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A generally adoptable radiotracing method for tracking carbon nanotubes in animals

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Abstract

Carbon nanotube (CNT) mediated drug delivery systems have currently aroused a great deal of interest. Such delivery systems for drugs, proteins and genes have been preliminarily studied using cellular and animal models. For the further study of the pharmacokinetics and related biological behaviours of CNTs *in vivo*, a fast and convenient tracing method is particularly demanded. In this paper, we developed a generally adoptable tracing method for the biodistribution study of functionalized CNTs *in vivo*. Taurine covalently functionalized multi-walled carbon nanotubes (tau-MWNTs) and Tween-80 wrapped MWNTs (Tween-MWNTs) were labelled with ^{125}I, and then their distribution in mice was determined. It is interesting that Tween-80 can reduce the RES uptake of MWNTs remarkably. The resulting distribution of ^{125}I-tau-MWNTs was very consistent with that using ^{14}C-taurine-MWNTs as the CNTs tracer, which means the easy ^{125}I labelling method is reliable and effective.

1. Introduction

Interesting biomedical applications of carbon nanotubes (CNTs), such as a delivery vehicle for drugs [1, 2], proteins [3, 4], genes [5, 6] and vaccine [7], have been proposed and great progress has been recently achieved in *in vitro* studies. However, these applications in animals and humans are held back, because basic knowledge of their behaviour *in vivo* is seriously scarce.

As an important indication of the *in vivo* behaviour, pharmacokinetics has not been extensively reported yet, likely due to the lack of suitable analytical methods. Most of these studies so far were carried out by using radioisotopes, including ^{125}I, ^{111}In, ^{64}Cu, ^{99m}Tc and ^{14}C. Indubitably, radioisotope tracing is an effective and indispensable technique to study the *in vivo* absorption, distribution, metabolism, excretion and toxicity (ADME/T) of xenobiotics [8]. We first reported the biodistribution of hydroxylated SWNTs in mice using ^{125}I-labelling [9]. Then, Singh *et al* reported the biodistribution of ^{111}In-DTPA (diethylenetriaminepentaacetic acids)-CNTs in mice [10]. Dai *et al* reported the biodistribution of ^{64}Cu-DOTA (1,4,7,10-tetra-azacyclododecane N,N',N'',N'''-tetraacetic acid)-PEG (polyethylene glycol)-CNTs in normal mice and tumor-bearing mice [11]. Recently, we successfully tracked the translocation pathways and target organs of MWNTs in mice using water-soluble ^{14}C-taurine-MWNTs as the CNT tracer [12]. Finally Guo *et al* reported the biodistribution of glucosamine-modified MWNTs using a ^{99m}Tc-MWNT-G tracer [13].

For further study of pharmacokinetics and related biological behaviour of different functionalized CNTs in animals, a generally adoptable tracing method is particularly demanded to accelerate these related studies.

In this paper, ^{125}I-radiolabelling is used as a generally adoptable method to track CNTs *in vivo*. The absorption and distribution of ^{125}I-taurine-MWNTs (tau-MWNTs) in mice were quantitatively determined and compared with those of ^{14}C-tau-MWNTs published recently. Very analogous

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distribution profiles of the two radiolabels are presented. Further, we extended the ^{125}I-radiolabelling method to determine the biodistribution of Tween-80 dispersed MWNTs (Tween-MWNTs) *in vivo*.

2. Experimental details

2.1. Materials

The raw MWNTs were purchased from Shenzhen Nanoharbor Company, China, and produced by the chemical vapour deposition (CVD) method with a diameter of 10–20 nm, lengths ranging from several microns to tens of microns, and a purity of >95%. Transmission electron microscopy (TEM), thermogravimetric analysis (TGA) and ICP-MS were employed to further characterize the MWNT samples.

The carrier-free Na ^{125}I was purchased from the China Institute of Atomic Energy, Beijing, and other chemicals were purchased from Beijing Chemical Co., Beijing.

2.2. Synthesis of ^{125}I-tau-MWNTs

Tau-MWNTs used in this work were prepared and characterized as described in [12]. The tau-MWNTs were easily labelled with ^{125}I by the iodogen (1, 3, 4, 6-tetrachloro-3α-6α-diphenylglycouril) method. The iodine atom can easily covalently bind to the position on the analogous phenyl structure on the sidewall of the CNTs, but not on the taurine molecule $H_2N(CH_2)_2SO_3H$. In brief, iodogen (25 μl in a 2 mg ml^{-1} chloroform solution) was coated on the inner wall of an Eppendorf tube by evaporating chloroform. To start the labelling, 100 μl tau-MWNTs (ca. 200 μg) and 20 μl carrier-free Na ^{125}I (5 μCi ^{125}I) were added to the iodogen tube. After incubation under ultrasonication for a certain time at room temperature, the mixture in the tube was transferred to another tube to stop the labelling process. The labelling efficiency was examined by thin layer chromatography (TLC, silica gel, eluting solvent: V_{acetone}:V_{water} = 10:1, $\text{Rf}_{\text{I-tau-MWNTs}}{}^{125}$ = 0). Seven different reaction times (5 min, 10 min, 30 min, 1 h, 2 h, 4 h and 8 h) were tested to determine the best labelling condition.

Radioiodinated tau-MWNTs were separated from free ^{125}I ions via dialysis against deionized water for several days until little radioactivity was detected in the dialysate. The final solution was measured on a γ detector (E 5002, Packard Bioscience, USA) with an energy range of 15–75 keV. The radioactivity was represented as counts per min (cpm). The radiochemical purity of ^{125}I-tau-MWNTs was examined by TLC.

The stability of the ^{125}I-tau-MWNTs in serum was assayed as follows: 50 μl ^{125}I-tau-MWNTs was added to 300 μl mouse serum and incubated. After a certain interval, the radiochemical purity of ^{125}I-tau-MWNTs was examined by TLC.

The non-radioactive iodinated tau-MWNTs (I-tau-MWNTs) were synthesized by the same method, in which stable iodine was substituted for ^{125}I. I-tau-MWNTs were characterized by x-ray photoelectron spectroscopy (XPS, Kratos Axis Ultra, UK) at 293 K. The chamber pressure was kept below 10^{-8} Torr. A binding energy of 284.8 eV for the C_{1s} level was used as an internal reference.

2.3. Biodistribution of ^{125}I-tau-MWNTs

All animal experiments were performed in compliance with the local ethics committee. Male Kunming mice (~25 g) were obtained from the Animal Center of Peking University. Mice were housed in polycarbonate cages and kept on a 12 h light/dark cycle. Food and water were provided *ad libitum*. They were cared for and used humanely according to the Animal Care and Use Program Guidelines of Peking University. Following acclimation, mice were randomly divided into groups (five mice per group) for the following experiments.

Each mouse was exposed to ^{125}I-tau-MWNTs at a single dose of 100 μl solution via intravenous administration (i.v.) through a tail vein. The animals were sacrificed at different intervals post-dosing. The blood was collected and then the tissues, including thyroid gland, heart, lungs, liver, kidneys, spleen, stomach and large intestine (full) and small intestine (full), were immediately dissected. Each tissue was wrapped in aluminium foil and counted for ^{125}I activity on the γ detector. The distribution of tau-MWNTs in each tissue was represented by the percentage of the injected dose (tissue activity/total activity dosed, %ID).

2.4. Synthesis of ^{125}I-Tween-MWNTs

The non-covalent attachment of Tween-80 was achieved by means of anchoring the hydrophobic aliphatic chain onto the sidewall of CNTs. MWNTs were H_2SO_4/HNO_3 oxidized first to create the hydroxyl and carboxyl groups on MWNTs. Suspended oxidized MWNTs were labelled with ^{125}I and separated from the free ^{125}I ions with dialysis, the same as the protocol used with ^{125}I-tau-MWNTs. The radiochemical purity and serum stability of ^{125}I-MWNTs was examined by TLC too. The aqueous suspension of ^{125}I-Tween-MWNTs (containing 1 wt% of Tween-80) was prepared according to [14].

2.5. TEM observation of tau-MWNTs and Tween-MWNTs in serum

One hundred μl of tau-MWNTs (100 μg ml^{-1}) or Tween-MWNTs were mixed with 400 μl serum and, after 30 min incubation, the mixture was dropped onto the copper grid for TEM observation.

2.6. Biodistribution of ^{125}I-Tween-MWNTs

Each mouse (five per group) was exposed to ^{125}I-Tween-MWNTs at a single dose of 100 μl solution via i.v. administration. The animals were sacrificed at different intervals post-dosing. Samples were collected and the radioactivity was measured following the same methods as for ^{125}I-tau-MWNTs. The distribution of Tween-MWNTs in each tissue was represented by the percentage of the injected dose (tissue activity/total activity dosed, %ID).

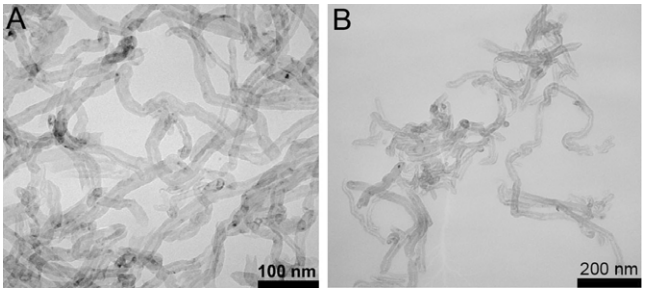

Figure 1. Typical TEM photos of MWNTs (A) and I-tau-MWNTs (B).

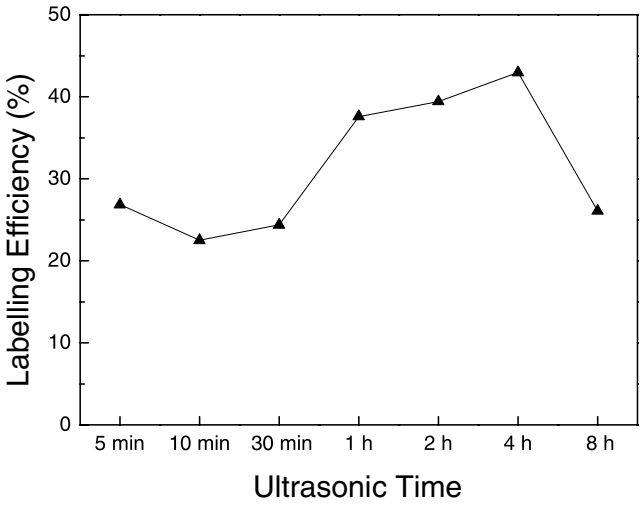

Figure 2. The labelling efficiency changes along with the ultrasonication time.

3. Results and discussion

3.1. Synthesis of ^{125}I-tau-MWNTs

TEM observation, TGA and ICP-MS were performed to further characterize the pristine MWNTs samples. TEM investigation shows that MWNTs samples are very clean (figure 1(A)). No MWNT bundles, carbon particles or metal particles were observed. The result of TGA demonstrates that the purity of the pristine MWNTs is over 95 wt%. ICP-MS analysis shows that MWNT samples contain very small amounts of metal impurities, mainly Ni (0.86 wt%), Fe (0.06 wt%) and Co (0.04 wt%). Tau-MWNTs were synthesized and characterized as reported in [12].

Due to its mildness, the iodogen method is widely used to label bioactive molecules, such as proteins, DNA, etc [15]. In general, the biomacromolecule can be labelled with ^{125}I by the iodogen method within 10 min. In order to gain a best labelling condition and higher labelling efficiency, a series of experiments with various labelling periods was carried out. After removing the adsorbed ^{125}I with dialysis, the labelling efficiency is reasonably considered as for the ^{125}I covalently bound tau-MWNT (figure 1(B)). The results show that the labelling efficiency of the covalently bound tau-MWNTs varies with ultrasonication time, from 26.8% (at 5 min) to 42.9% (at 4 h), but it decreases to 26.1% (at 8 h) (figure 2), which

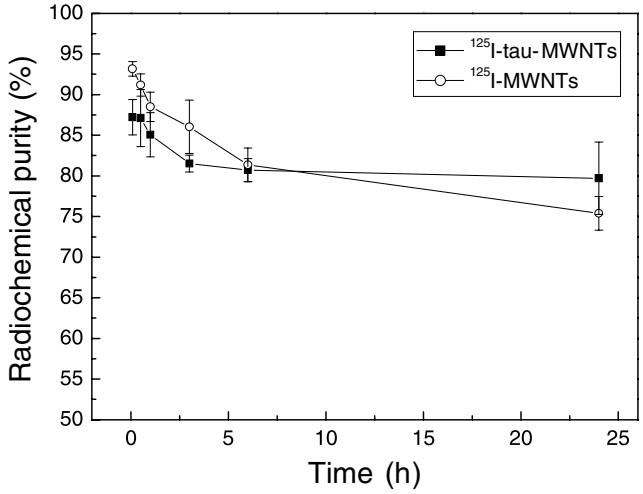

Figure 3. The stability of ^{125}I-tau-MWNTs and ^{125}I-MWNTs in serum.

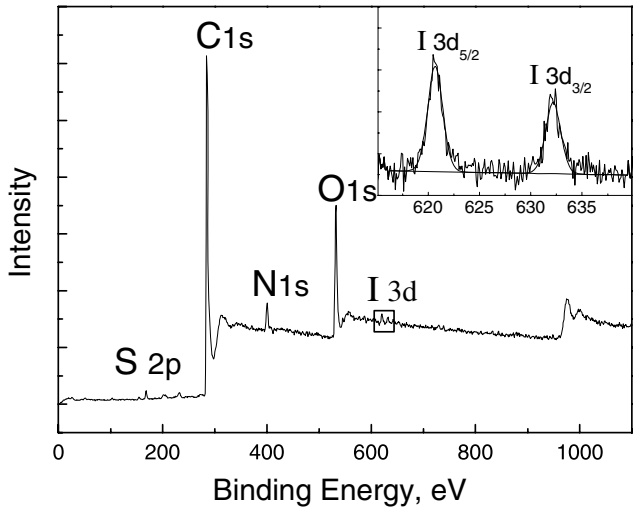

Figure 4. Representative XPS spectra of I-tau-MWNTs. The inset illustrates that the binding energies of I 3d are 620.7 and 632.0 eV.

presumably comes from the dissociation of ^{125}I from tau-MWNTs.

After dialysis against deionized water for three days, the radiochemical purity of ^{125}I-tau-MWNTs was above 87% according to TLC. Figure 3 shows both ^{125}I-tau-MWNTs and ^{125}I-MWNTs are stable in serum. Within 24 h, the radiochemical purity remained over 78% when ^{125}I-tau-MWNTs or ^{125}I-MWNTs were incubated in serum.

In order to confirm the formation of a covalent bond C–I between iodine and MWNTs, non-radioactive I-tau-MWNTs were synthesized. The iodine 3d XPS spectra of I-tau-MWNTs are shown in figure 4. The peak split corresponds to the orbital split of iodine 3d into $3d_{3/2}$ and $3d_{5/2}$ levels. The binding energies of 620.7 and 632.0 eV are in quite good agreement with those of the reference compounds *p*-I-$C_6H_4NO_2$ (620.4 and 631.9 eV) and *m*-I-C_6H_4COOH (620.7 and 632.2 eV) [16]. No ionic iodine ($3d_{5/2}$, 618.6 eV and $3d_{3/2}$, 630.1 eV) is observed in the sample [17]. Thus, we conclude that iodine atoms are covalently bound to nanotubes at the *meta* position

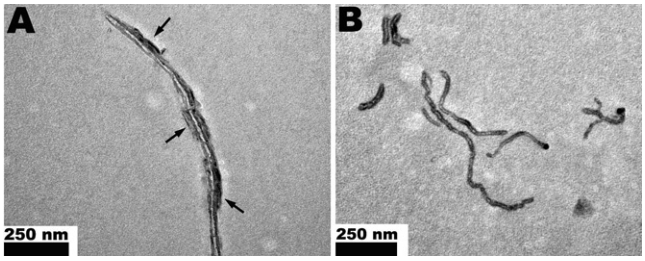

Figure 5. Typical TEM photos of tau-MWNTs (A) or Tween-MWNTs (B) in mouse serum. The arrows in (A) indicate proteins absorbed on tau-MWNTs.

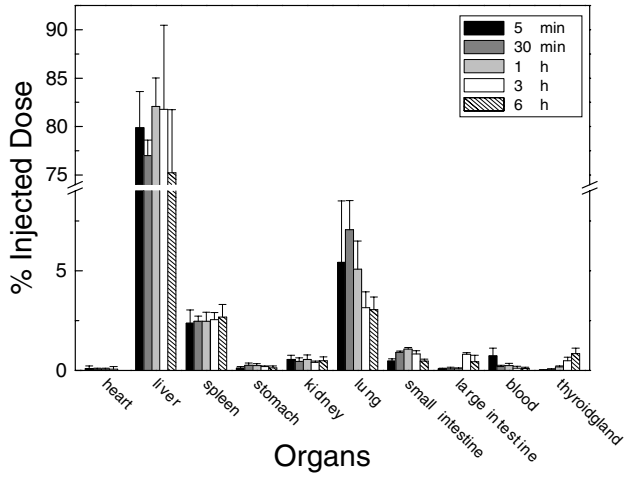

Figure 6. Biodistribution histogram of ^{125}I-tau-MWNTs in mice at five different time intervals by i.v. injection. Data represent mean ± S.D. ($n = 5$).

of the carboxyl groups on MWNTs. In general, the covalent bond is remarkably stronger than the other weak binding forms *in vivo*.

3.2. TEM observation of tau-MWNTs and Tween-MWNTs in serum

Figure 5 shows the TEM photos of two kinds of MWNTs in serum. The result shows that tau-MWNTs and Tween-MWNTs remain debundled in serum, and also clearly shows that tau-MWNTs adsorb some proteins, while Tween-MWNTs do not.

3.3. Biodistribution of ^{125}I-tau-MWNTs

At 5 min after i.v. administration, about 80% of injected tau-MWNTs arrives at the liver, while 5.4% and 2.4% go to the lung and spleen respectively, but not the other organs, i.e., blood, brain, stomach, muscle, bone and intestine (figure 6) [18]. The activity in the liver and spleen remains ca. 75% and ca. 2.6%, respectively, at 6 h post-dosing, whereas it reduces gradually in the lung. The low radioactivity detected in the thyroid gland, the major target organ of iodine, indicates that the tracer is stable and only a very few ^{125}I atoms drop down from tau-MWNTs.

The biodistribution data of ^{125}I-tau-MWNTs in the liver, spleen and lung are very well compatible with those of

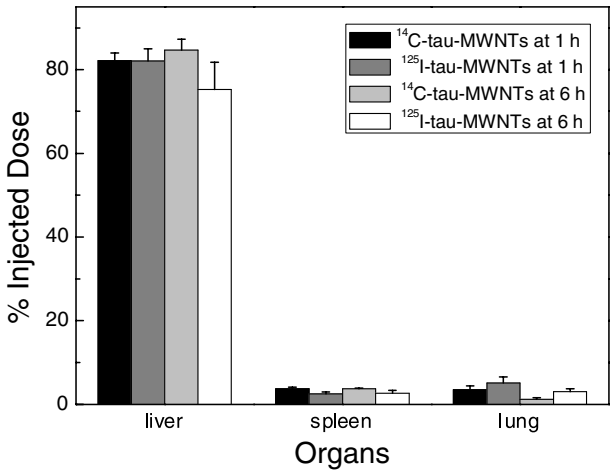

Figure 7. Comparison of distribution of ^{14}C-tau-MWNTs and ^{125}I-tau-MWNTs in mice at 1 h and 6 h post i.v. dosing. Only three main target organs, liver, spleen and lungs, are displayed. Data represent a mean value of five mice.

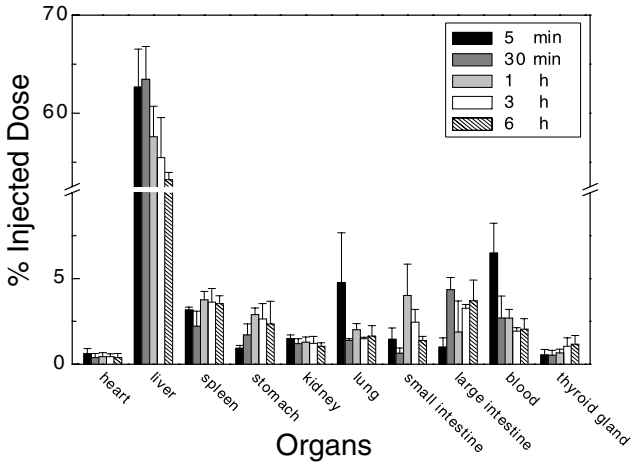

Figure 8. Biodistribution histogram of ^{125}I-Tween-MWNTs in mice at three different time intervals by i.v. injection. Data represent mean ± S.D. ($n = 5$).

^{14}C-taurine-MWNTs (figure 7) [12]. This is also good evidence showing that the ^{125}I-labelling method is reliable for tracing tau-MWNTs *in vivo*.

3.4. Biodistribution of ^{125}I-Tween-MWNTs

As displayed in figure 8, different from tau-MWNTs, Tween-MWNTs not only distribute into the liver, spleen and lung, but also into other organs like the stomach, kidney, large intestine and small intestine. At four time points after 30 min post-dosing, the lung uptake of Tween-MWNTs all declines remarkably. The reduction maybe comes from the well-dispersed property of Tween-nanotubes, which enables the nanotubes to easily evade RES capture.

In table 1, all liver uptakes of tau-MWNTs at three time intervals, 5 min, 30 min and 1 h, are larger than those of Tween-MWNTs. In addition, from the blood uptake data of

Table 1. Comparison of distribution of ^{125}I-tau-MWTNs and ^{125}I-Tween-MWTNs in mice at different intervals. Data represent a mean ± S.D. of % ID in each case ($n = 5$).

	5 min		30 min		1 h	
	Tau-MWNTs	Tween-MWNTs	Tau-MWNTs	Tween-MWNTs	Tau-MWNTs	Tween-MWNTs
Liver	79.9 ± 3.8	62.6 ± 3.9	77.0 ± 1.6	63.4 ± 3.3	82.1 ± 2.9	57.6 ± 3.1
Lung	5.4 ± 3.1	4.8 ± 2.9	7.1 ± 1.5	1.4 ± 0.1	5.1 ± 1.4	2.0 ± 0.4
Blood	0.7 ± 0.4	6.5 ± 1.7	0.2 ± 0.1	2.7 ± 1.3	0.2 ± 0.1	2.7 ± 0.5

the two different ^{125}I-MWNTs at 5 min post-dosing, 6.5% of Tween-MWNTs remains in the blood, whereas only 0.7% of tau-MWNTs remains in the blood. These indicate that Tween-MWNTs have a higher ability for evading RES capture. It is interesting to note that this phenomenon conforms very well to the case of Tween-^{13}C-SWNTs, in which the liver uptake is also remarkably reduced [19].

3.5. Evaluation of ^{125}I-labelling method

Among the radioisotopes used in the tracing studies, iodine-125 (^{125}I) is the most convenient, because of the easy labelling process and simple biosampling. The iodine atoms can be bound to CNTs easily and directly through a mild one-step reaction. The tracing is achieved by counting the radioactivity of biosamples without further treatment. The waste with ^{125}I-containing samples can be disposed of normally after storing for a period of several months, due to its half-life ($T_{1/2}$ = 60.2 d). In contrast, other radioisotopes, such as ^{64}Cu, ^{99m}Tc and ^{111}In, require special chelating agents attached to CNTs to chelate them. As for the ^{14}C-labelling method, a complicated digestion process of biosamples is inevitable for ^{14}C detection using a liquid scintillation counter, and the long half-life of ^{14}C (5760 years) makes the radioactive waste management troublesome.

There are two non-radioactive methods. One was performed by detecting the near-IR of single-walled CNTs (SWNTs) [20]. This non-radioactive method is only valid in detecting debundled SWNTs. However, most pristine and functionalized SWNTs naturally form bundles that remarkably restricts the application. Another method was by using skeleton ^{13}C-enriched SWNTs as the tracer [19]. The preparation of ^{13}C-enriched SWNTs is complicated and the detection sensitivity by mass spectrometry is quite low. Also, a pulverizing and freeze-drying process is required to prepare the tissue samples. However, this ^{13}C tracing method is undoubtedly valuable, because it keeps the original nature of SWNTs intact and is suitable for long-term tracing owing to its high stability *in vivo*.

Although the I-labelling method is generally adoptable and convenient, we have to mention its limitation here. The binding of the covalent bond of I–C is much stronger than that of the physical adsorption of I ions on CNTs, but the I–C bond is rather easy to break, more or less, in physiological conditions. In fact, we found a small amount of radioactivity, which was dissociated from CNTs, increasing gradually in the thyroid gland over the course of time. We may conclude that it is suitable for a short-term tracing study, e.g. less than 24 h, but not good for a long course of time. Nevertheless, several previous studies on distribution of CNTs have shown that CNTs distribute and translocate very rapidly in animals, and thus a time course of 24 h seems enough for most cases of the distribution study. On this account, we contend that the rather simple ^{125}I-labelling method can be adopted as an easier and applicable one for the distribution and translocation studies of CNTs.

4. Conclusion

For determining the biodistribution of CNTs *in vivo*, we have developed a convenient and general method to label ^{125}I onto the shortened tau-MWNTs. The nature of the covalent bond between the I and C atoms of CNTs in tau-MWNTs was proved. The very analogous biodistribution of ^{125}I-tau-MWNTs and ^{14}C-tau-MWNTs demonstrates the radiotracing method is reliable. This method was also successfully used to determine the biodistribution of Tween-MWNTs *in vivo*. Our results suggest that this ^{125}I-radiolabelling method can be generally adoptable as an effective and convenient technique for *in vivo* tracing diverse CNT derivatives, though it is unsuitable for long-term tracing studies.

Acknowledgments

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