Radiosynthesis of A Novel Antisense Imaging Probe Targeting LncRNA HOTAIR in Malignant Glioma.

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Research Article

Keywords: LncRNA HOTAIR, antisense oligonucleotide probe, malignant glioma, antisense imaging

Posted Date: February 3rd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-152169/v1

Abstract

Background: Long non-coding RNA (lncRNA) HOTAIR was manifested overexpressing and amplifying in many human carcinomas, which could serve as a useful target for cancer therapy. The ^{99m}Tc radiolabeled antisense oligonucleotides (ASON) could visualize the expression of HOTAIR and provide a diagnostic and therapeutic value for malignant tumors. The aim of this study was to radiosynthesis ^{99m}Tc with HOTAIR ASON and investigate the in vivo imaging in malignant glioma xenografts.

Methods: The ASON targeting lncRNA HOTAIR as well as mismatched ASON probe (ASONM) were designed and modified. The radiolabeling of ^{99m}Tc with two probes was via the conjugation of bifunctional chelator HYNIC. Then the probes were purified by Sephadex G25 and tested for its radiolabeling efficiency and purity, as well as stability by iTLC and gel electrophoresis. Then the radiolabeled probes were transfected with lipofectamine 2000 for cellular uptake test and the next experimental use. Furthermore, biodistribution study and SPECT imaging was performed at different time after probes were intravenously injected in U87 tumor bearing nude mice models. All data were analyzed by statistical software.

Results: The labeling efficiencies of ^{99m}Tc-HYNIC-ASON and ^{99m}Tc-HYNIC-ASONM measured by iTLC were (91 ±1.5) % and (90 ±0.6) %, respectively, and the radiochemical purity were more than 89%. The probes showed good stability within 12 hours for high radiochemical purity. Gel electrophoresis confirmed that the oligomers were successfully radiolabeled no significant degradation were found. Cellular uptake experiment showed that liposomes had ability to carry probes into cells. Biodistribution study demonstrated that liposome coated ^{99m}Tc-HYNIC-ASON had significantly higher uptake in the tumor and higher tumor to muscle ratio than mismatched group. Meanwhile tumor was clearly shown at 1 hours post probe injection of Liposome coated ^{99m}Tc-HYNIC-ASON on SPECT/CT imaging, compared with mismatched and blocking group.

Conclusion: The Liposome encapsulated ^{99m}Tc-HYNIC-ASON probe can be radiosynthesized and used in the in vivo, real-time imaging of lncRNA HOTAIR expression in malignant glioma.

Background

Glioma is the most common proliferative and reversible primary brain tumor in human beings[1]. Approximately 80% of the malignant tumors of the central nervous system are gliomas[2]. Although treatment approaches such as surgery, radiotherapy and chemotherapy have been widely used, the median survival time of glioma patients is still limited to about 14 months[3 4]. Therefore, new and more effective approaches are urgently needed. LncRNA, a kind of mRNA-like transcripts as 200 nt to 100 kilobases (kb), have no protein coding potential however its roles of gene transcription and translation, heredity and epigenetic regulation in tumors has gained increasing attention in recent years[4]. It was found that LncRNA are widely expressed in human cancers, indicating that it may be associated with

cancer onset and progression[5]. HOTAIR, as the first lncRNA with trans-regulatory function is overexpressed in glioma and silencing tumor suppressor genes by mediates recruitment of polycomb repressive complex 2 (PRC2) [6 7]. A previous literature reported that HOTAIR can affect the cell cycle, and the decrease of HOTAIR expression will lead to a significant increase in G0/G1 phase cells[3]. Meanwhile HOTAIR has been shown to be positively correlated with tumor cell proliferation, survival, invasion and resistance to treatment, via molecules such as chromatin modifiers, ubiquitin ligases and miRNAs[8]. These findings suggest that the high expression of HOTAIR is a biomarker of cancer diagnosis, metastasis, drug resistance and poor prognosis. The fact that HOTAIR is stable and measurable in body fluids has diagnostic and prognostic value for glioma[9 10].

Antisense imaging is an attractive and non-invasive method for detecting the expression of HOTAIR in tumors. It visually represents, characterizes and quantifies biological processes at the cellular or subcellular level by linking radionuclides to targeted antisense oligonucleotides[11 12].

Antisense oligonucleotide (ASON) technique uses a completely (or nearly complete) complementary single strand of 15 to 25 nucleotides that binds to the target RNA to produce DNA RNA heteroduplex, which can lead to the silencing of the target gene or interfere with the processing or translation of RNA[13,14]. Preclinical studies have shown that after chemical synthesis and modification of ASON, the nuclease degradation rate is decreased, while enhanced the affinity of plasma binding protein , the tissue biological distribution is rapid, thus makes ASON a good biological effect and stability[15 16 17], which can be used as a feasible human treatment strategy. As a molecular imaging technology, It has attracted much attention because of its high sensitivity, high resolution and short acquisition time and detect the dynamic changes of tumor-related molecules in real time, so it also has a great application prospect in early diagnosis [18].

Considering the role of HOTAIR in tumorigenesis and treatment, as well as the efficiency, specificity and irreversibility of antisense oligonucleotides binding to the target, the objective of the present study was to explore the ^{99m}Tc labeled antisense oligonucleotide probe targeting HOTAIR mRNA, evaluate its characteristics in vitro, and observe whether it can be used in the imaging of gliomas.

Materials And Methods

The 19-base oligonucleotide probe was chemically synthesized and modified from Shanghai Shangon Bioengineering Co., Ltd. The ASON sequence was designed as 5'- AATTCTTAAATTGGGCTGG -3', which was completely complementary to the HOTAIR fragment, and the ASONM□mismatched antisense oligonucleotides□ sequence was 5'-AATACTTAGATTAGGCAGG-3' (The underlined part is the substituted nucleosides).Two probes were modified with 2' methylation at both ends of the sequence and two bases at each end were thiomodificed to improve the stability. A primary amine structure is connected at the 3 'end, and $(CH_2)_6$ is used as the connector between the NH_2 and the skeleton. Ultimately, the synthetic

structure was 5'-CH_2 ASON/ASONM-$(CH_2)_6$-NH_2-3'. ^{99m}Tc is obtained from the ^{99m}Tc radionuclide generator produced by China Atomic Energy Research Institute.

Synthesis and labeling of the probe

4OD ASON dissolved in 50μl buffer (2mol/l NaCl, 0.5mol/l $NaHCO_3$, 2mmol/l EDTA), HYNIC (TriLink, US) dissolved in DMF solution (10mg/ml). Then mixed at a molar ratio of 25:1(HYNIC: ASON) and avoid light for 1 h. Then added 60% methanol to the total volume of 500μl, using ultrafilter tube(Sartorius, GER) 15000g/rcf to centrifuge for 10min (ensure that the volume after centrifugation was less than 50μl). Next, added 100μl Tricine(100mg/ml), 30μl ^{99m}Tc(222MBq) as well as 4μl fresh SnCl2•2H2O (1mg/ml) to the above reactants in turn, reacted for 60min. After the reaction, using Sephadex G25(GE, US) to separate and purify. 15 tubes of eluent were collected, then measured the radioactivity counts and nucleic acid concentration of each tube. Take the peak tube for the following up experiment.

Serum stability

Fresh human serum is provided by volunteers in our department. The institutional review committee of the General Hospital of Ningxia Medical University approved the study and all the volunteers obtained informed consent. And this experiment is carried out in accordance with the Helsinki Declaration. ^{99m}Tc-HYNIC-ASON was incubated in saline and fresh human serum at 37℃ and room temperature, respectively (the volume ratio of probe to serum / saline was 1:1). The radiochemical purity was detected by thin layer paper chromatography (iTLC) at 0, 2, 4, 6, 8, 12 h.

Agarose gel electrophoresis

To identify the integrity of probes and eliminate the degradation of antisense oligonucleotides after labeling. 1% agarose gel was configured, followed by unbonded ASON sample, ^{99m}Tc, ^{99m}Tc-HYNIC-ASON before and after purification. The voltage was 120V, electrophoresis for 20 minutes, then the band was observed under UV.

Cell culture and transfection.

U87 glioma cells were purchased from Chinese Academy of Sciences, and cultured in DMEM(Invitrogen,US) medium containing 15% fetal bovine serum and 1% antibiotics, in a CO2 incubator at 37 ℃ for 24 hours, then passaged when the cell density reached 90%.

For transfection (Lip-^{99m}Tc-HYNIC-ASON and Lip-^{99m}Tc-HYNIC-ASONM), 10μg purified ^{99m}Tc-HYNIC-ASON/ ^{99m}Tc-HYNIC-ASONM were added to 500μl DMEM without serum and antibiotics; 25μl Lipofectamine 2000 (Invitrogen,US) were added to 475μl DMEM, and the mixtures were placed at room temperature for 5min respectively, then mixed two of them for 20min. After added liposome-coated ^{99m}Tc-HYNIC-ASON/ ^{99m}Tc-HYNIC-ASONM to the walls and cultured in a 37℃ incubator for 6 hours, the

medium was replaced by DMEM contained 15% fetal bovine serum for 24-48 hours to complete transfection.

Cellular uptake

U87 cells were inoculated in 12-well plate at 1 × 10^5 density and cultured overnight in DMEM containing 15%FBS without antibiotics. The cells were divided into liposome transfected and non-transfected group. In the transfection group, 200μl DMEM, 500ng ^{99m}Tc-HYNIC-ASON or ^{99m}Tc-HYNIC-ASONM (37kBq) and 3μl Lipofectamine 2000 were added into each well, 500ng ^{99m}Tc-HYNIC-ASON or ^{99m}Tc-HYNIC-ASONM (37kBq) in the non-transfection group. After cultured in 37 ℃ incubator, each well of culture medium was collected and washed three times with 100μl PBS collected into the same EP tube, labeld Cout at 0.5h,1h, 2h, 4h, and 6h. Then the cell was collected with trypsin containing EDTA, 100μl PBS in each well was washed three times to EP tube labeled Cin. The radioactivity counts of Cin and Cout were measured by γ radioimmunoassay counter(Chinese Academy of Metrology), and the uptake rate of cells to the probe at each time was calculated. Calculation formula: cell uptake rate = Cin/ (Cin+Cout) × 100%.

Animal Xenograft Model

BALB/c nu/nu mice (female, weight ±SD, 206.0g, age 3~4wk) were fed in the Experimental Animal Center of Ningxia Medical University. Malignant glioma U87 cells (5×10^{11}) were subcutaneously injected into the right fore axilla of each mouse. When the diameter of the tumor reached 1.0-1.5cm, it was used in the follow-up experiment. All animal experiments have passed the ethical review of the Animal Experimental Center of Ningxia Medical University and are conducted in accordance with the guidelines of the Animal Welfare Committee.

Biodistribution Studie

Twenty nude mice were randomly divided into 5 groups with 4 mice in each group. Lip-^{99m}Tc-HYNIC-ASON 1μg, 2.59MBq (100μl) was injected into the tail vein. Then the mice were killed by cervical dislocation at 1, 2, 4, 6 and 8 hours after 100μl of blood was taken from ophthalmic vein. After that, tissues like Heart, liver, spleen, kidney, stomach, small intestine, bladder, muscle, bone and tumor were removerd and weighed, then radioactivity count was measured. The distribution results were recorded as the percentage of radioactivity per gram of tissue(% ID/g).

SPECT Imaging

Images were performed by a SPECT scanner at 1h, 2h, 4h, 6h and 8h respectively after 4μg, 14.8MBq (150μl) Lip-^{99m}Tc-HYNIC-ASON/ ^{99m}Tc-HYNIC-ASONM probe were injected into the tail vein. For blocking groups, 10μg liposome transfected unlabeled probes were injected 2 hours before Lip-^{99m}Tc-HYNIC-ASON injection.The collection counts was 100 KCous and stored as a 64×64 matrix at 3.2 zoom, then T/M (Tumor/Muscle) and T/A (Tumor/Abdomen) ratio of regions of interest were calculated.

Statistical analysis.

Establish a database, all data are processed by SPSS22.0 statistical software, variables are represented by average±SD. The statistical comparison between variables was carried out by analysis of variance (ANOVA). $P<0.05$ is considered to be statistically significant.

Results

Synthesis and labeling of the probe

iTLC paper chromatography used saline as the developer, the radioactivity of the antisense probe before and after purification remained in the same position(1.18 Minutes)(Fig 1). The labeling rate of ^{99m}Tc-HYNIC-ASON and ^{99m}Tc-HYNIC-ASONM measured by iTLC were (91±1.5)% and (90±0.6)%, respectively and both radiochemical purity were more than 89% after purification.

Radioactivity countsand nucleic acid concentration

After separated and purified by Sephadex G25, the mixture was collected in one tube every five drops, and then the radioactivity counts and nucleic acid concentration in each tube were measured. In Fig 2b, the unbonded ASON fragments were filtered out by 1-4 tubes, and the eluent of 4-8 tubes was ^{99m}Tc-HYNIC-ASON, which coincided with the peak of radioactivity counts in Fig 2a.

Serum stability and agarose gel electrophoresis

The radiochemical purity of ^{99m}Tc-HYNIC-ASON reached more than 80% in 12 hours, and there was no significant difference between saline and fresh human serum (Fig 3a). Figure 3b shows brighter bands before and after purification, indicating that the oligonucleotide probe has integrity and there is no obvious degradation and miss target. The results showed that ^{99m}Tc-HYNIC-ASON had good tolerance and stability in serum at 37 ℃, which was similar to that in vivo.

Cellular uptake

At each time point, the uptake rate of transfection groups were higher than that of the non-transfection group, and reached the peak at 2 hour. The cellular uptake of Lip-^{99m}Tc-HYNIC-ASON at 2 h was 3.0%, while that of Lip-^{99m}Tc-HYNIC-ASONM was only 0.6% (Fig 4) .

Biodistribution Studies

Lip-^{99m}Tc-HYNIC-ASON and mismatched probes had similar biological distribution after injection (listed in Tables 1 and 2). The two probes were mainly concentrated in the kidney and bladder, followed by the stomach and small intestine, indicating that the probes were cleared through the urinary and digestive system. Secondly, for the organs with rich blood supply, such as heart, liver, spleen and lung, the uptake of the two probes decreased gradually due to the effect of blood clearance. While the uptake rate of probe

by other organs such as skeletal muscle and bone is low. With the extension of time, after injection of Lip-^{99m}Tc-HYNIC-ASON the radioactivity in the kidney decreased rapidly, from (6.25 ±1.31)% to (2.63 ±0.55)%, while the radioactivity in the tumor decreased slowly in 8 hours, from (1.73 ±0.13)% to (0.58 ±0.1)%. It suggested that the Lip-^{99m}Tc-HYNIC-ASON targeted probe can be specifically aggregated in the tumor. This result was also confirmed by the ratio of tumor to non-tumor (T/NT) of Lip-^{99m}Tc-HYNIC-ASON (Fig 5).

SPECT Imaging

The static images were obtained within 8 hours after injection. According to the prediction of the experimental results of biological distribution, radioactivity is mainly concentrated in the abdomen. After injection of Lip-^{99m}Tc-HYNIC-ASON, the tumor was clearly visible at 1-8 h. In contrast, the tumor was not shown within 8 hours after injection of the mismatched probe group and blocking group(Fig 6).The ratio of T/M and T/A in antisense group was significantly higher than that in the mismatched group. 2h after injection, the maximum of T/M ratio in antisense group was 4.77, while that in the mismatch group was only 2.59. (Fig 7).

Discussion

With the successful application of antisense oligonucleotide technique combined with radionuclide tracer technique, precise tracking method at molecular level is used for tumor localization diagnosis and therapy. In the present study, we successfully labeled antisense oligonucleotide probe targeting LncRNA HOTAIR with ^{99m}Tc to track the expression of HOTAIR in glioma cells. We found that, in the labeling experiment, the chemically synthesized and modified probes can successfully connect with ^{99m}Tc and obtain a higher labeling rate; At cellular level, liposomes can effectively assist the probe to enter tumor cells; From imaging results, antisense probes can detect specific radioactivity uptake in the tumor site, while no radioactivity uptake was found in the mismatched group.

In this study, we try to adjust the ratio of HYNIC to ASON(25:1) and replace the buffer solution(2mol/l NaCl, 0.5mol/l $NaHCO_3$, 2mmol/l EDTA) that dissolves ASON to improve the labeling rate. The effect is obvious. The average labeling rate of antisense probe and mismatched probe is more than 90%, but the radiochemical purity is lower than that of other literatures [19 20 21].

Comparing the differences of the two probes between liposome transfection and non-transfection, we found that the uptake rate of the transfected antisense probe in tumor cells was the highest which indicated that the antisense probe sequence was specifically targeted. Meanwhile it can be concluded that liposomes can effectively carry probes through the phospholipid bilayers on the cell surface. However this metastasis is not an one-way street, as the mismatched probes encapsulated by liposomes do not bind specifically, while it does not accumulate a large number of radioactivity in the cells can explain this problem.

According to the biological distribution study, the antisense probe has specific uptake in the tumor, meanwhile the most radioactivity is accumulated in the kidney and bladder, indicating that the probe is mainly excreted through the urinary and digestive system. However, there is not much radioactivity accumulation in the liver, which is different from other reports that the liver has the highest radioactivity uptake in all organs [11 12 21 22]. The reason might be that the molecular of Lip-^{99m}Tc-HYNIC-ASON probe is small and does not need to be digested by macrophages when passing through the liver, hense, caused a short retention time and a mild potential toxicity to the liver.

In the imaging experiment, tumor was clearly visualized in antisense group, and there is a large amount of radioactivity in the abdomen and bladder, which is consistent with the biological distribution. No tumor imaging was seen in the blocking group within 8 hours, indicated that unlabeled ASON could block the binding of ^{99m}Tc-HYNIC-ASON to HOTAIR, and the binding was specific. The fact that there is no imaging in the mismatch group also illustrates this problem.

In summary, these results demonstrated that ^{99m}Tc-HYNIC-ASON can be successfully synthesized and used for glioma-specific imaging via transfected with lipofectamine. However, there are still some limitations in this study that need to be addressed in future investigations. First of all, the high uptake of radioactive probes in the bladder and kidney will affect the image quality and blur the display of the tumor. The application of furosemide, a highly effective diuretic which has a strong diuretic effect can dilate the blood vessels of the kidney quickly and briefly, may be helpful to accelerate the excretion of radioactivity. Secondly the higher uptake of stomach and small intestine , which will cause radioactive damage to tissue and lead to dysfunction, so we speculate whether gastric mucosal protective agent could help to reduce mucosal radioactive damage and higher uptake. These are the problems that we need to solve next.

Conclusion

The liposome coated ^{99m}Tc-HYNIC-ASON probe can be used for real-time imaging of lncRNA HOTAIR expression in malignant gliomas in vivo. The probe has good stability and targeting ability, which is a new type of non-invasive probe.

Declarations

ACKNOWLEDGMENT

We thank He Yong and Li Chongjiao from the people's Hospital of Wuhan University for their help. This study was supported by the National Natural Science Foundation of China (NSFC 81560292).

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection were performed by [Xiyuan Zhang]□[Jinag Cao]□[Jiongyu Ren]□[Jiali Tian]□[Jin Luo]□[Yaping Yu]□[Jiamiao Li].

Analysis were performed by[Qian Zhao][Fengkui Wang]. The first draft of the manuscript was written by [Jiongyu Ren] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflicts of interest/Competing interests

There are no conflicts of interest to disclose to this article.

Disclosure of potential conflicts of interest

There are no conflicts of interest to disclose to this article.

Approval for animal experiments

BALB/c nu/nu mice (female, weight ±SD, 206.0g, age 3~4wk) were purchased and fed in the Experimental Animal Center of Ningxia Medical University. All animal experiments have passed the ethical review of the Animal Experimental Center of Ningxia Medical University and are conducted in accordance with the guidelines of the Animal Welfare Committee.

All the experimenters follow the ARRIVE guidelines. The experimental operation is described in detail in the method section of the article. All animals were killed by cervical dislocation.

Human Subjects

The institutional review committee of the General Hospital of Ningxia Medical University approved the study. The human serum came from volunteers, and all the volunteers obtained informed consent. All experiments involving humans are carried out in accordance with the Helsinki Declaration.

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Tables

TABLE 1. Biodistribution (%ID/g) of Lip-^{99m}Tc-HYNIC-ASON in Tumor-Bearing Mice

Tissue	1h	2h	3h	4h	6h
Heart	1.68±0.38	1.73±0.33	1.32±0.1	0.82±0.15	0.59±0.21
Blood	1.37±0.5	0.81±0.58	0.56±0.4	0.66±0.17	0.18±0.27
Liver	1.22±0.42	1.28±0.52	0.83±0.32	0.88±0.08	0.61±0.29
Spleen	0.61±0.05	0.45±0.05	0.38±0.14	0.29±0.04	0.21±0.04
Lung	1.5±0.24	1.37±0.35	1.01±0.37	0.58±0.08	0.37±0.22
Kidney	6.25±1.31	5.36±1	5.38±1.52	3.95±0.67	2.63±0.55
Stomach	2.3±1.29	2.86±0.46	2.7±0.57	1.46±0.14	1.31±0.43
Small intestine	3.43±0.82	3.17±0.35	3.06±0.82	2.21±0.66	2.14±0.67
bladder	11.89±0.9	11.7±0.75	9.81±1.62	9.12±1.66	8.75±0.95
Skeletal muscle	0.81±0.06	1.73±0.23	2.68±2.35	1.01±0.48	1.76±2.37
Bone	0.63±0.32	1.96±1.53	0.46±0.32	0.87±0.6	0.66±0.6
Tumor	1.73±0.13	1.22±0.1	1.06±0.22	0.82±0.22	0.58±0.1
Each value represents average of 4 mice ± SD.					

TABLE 2. Biodistribution (%ID/g) of Lip-^{99m}Tc-HYNIC-ASONM in Tumor-Bearing Mice		
Tissue	2h	4h
Heart	0.31±0.13	0.35±0.16
Blood	0.03±0.01	0.06±0.04
Liver	0.52±0.25	0.6±0.13
Spleen	0.19±0.1	0.31±0.07
Lung	0.43±0.38	0.54±0.14
Kidney	3.21±0.54	2.73±0.48
Stomach	0.42±0.18	0.64±0.19
Small intestine	0.21±0.1	0.22±0.13
bladder	2.62±3.82	1.26±0.77
Skeletal muscle	0.2±0.13	0.45±0.13
Bone	0.31±0.14	0.3±0.19
Tumor	0.43±0.28	0.3±0.1
Each value represents average of 4 mice ± SD.		

Figures

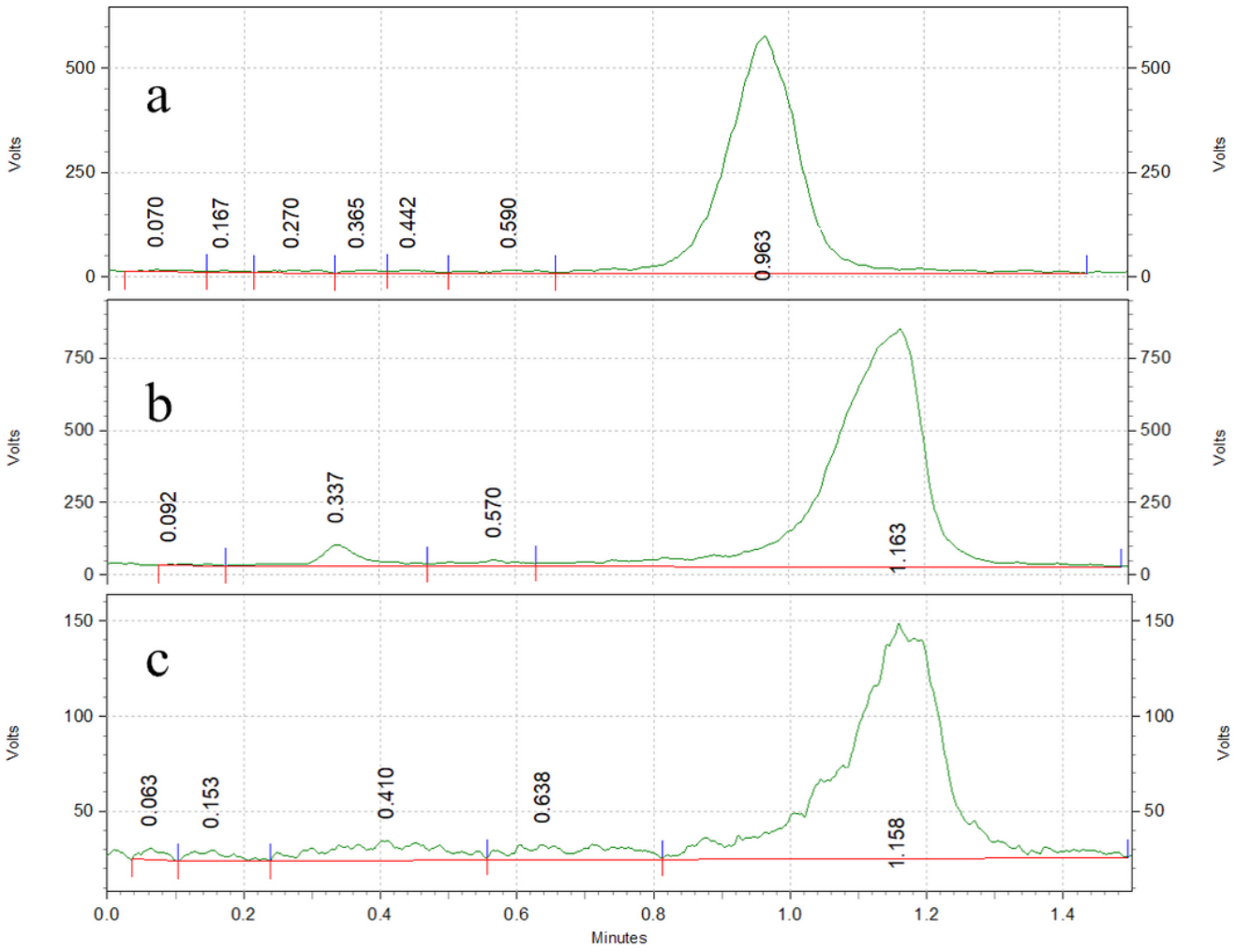

Figure 1

iTLC (a: 99mTc; b: 99mTc-HYNIC-ASON before purification; c: 99mTc-HYNIC-ASON after purification)

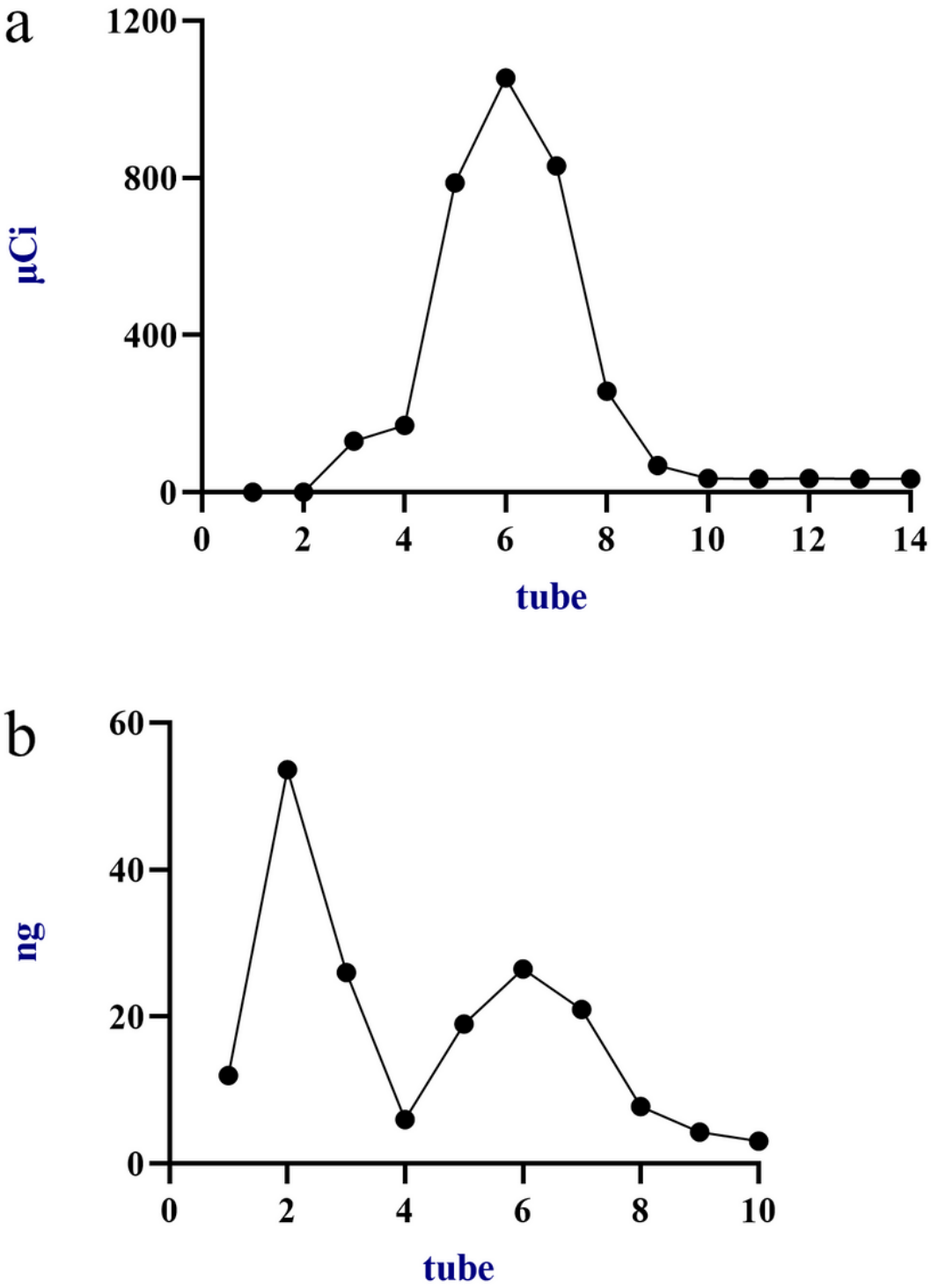

Figure 2

radioactivity counts (a) and nucleic acid concentration (b) per tube purified by Sephadex G25.

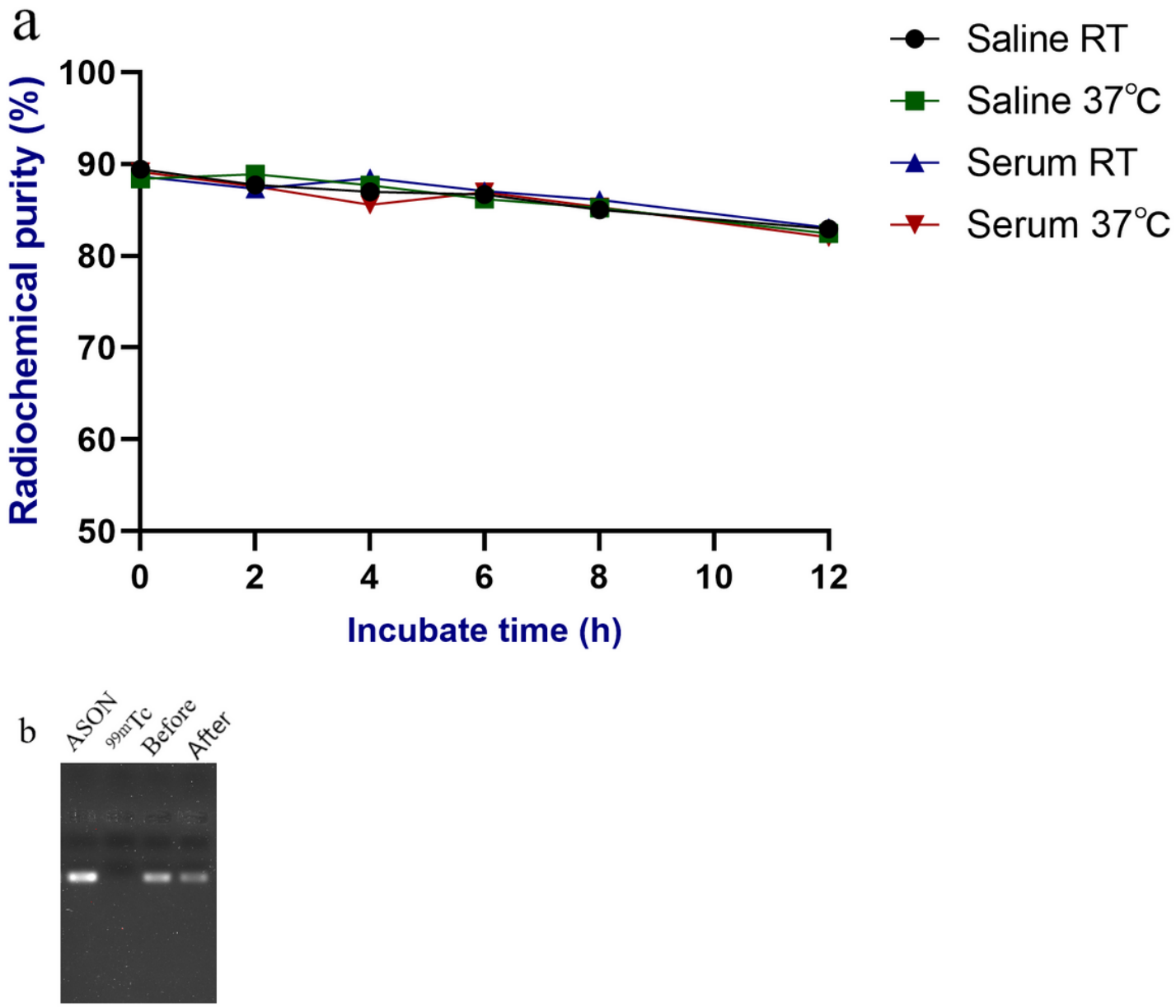

Figure 3

Probe stability identification a radiochemical purity of 99mTc-HYNIC-ASON incubated in saline and fresh serum during 6h, at room temperature (RT) and 37℃. b: the probe integrity was detected by agarose gel (from left to right: ASON sample, 99mTc, 99mTc-HYNIC-ASON before purification, 99mTc-HYNIC-ASON after purification). (full-length gel is presented in Supplementary Figure 1.)

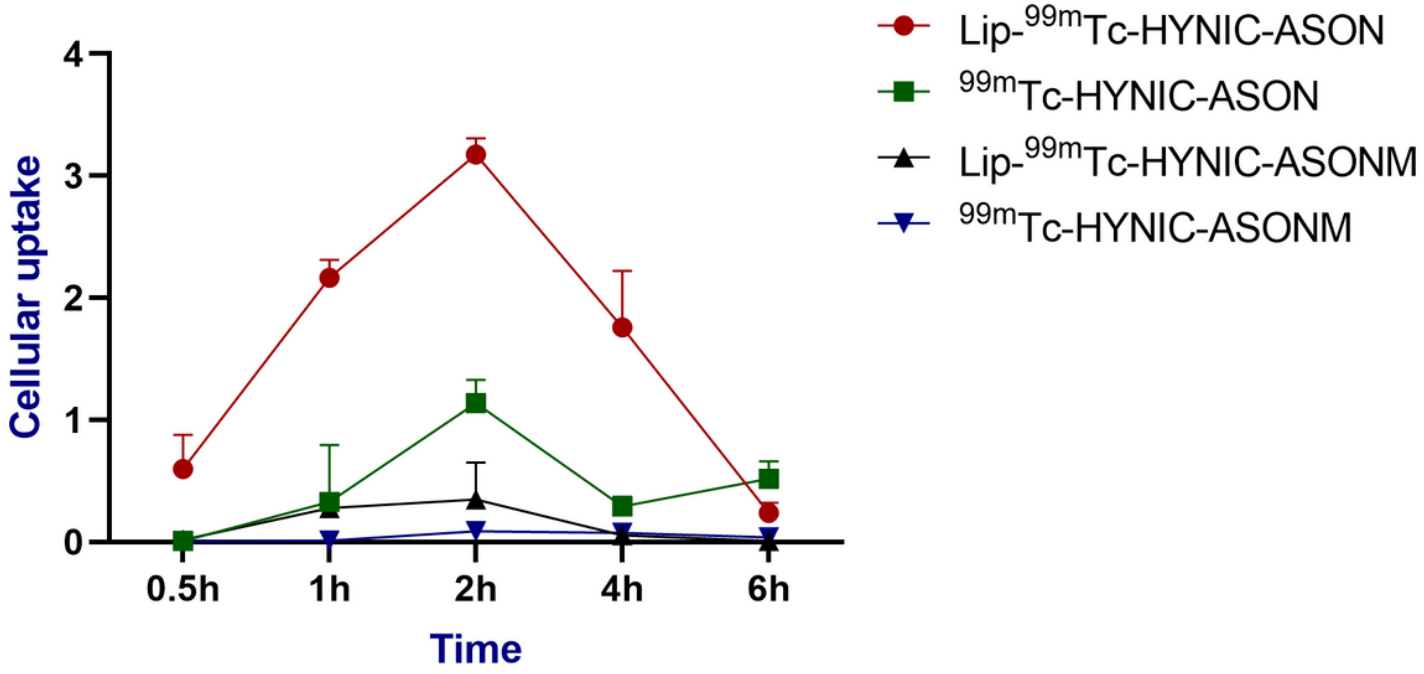

Figure 4

The in vitro cell uptake rates of 99mTc-HYNIC-ASON and mismatch groups with or without transfection at 30min, 1h, 2h, 4h and 6h.

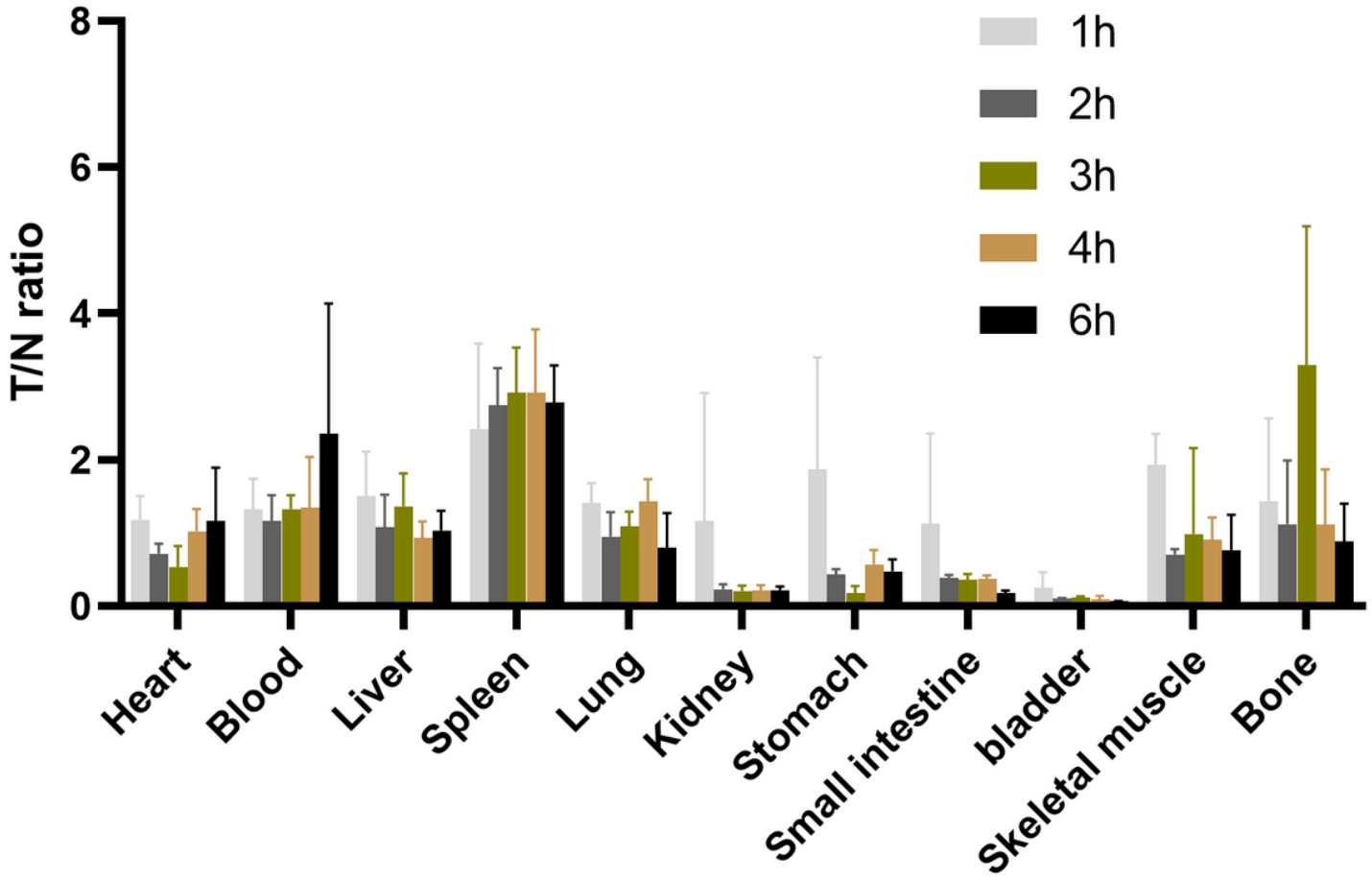

Figure 5

T/NT(Tumor and Non tumor tissues) ratio of Lip-99mTc-HYNIC-ASON in different tissues of glioma nude bearing mice xenografts at 0.5, 1, 2, 4, and 6 h after injection.

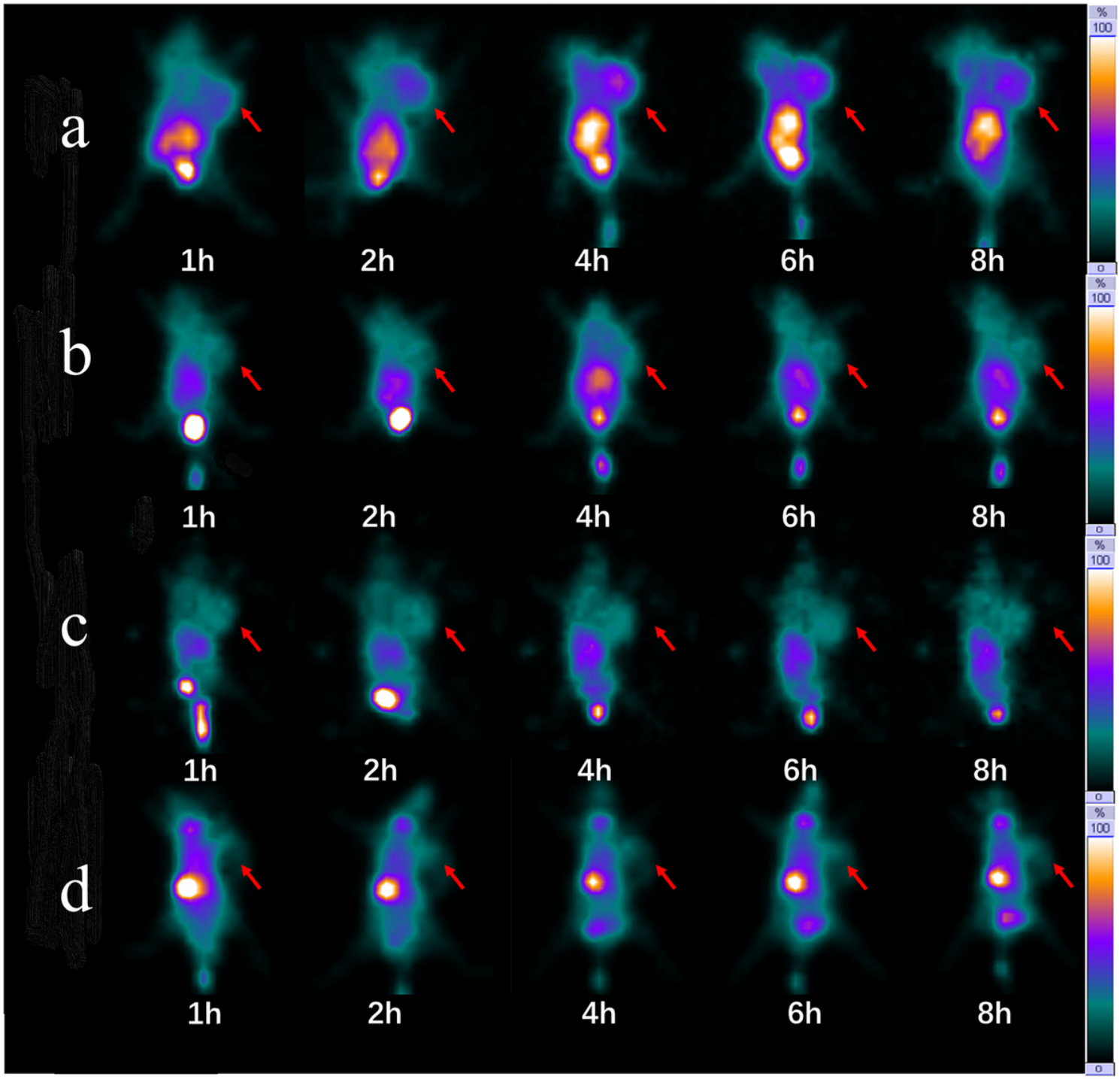

Figure 6

SPECT Imaging at 1 h, 2 h, 4 h, 6 h and 8 h after injected to glioma nude bearing mice xenografts. a: Lip-99mTc-HYNIC-ASON; b: Lip-99mTc-HYNIC-ASONM; c: Blocked; d: Tc-control. Tumor (arrow) was clearly visualized after injected with Lip- 99mTc-HYNIC-ASON, while is not visualized in mismatched□Blocked as well as Tc-control group.

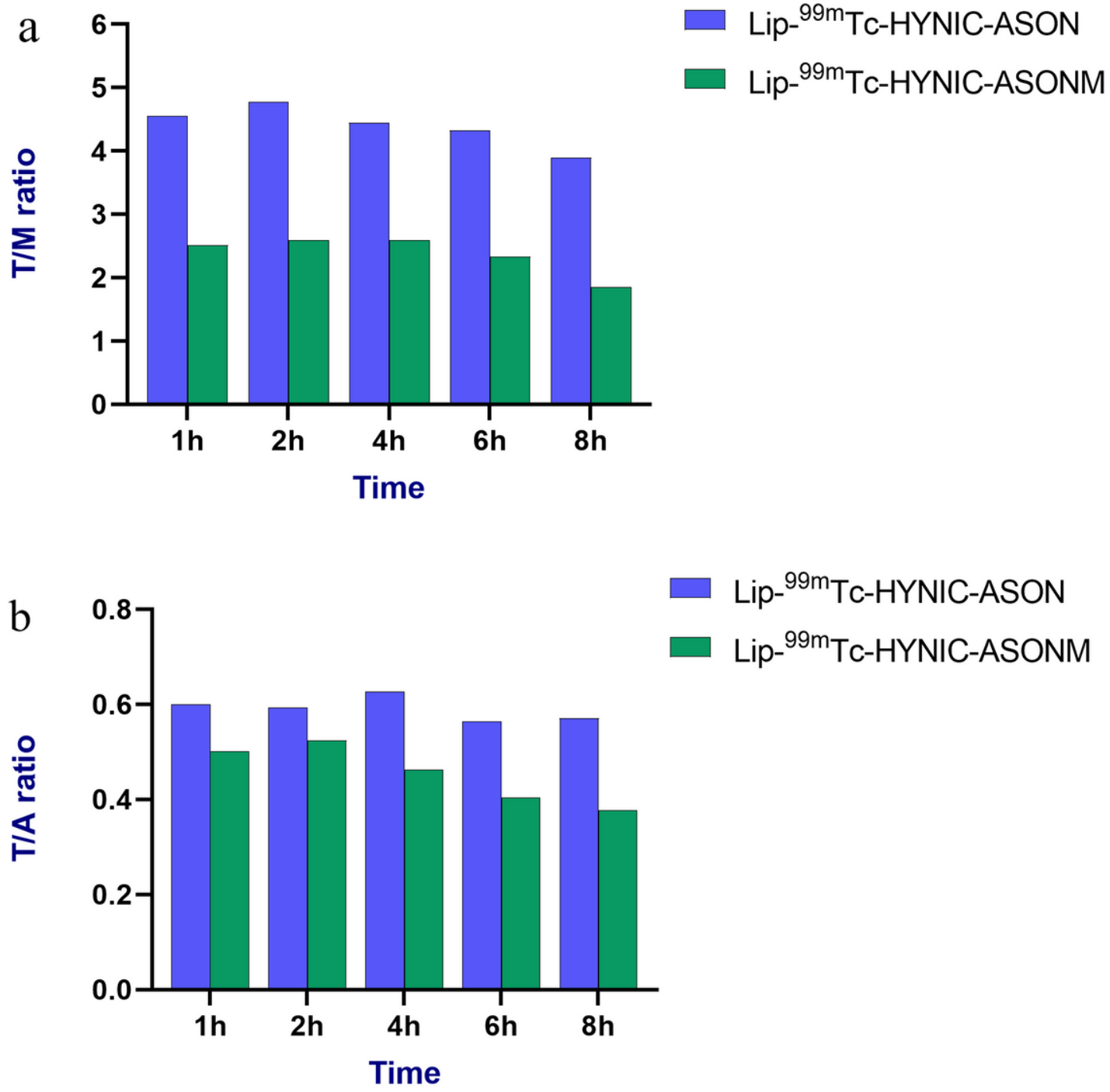

Figure 7

T/M(Tumor and Muscle)、T/A(Tumor and Abdomen) ratio of Lip- 99mTc-HYNIC-ASON and Lip-99mTc-HYNIC-ASONM. At all time, ratio of T/M and T/A is significantly higher in antisense groups than mismatched groups.

Supplementary Files

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- Supplementaryinformation.docx