, T.; Okuno, H.; Naitoh, Y., et al *Jpn. J. Nucl. Med.* **1986**, *23*, 899.
3. Stadalnik, R. C.; Vera, D. R. *Nucl. Med. Biol.* **2001**, *28*, 499.
4. Kokudo, N.; Vera, D. R.; Makuuchi, M. *Nucl. Med. Biol.* **2003**, *30*, 845.
5. Ono, M.; Arano, Y.; Uehara, T.; Fujioka, Y.; Ogawa, K.; Namba, S.; Mukai, T.; Nakayama, M.; Saji, H. *Bioconjugate Chem.* **1999**, *10*, 386.
6. Yang, W.; Mou, T.; Zhang, X.; Wang, X. *Appl. Radiat. Isot.* **2010**, *68*, 105.
7. Jeong, J. M.; Hong, M. K.; Lee, J.; Son, M.; So, Y.; Lee, D. S.; Chung, J. K.; Lee, M. C. *Bioconjugate Chem.* **2004**, *15*, 850.
8. Wakisaka, K.; Arano, Y.; Uezono, T.; Akizawa, H.; Ono, M.; Kawai, K.; Ohomomo, Y.; Nakayama, M.; Saji, H. *J. Med. Chem.* **1997**, *40*, 2643.
9. Arano, Y.; Mukai, T.; Akizawa, H.; Uezono, T.; Motonari, H.; Wakisaka, K.; Kairiyama, C.; Yokoyama, A. *Nucl. Med. Biol.* **1995**, *22*, 555.
10. Vera, D. R. *J. Nucl. Med.* **1992**, *33*, 1160.
11. Yang, W.; Mou, T.; Peng, C.; Wu, Z.; Zhang, X.; Li, F.; Ma, Y. *Bioorg. Med. Chem.* **2009**, *17*, 7510.
12. Onishi, H.; Machida, Y. *Biomaterials* **1999**, *20*, 175.
13. Park, I.-K.; Yang, J.; Jeong, H.-J.; Bom, H.-S.; Harada, I.; Akaike, T.; Kim, S.-I.; Cho, C.-S. *Biomaterials* **2003**, *24*, 2331.
14. Kim, E. M.; Jeong, H. J.; Park, I. K.; Cho, C. S.; Kim, C. G.; Bom, H. S. *J. Nucl. Med.* **2005**, *46*, 141.
15. Kim, E. M.; Jeong, H. J.; Kim, S. L.; Sohn, M. H.; Nah, J. W.; Bom, H. S.; Park, I. K.; Cho, C. S. *Nucl. Med. Biol.* **2006**, *33*, 529.
16. Baldrick, P. *Regul. Toxicol. Pharm.* **2010**, *56*, 290.
17. Carreño-Gómez, B.; Duncan, R. *Int. J. Pharm.* **1997**, *148*, 231.