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Quantification of Carbon Nanomaterials *in Vivo*

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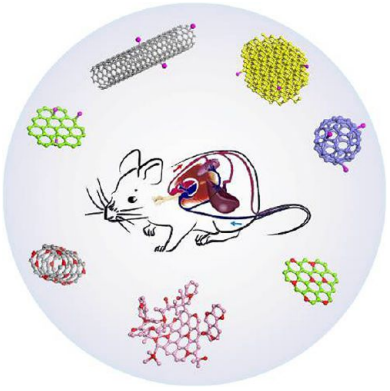
CONSPECTUS

A diverse array of carbon nanomaterials (NMs), including fullerene, carbon nanotubes (CNTs), graphene, nanodiamonds, and carbon nanoparticles, have been discovered and widely applied in a variety of industries. Carbon NMs have been detected in the environment and have a strong possibility of entering the human body. The safety of carbon NMs has thus become a serious concern in academia and society. To achieve strict biosafety assessments, researchers need to fully understand the effects and fates of NMs in the human body, including information about absorption, distribution, metabolism, excretion, and toxicity (ADME/T).



To acquire the ADME data, researchers must quantify NMs, but carbon NMs are very difficult to quantify *in vivo*. The carbon background in a typical biological system is high, particularly compared with the much lower concentration of carbon NMs. Moreover, carbon NMs lack a specific detection signal. Therefore, isotopic labeling, with its high sensitivity and specificity, is the first choice to quantify carbon NMs *in vivo*. Previously, researchers have used many isotopes, including ^{13}C, ^{14}C, ^{125}I, ^{131}I, ^{3}H, ^{64}Cu, ^{111}In, ^{86}Y, ^{99m}Tc, and ^{67}Ga, to label carbon NMs. We used these isotopic labeling methods to study the ADME of carbon NMs via different exposure pathways in animal models.

Except for the metabolism of carbon NMs, which has seldom been investigated, significant amounts of data have been reported on the *in vivo* absorption, distribution, excretion, and toxicity of carbon NMs, which have revealed characteristic behaviors of carbon NMs, such as reticuloendothelial system (RES) capture. However, the complexity of the biological systems and diverse preparation and functionalization of the same carbon NMs have led to inconsistent results across different studies. Therefore, the data obtained so far have not provided a compatible and systematic profile of biosafety. Further efforts are needed to address these problems.

In this Account, we review the *in vivo* quantification methods of carbon NMs, focusing on isotopic labeling and tracing methods, and summarize the related labeling, purification, bio-sampling, and detection of carbon NMs. We also address the advantages, applicable situations, and limits of various labeling and tracing methods and propose guidelines for choosing suitable labeling methods. A collective analysis of the ADME information on various carbon NMs *in vivo* would provide general principles for understanding the fate of carbon NMs and the effects of chemical functionalization and aggregation of carbon NMs on their ADME/T *in vivo* and their implications in nanotoxicology and biosafety evaluations.

1. Introduction

Since the discovery of fullerene in 1985, carbon nanotubes (CNTs) in 1991, and graphene in 2004, various carbon nanomaterials (NMs) have been extensively studied. The huge application potentials of these promising NMs in diverse areas, such as materials, electronic, environmental, and biomedical areas, have strongly stimulated the production and consumption of carbon NMs.[1,2] Inevitably, carbon NMs have come into our daily life and environment. Thus, the related safety issue has been raised and attracted serious concerns from academia, governments and our society.[3−5]

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To date, the environmental health and safety (EHS) issues of carbon NMs have not been well assessed. Taking CNTs as an example, the first toxicity study was performed *in vitro* in 2003;[6] early *in vivo* evaluations were reported in 2004, concerning on their pulmonary toxicity.[7,8] In the same year, we published the first paper reporting the biodistribution of CNTs in mice,[9] where the concept of absorption, distribution, metabolism, and excretion (ADME) was introduced to the safety evaluation of CNTs. Since then, hundreds of papers have been published on the ADME and toxicity (ADME/T) of carbon NMs.[10,11] Although ADME information is fundamental and necessary for the toxicity and biosafety evaluations of the xenobiotics,[12,13] the present knowledge could not provide a compatible and systematic profile for the biosafety of carbon NMs. Therefore, systematic and reliable studies on the ADME/T of carbon NMs are highly demanded.

To acquire ADME information, the quantitative analysis of carbon NMs is definitely indispensable. However, it is very difficult to quantify how much of carbon NMs enter the body, given the fact that normally the concentration of the carbon NMs is quite low while the background of carbon is rather high, and there is a lack of specific detection signal.[10] Therefore, isotopic labeling becomes the best and almost the only choice, due to its high sensitivity and specificity. In recent years, we have successfully developed a series of isotopic labeling and tracing methods to quantify carbon NMs *in vivo*.[9,14−18]

In this Account, we review the advantages, applicable situations and limits of isotopic labeling and tracing methods for *in vivo* quantification of carbon NMs. The knowledge from quantitative data of carbon NMs *in vivo* is summarized. The implications to nanotoxicology and biosafety evaluation are extensively discussed.

2. Isotopic Labeling of Carbon NMs

To adopt isotopic tracing methods, carbon NMs should be well labeled using proper methods with suitable isotopes.

2.1. Isotopes and Labeling Methods. Currently, isotopes including carbon (^{13}C and ^{14}C) and other elements (^{125}I, ^{131}I, ^{3}H, ^{64}Cu, ^{111}In, ^{86}Y, ^{99m}Tc, and ^{67}Ga) have been used for labeling carbon NMs for *in vivo* studies. The basic properties of these isotopes are summarized in Table 1.[9,14−32]

2.1.1. Non-Carbon Isotopes. The most used isotopes in labeling carbon NMs are non-carbon radioisotopes, due to the ease of labeling procedures and detection. These isotopes can label materials via covalent bonding, chelation, or encapsulation. ^{125}I and ^{131}I can label carbon NMs covalently by direct addition onto the skeleton. In a typical ^{125}I labeling reaction, the carbon NMs are mixed with Na^{125}I, and an oxidative reagent (chloramine-T or iodogen) is added to generate free ^{125}I atoms from Na^{125}I (Figure 1a).[9,32] The as-prepared ^{125}I atom then interacts with the carbon atom of the carbon NMs to form ^{125}I−C bond, which is evidenced by X-ray photoelectron spectroscopy (XPS) (Figure 1b).[32] ^{3}H labeling is achieved by incorporating ^{3}H atom into a functional group during the surface functionalization.[19] Metal isotopes, such as ^{64}Cu, ^{86}Y, and ^{111}In, can be attached to carbon NMs by chelation with the surface-functionalized chelators, such as diethylene triamine pentaacetic acid (DTPA) and tetraaza-cyclododecane tetraacetic acid (DOTA).[22,24,27] In addition, metal isotopes can also be encapsulated or trapped inside the carbon NMs,[33] which is particularly useful in studying metal@C_n-cage derivatives.

TABLE 1. The Basic Properties of Isotopes for Labeling Carbon NMs

isotope	radioactivity/detector	half-life	sampling	reference
		Non-carbon		
^{125}I	γ-photon/γ-counter	59.4 d	none	9, 17, 18, 28
^{131}I	γ-photon/γ-counter	8.02 d	none	29
^{64}Cu	γ-photon /γ-counter	12.70 h	none	22
^{67}Ga	γ-photon/γ-counter	3.2616 d	none	23
^{86}Y	γ-photon/γ-counter	14.74 h	none	24
^{99m}Tc	γ-photon/γ-counter	6.008 h	none	25
^{110m}Ag	γ-photon/γ-counter	249.95 d	none	26
^{111}In	γ-photon/γ-counter	2.8047 d	none	27
^{188}Re	γ-photon/γ-counter	16.9 h	none	30
^{3}H	β-ray/LSC	12.33 y	digestion	19
		Carbon		
^{14}C	β-ray/LSC	5730 y	digestion	14, 20, 21
^{13}C	none/IRMS	stable	homogenization	15, 16, 31

2.1.2. Carbon Isotopes. There are two carbon isotopes (^{14}C and ^{13}C) for labeling carbon NMs. Radioactive ^{14}C can be incorporated into functional groups to label the carbon NMs. We have labeled multiwalled CNTs (MWCNTs) with ^{14}C-taurine by this approach.[14] Meanwhile, ^{14}C can also be incorporated into the skeleton of carbon NMs by proper synthesis procedures.[20,21]

Given the disadvantages of radioactive labeling on the generation of radioactive wastes and strict requirement of radioactivity protection, nonradioactive ^{13}C skeleton labeling is a good alternative.[15,16,34,35] Using ^{13}C-enriched amorphous carbon as the precursor, Sun et al. prepared ^{13}C skeleton labeled single-walled CNTs (SWCNTs), carbon nanoparticles, and graphene by laser ablation or arc discharge (Figure 2a).[15,16,31] The enrichment of ^{13}C in SWCNTs was evidenced by Raman spectrum, where a typical shift of G-band for ^{13}C-enriched SWCNTs was observed (Figure 2c). The content of ^{13}C in carbon NMs usually is around 30%. As a distinct merit, skeleton labeling keeps the original property

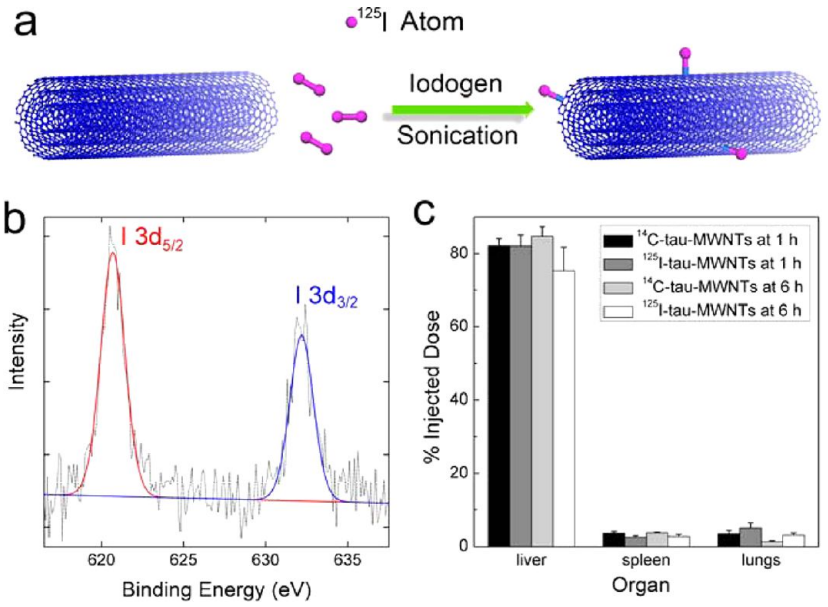


FIGURE 1. Labeling of oxidized multiwalled CNTs (O-MWCNTs) and taurine-multiwalled CNTs (tau-MWCNTs) with ^{125}I. (a) Schematic labeling procedure; (b) XPS spectrum of I-tau-MWCNTs; the binding energies of I peaks indicate the formation of I−C bond (referring to *p*-I-$C_6H_4NO_2$); (c) comparison of distribution of ^{125}I- and ^{14}C-labeled tau-MWCNTs in mice post-intravenous (iv) exposure. The profiles demonstrate the reliability of methods. Adapted from ref 32 with permission. Copyright 2008 IOP Publishing.

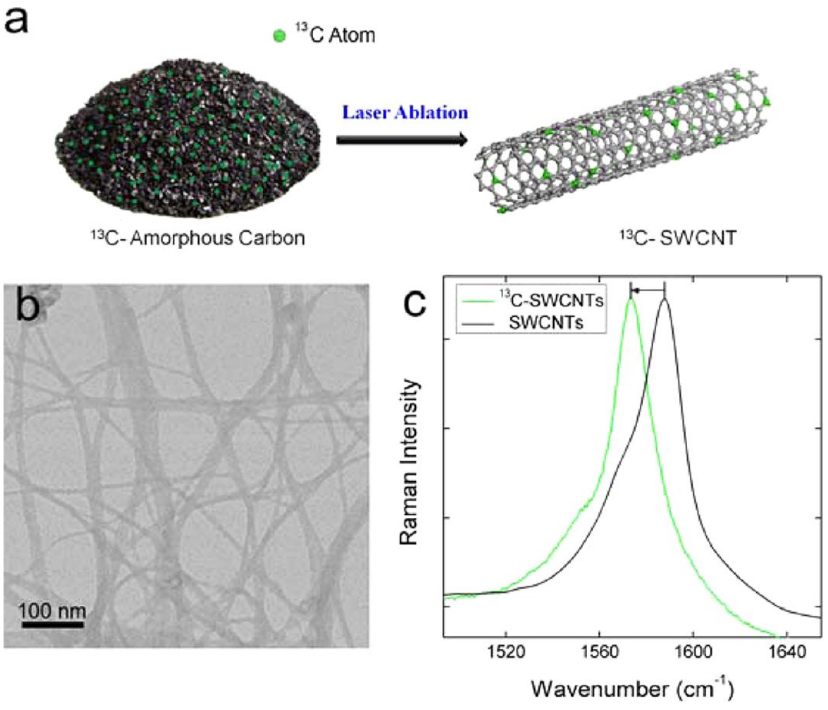
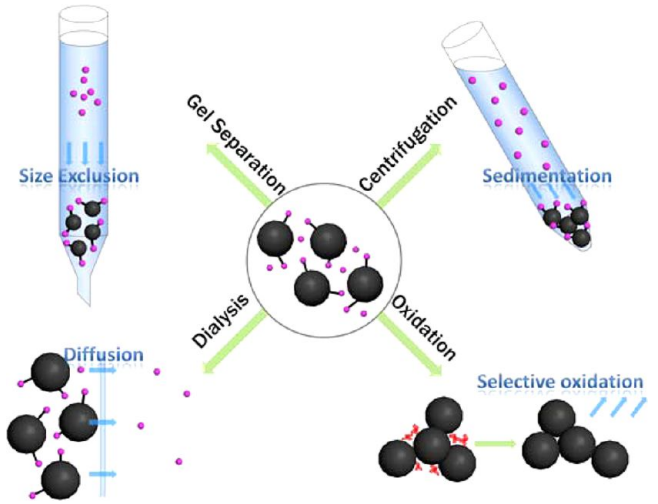


FIGURE 2. ^{13}C-enriched SWCNTs. (a) Schematic labeling procedure; (b) a transmission electron microscope (TEM) image; (c) Raman spectra of SWCNT samples with and without ^{13}C labeling, showing a distinct shift of G-band corresponding to ^{13}C content of SWCNTs. Adapted from ref 15 with permission. Copyright 2007 American Chemical Society.

of carbon NMs, whereas labeling on the functional group may more or less alter the original property of the targeted NMs.

2.2. Purification of Labeled Carbon NMs. After labeling, the separation of the labeled carbon NMs from the free isotopes is essential to guarantee that the detected signal stands for carbon NMs. If applicable, centrifugation is apparently the first choice, due to its simplicity. Otherwise, dialysis could be a good alternative. But it takes a longer time

SCHEME 1. Purification Approaches of the Labeled Carbon NMs[a]



[a]Purple dots, isotopes; black balls, carbon NMs.

(at least 2−3 days) and generates a large volume of radioactive waste. Size exclusion gel chromatography is another choice. A sephadex G-25 column has been employed to separate ^{125}I-labeled hydroxylated SWCNTs (SWCNTols) and free Na^{125}I.[9] For skeleton labeling, the conventional purification process can be used to remove amorphous carbon materials.[15,21] For example, ^{13}C source powder could be selectively burned during the purification with the purified ^{13}C-SWCNTs left.[15] These separation approaches are summarized in Scheme 1.

2.3. Biosample Treatment and Detection. The biosample treatment and detection of labeled carbon NMs depend on the isotopes used (Table 1). Generally, for radioisotopes emitting γ-rays, the tissues or organs can be detected directly and sensitively by a γ-counter.[9,22,27] ^{14}C and ^{3}H emitting β-rays can be very sensitively detected using a liquid scintillation counter (LSC),[14,19,21] but the biosamples have to be predigested into a clear solution.[14] Beyond these quantitatively analytical methods, radioactive imaging techniques, including positron emission tomography (PET) and single photon emission computed tomography (SPECT), are widely adopted to provide semiquantitative dynamic translocation information *in vivo* with spatial resolution.[22,24] In particular, radioactive tracing requires a very small amount of radioisotopes that would neither cause serious radioactive damage nor influence the properties of carbon NMs labeled. Taking ^{125}I-SWCNTol as an example, the atom ratio of ^{125}I/C is only 9:10^{7}.[9]

The abundance of ^{13}C in samples can be measured by isotope ratio mass spectrometry (IRMS) after the biosamples are homogenized and lyophilized. Yet, the sensitivity of ^{13}C labeling is much lower than that of the radioisotopic labeling.

2.4. Guidelines for Choosing a Suitable Isotope. To select a suitable isotope, the first thing to consider is the requirement of the object to be studied, such as the time scale (related to the half-life of the isotope and the label stability), sensitivity, and reliability (related to the content and detection method). When there are multiple choices, the next thing to consider is the experimental procedures to be taken, such as the labeling, sampling, and waste handling. Based on these criteria, Scheme 2 shows the pros and cons of various isotopic labeling methods for carbon NMs.

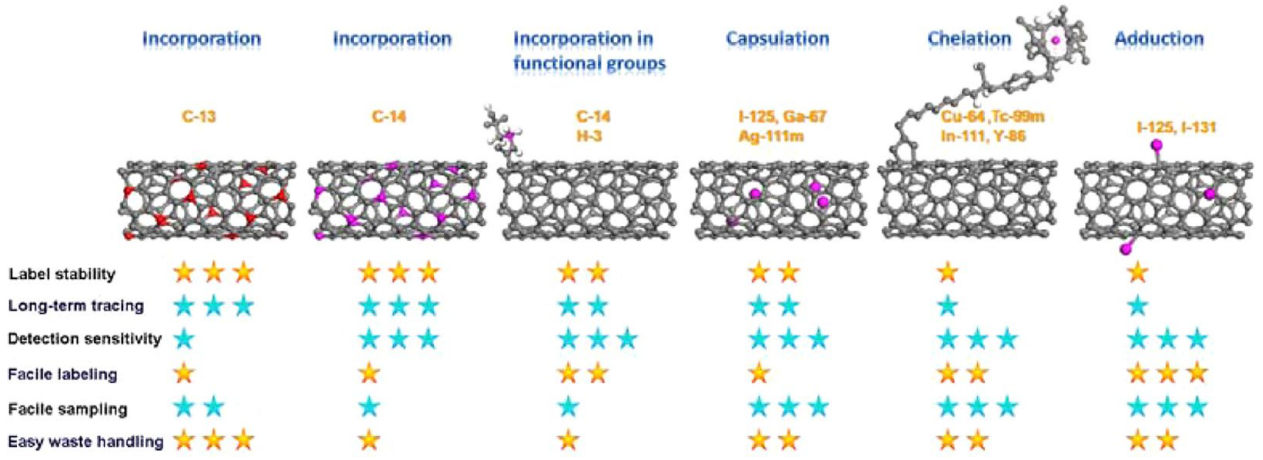
For short-term (a few days) ADME studies, all isotopes in Table 1 are eligible. However, considering the sensitivity and the ease of labeling and sampling, isotopes emitting γ-photons are recommended. In addition, their short half-life makes it easier to handle the waste. Particularly, ^{125}I labeling via adduction is highly favorable. First, it is an easy and convenient labeling/detecting protocol (Figure 1a), where ^{125}I can directly attach to the carbon NMs by forming C−I bonds, unlike metal isotopes, which need a special functional group for chelation (metal encapsulation only works for rare circumstances). Second, ^{125}I labeling is generally available for various carbon NMs with the same protocol. We have achieved ^{125}I labeling of SWCNTol, O-MWCNT, tau-MWCNT, fullerenol, nanodiamond, and graphene oxide (GO) to study their distribution and toxicokinetics.[9,17,18,32,36] We have also proven the reliability of ^{125}I labeling by the consistent distribution data of tau-MWCNT obtained by ^{125}I and ^{14}C labeling (Figure 1c).[32]

For long-term tracing studies, we must choose the isotopes with a longer half-life and a higher labeling stability, that is, ^{13}C, ^{14}C, and ^{3}H. Although ^{14}C and ^{3}H labeling have a high sensitivity, their sampling and waste handling are pretty laborious. Comparatively, nonradioactive ^{13}C is a compromising choice with relatively lower detection sensitivity. We adopted ^{13}C labeling in our studies of CNTs, GO, and carbon nanoparticles. ^{13}C skeleton labeling avoids not only the possible detachment of labeled isotopes from carbon NMs toward the long-term evaluations,[15,16] but also the additional functionalization to keep the original nature of carbon NMs intact.

3. Quantitative ADME Information from Isotopic Labeling Studies

The ADME/T studies of carbon NMs have been performed with various exposure pathways on animal models. The general

SCHEME 2. Strategies for Labeling Carbon NMs[a]



[a]The number of stars indicates the favorable level of the corresponding process. The label stability corresponds to the detachment of labeled isotopes; thus the favorite sequence is incorporation (skeleton) > encapsulation ≈ covalent binding > chelation. Correspondingly, the more stable it is, the more preferable for the long-term tracing. Radioactivity detection is very sensitive; thus, all of the radiolabelings are favorable in the detection sensitivity. The labeling methods are judged by the convenience in operation. The sampling is based on how the samples are prepared for determination. The waste handling is mainly dependent on the half-life of radioisotopes. ^{13}C is non-radioactive; hence it is the most favorable here.

ADME/T information of carbon NMs is summarized in Scheme 3, which is achieved mainly by isotopic labeling studies.

3.1. Absorption. Absorption is the first issue of concern when the human body is exposed to carbon NMs. In animal experiments, NMs are generally administrated via oral dosing, inhalation and iv injection, corresponding to different exposure pathways. Intravenous dosing means 100% absorption into the circulation system. Other administration methods only yield a small amount entering the body, depending on the exposure pathways and physicochemical properties of carbon NMs.

Upon absorption from pulmonary system, the spherical carbon NMs tend to enter the blood circulation,[25,34,35,37,38] while fibrous CNTs are generally retained in lungs with minor migration into lymph nodes.[14,39] The clearance of carbon NMs from lungs would be a long process. We found that there were still 20% of tau-MWCNTs retained in lungs at 28 days post intratracheal (i.t.) instillation (Figure 3b).[14]

After oral dosing (gavage), the absorption of carbon NMs depends on their properties, for example, surface chemistry. SWCNTols were absorbed easily and migrated freely in animal's bodies, except the brain.[9] Tyrosine-functionalized MWCNTs had similar behavior.[40] In contrast, tau-MWCNTs did not enter the blood circulation but excreted directly in feces (Figure 3c).[14]

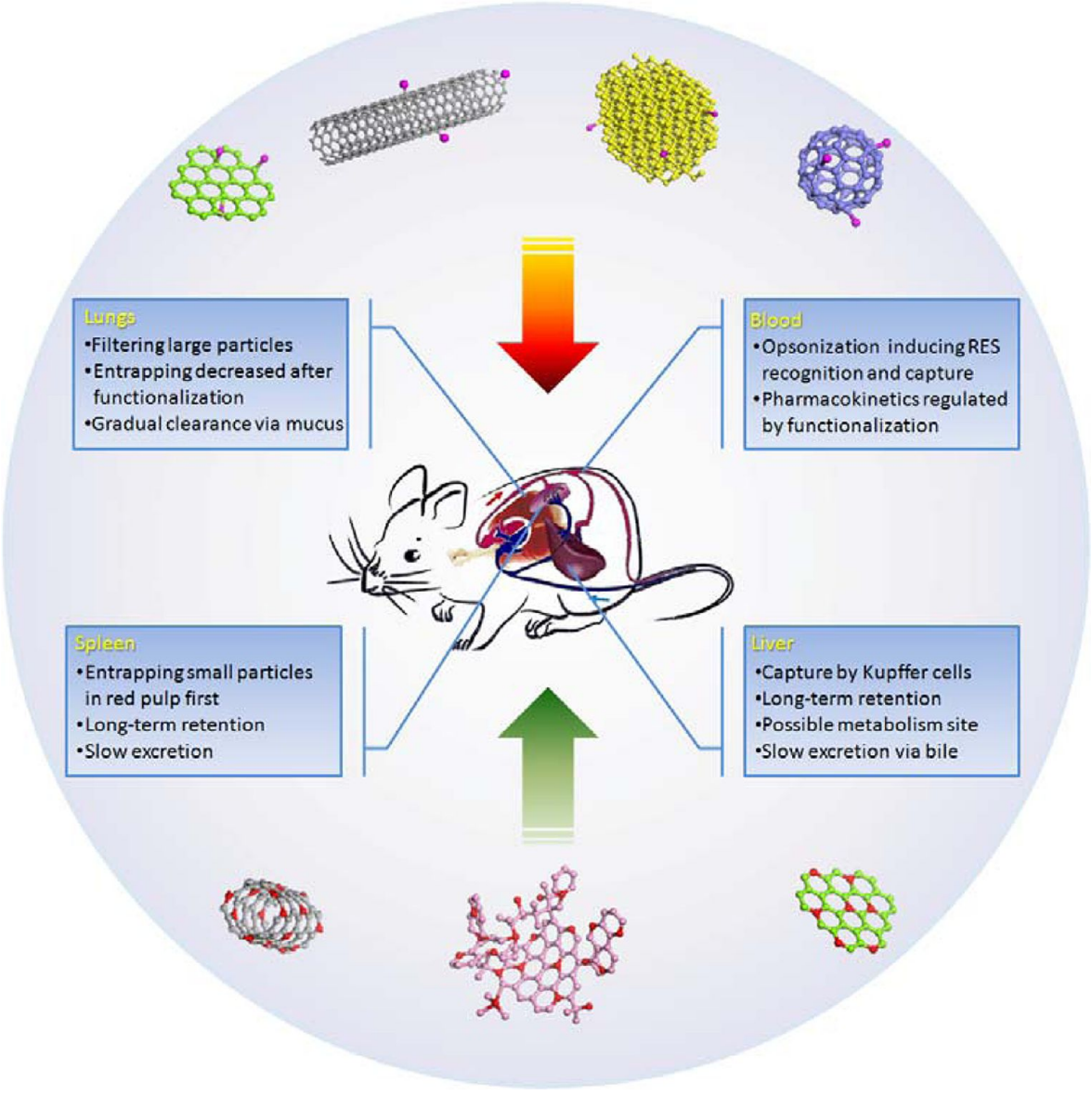
Overall, the absorption of carbon NMs is influenced by many factors. After entering the blood circulation, carbon NMs distribute to organs following the bloodstream.

3.2. Distribution. Despite the differences among specific carbon NMs, the most distinct distribution behavior of carbon NMs is reticuloendothelial system (RES) capture. The lungs, liver, and spleen are the important components of the RES.

A large amount of data have demonstrated that carbon NMs are cleared in part from the blood circulation by the remarkable capture through the RES.[14,15] Opsonization is regarded as a process in the RES capture of xenobiotics.[41] Since carbon NMs are prone to protein adsorption, opsonins are adsorbed leading to the subsequent recognition and capture by RES. This phenomenon is widely observed on carbon NMs, regardless of their physiochemical properties. After iv injection, 80% of tau-MWCNTs and 60% of O-MWCNTs (dispersed in tween 80) accumulated in the liver within 5 min.[14,32] Other carbon NMs, such as fullerene, nanodiamond, carbon nanoparticle, and graphene were also readily captured by RES.[16,18,20,36] The RES uptake was further proven by complementary techniques, such as TEM and Raman spectroscopy.[14,15,42,43] For example, around 37% of nanodiamond was entrapped in liver after iv injection.[18] This was evidenced further with ultrastructural observation and Raman analysis (Figure 4). If they escaped from the RES capture, carbon NMs could further distribute to other tissues.

3.3. Metabolism. The metabolism of carbon NMs *in vivo* could be divided into two categories: One is the carbon skeleton, which might be destroyed, and the other is the surface functional groups, which might be added or removed. In principle, carbon NMs are quite stable *in vivo*.

SCHEME 3. Translocation and Biodistribution of Carbon NMs from Isotopic Labeling Studies[a]



[a]After carbon NMs enter the body, blood will make them travel in the body. The lungs, liver, and spleen are the most probable organs for accumulation. The related distribution and excretion information on these sites is summarized in the corresponding squares.

If there is any metabolism of carbon NMs, it is likely to be a slow process requiring a long-term observation, which is one of the reasons that metabolism data are scarce. Unfortunately, isotopic labeling has not yet been applied in metabolism studies of carbon NMs.

By monitoring the recovery of Raman peaks of SWCNTs, we observed the slow removal of PEG [poly(ethylene glycol)] fragments from PEGylated SWCNTs (PEG-SWCNTs) *in vivo*.[44] The defunctionalization of PEG from PEG-SWCNTs only occurred in the liver at 28 days postexposure, whereas PEG-SWCNTs remained stable in spleen (Figure 5). The functionalization of C_{60} in mice was observed by Moussa et al.[45] At 7 days post-intraperitoneal (ip) administration, the cycloaddition of retinol on C_{60} in liver was detected by high-performance liquid chromatography−mass spectrometry (HPLC-MS), suggesting that the fullerene cage was transformed.

3.4. Excretion. The excretion of carbon NMs is of great interest and importance. However, quantitative excretion data are scarce. Because the excretion of most carbon NMs is rather slow, requiring a long-term and highly sensitive observation. ^{13}C labeling is not sensitive enough for quantifying carbon NMs in feces.[15,16] There are several studies on the excretion of carbon NMs either quantitatively

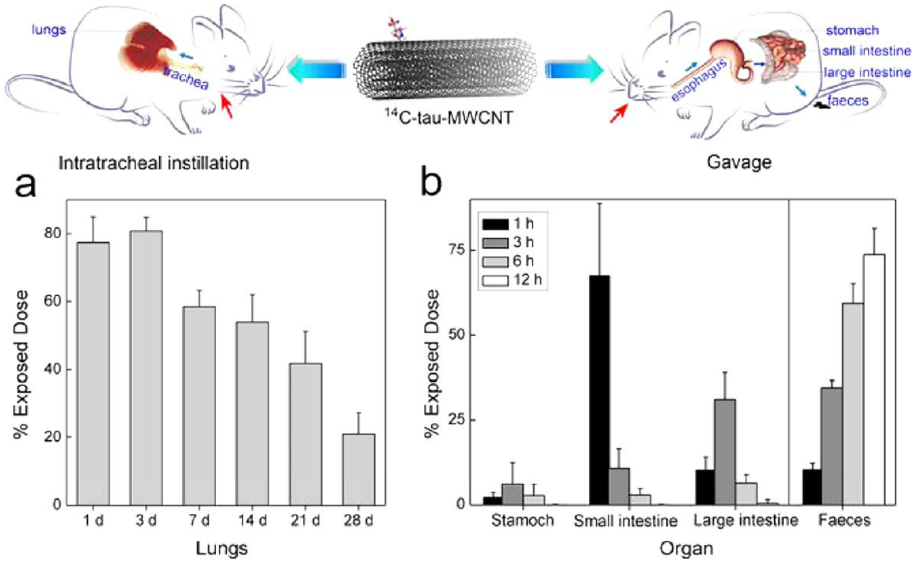


FIGURE 3. Accumulation of ^{14}C-tau-MWCNTs in mice post i.t. and gavage exposure: (a) i.t. exposure, showing that the content of tau-MWCNTs decreases slowly with time; (b) gavage exposure, indicating the movement of tau-MWCNTs in the gastrointestinal tract. At 12 h postexposure, over 75% of tau-MWCNTs have been excreted in feces. Adapted from ref 14 with permission. Copyright 2007 Elsevier.

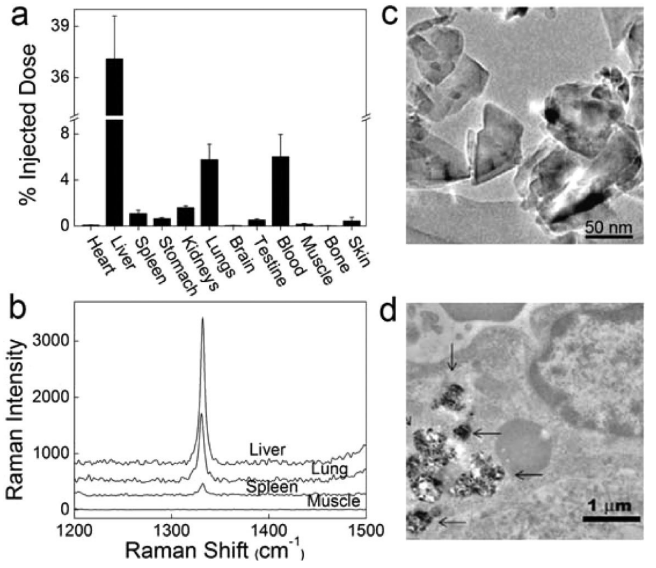


FIGURE 4. Distribution of nanodiamond in mice after iv injection. (a) Biodistribution histogram obtained by ^{125}I-labeling method; (b) Raman spectra of different organs; (c) TEM image of nanodiamonds in digested liver; (d) ultrastructure of a mouse liver (nanodiamonds are indicated by arrows). The results in panel b, c, and d collectively support the reliability of data from ^{125}I-labeling in panel a. Adapted from ref 18 with permission. Copyright 2009 Elsevier.

or qualitatively. We have reported SWCNTs entrapped in liver without discernible metabolism for more than three months, indicating the very slow excretion from mice.[42] In contrast, the carbon NMs trapped in lungs would be cleared gradually, partly through mucus.[15,38] However, the thorough clearance of the trapped carbon NMs is quite difficult.[14]

3.5. Effects of Chemical Functionalization. Chemical functionalization is a crucial factor regulating the ADME behavior of carbon NMs. Chemical functionalization regulates the distribution of carbon NMs by regulating the protein adsorption ability of carbon NMs.[41,46] A good example comes from the PEGylation of SWCNTs. PEG is particularly powerful in reducing opsonization;[41] PEGylation is therefore regarded as a most efficient means to improve the pharmacokinetics of xenobiotics. Lin et al. reported that PEG-SWCNTs were protein resistant.[47] In the isotopic tracing, more than 37% of tween 80 suspended SWCNTs were entrapped in lungs, liver, and spleen,[15] while only 28% of PEG-SWCNTs accumulated in these organs (Figure 6).[48] Meanwhile, PEGylation significantly prolonged the half-life of SWCNTs in the blood circulation to 15.3 h.[48] Another example comes from hydroxylated carbon NMs. After hydroxylation both SWCNTs and C_{60} became soluble sharing nearly the same benefit from the surface chemistry. They had very similar distribution profile, though the accumulation of SWCNTols is much higher (Figure 7).[17,29]

The excretion of carbon NMs is regulated by chemical functionalization, too. Well-designed functionalization can avoid RES uptake and consequently facilitate the excretion of carbon NMs. For example, pristine and taurine functionalized CNTs were excreted very slowly after iv injection.[14,15] Upon PEGylation, the excretion via urine and feces of CNTs became faster.[43,48]

3.6. Effects of Aggregation. Aggregation remarkably affects the ADME of carbon NMs. Generally, if the diameter

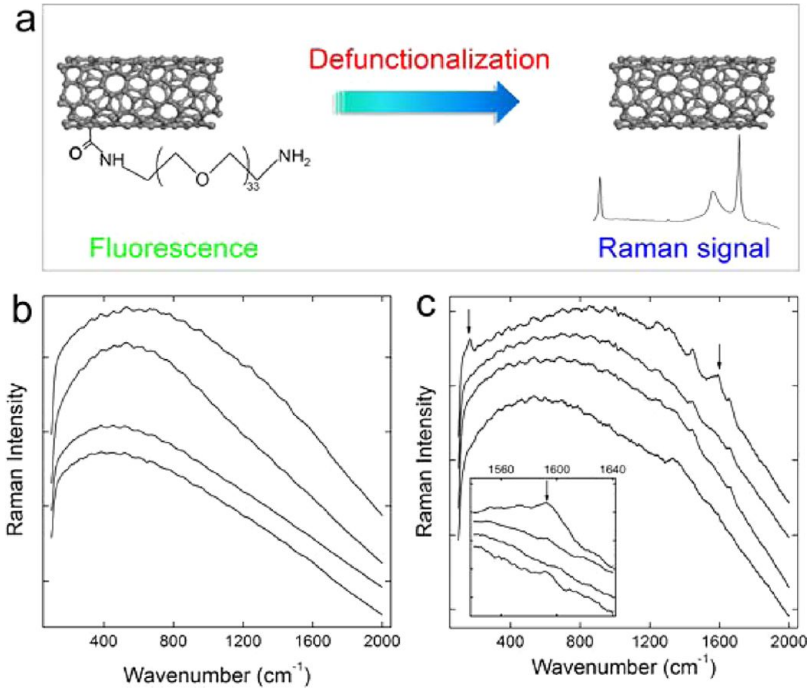


FIGURE 5. Metabolic defunctionalization of PEG from PEG-SWCNTs: (a) schematic detection principle; (b) stability in spleen; (c) defunctionalization in liver at 4 weeks postexposure (Raman signals were denoted by arrows). Adapted from ref 44 with permission. Copyright 2009 American Chemical Society.

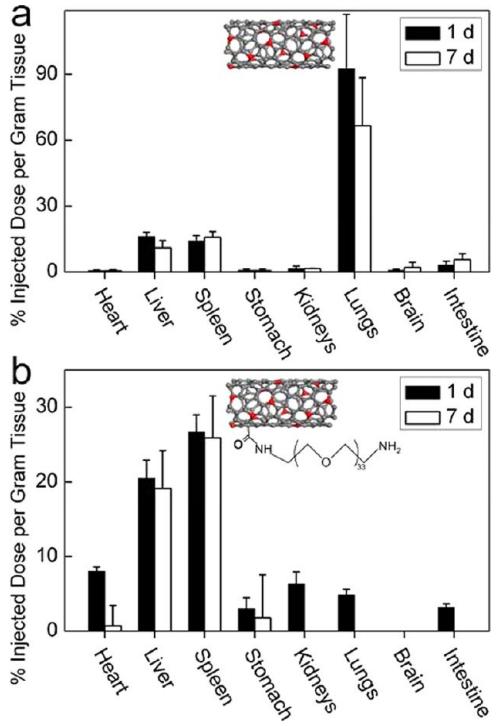


FIGURE 6. Biodistribution of (a) ^{13}C-SWCNTs and (b) PEG-^{13}C-SWCNTs in mice after iv injection. PEGylation improves the distribution of SWCNTs. Adapted from refs 15 and 48 with permission. Copyright 2007 American Chemical Society and Copyright 2008 Wiley-VCH.

of aggregates is larger than 2 μm, they will be entrapped in the pulmonary capillary vessels; while when the diameter is decreased, entrapping in liver and spleen will dominate. Therefore, the less dispersed carbon NMs are easily trapped in lungs. For example, SWCNTs accumulate largely in lungs (15%, corresponding to 92.6% injected dose per gram tissue) (Figure 6).[15] Serum albumin suspended nanodiamonds are slightly entrapped in lungs (5.7%) (Figure 4).[18] Very recently, we found that the size of GO–protein complex regulated the distribution of GO in mice.[36] GO with a large size or a high dose formed large GO–protein complexes in blood, leading to a high accumulation in lungs after iv injection; while GO with a small size at a low dose preferred to stay in liver. Generally, when the carbon NMs are well functionalized and dispersed, the pulmonary uptake is negligible.[9,16,17]

When well-dispersed carbon NMs are trapped in liver and kidneys, they will be excreted via bile and urine. Their aggregates in a larger size are mainly trapped in lungs and excreted through mucus. Therefore, to facilitate the excretion of carbon NMs, the dispersion state should be seriously considered.

4. Implications of Quantitative Information for Toxicity Studies in NanoEHS

The ADME information and its relevance to the physicochemical properties of carbon NMs obtained by isotopic labeling are rather interesting and guide the ongoing NanoEHS evaluations.

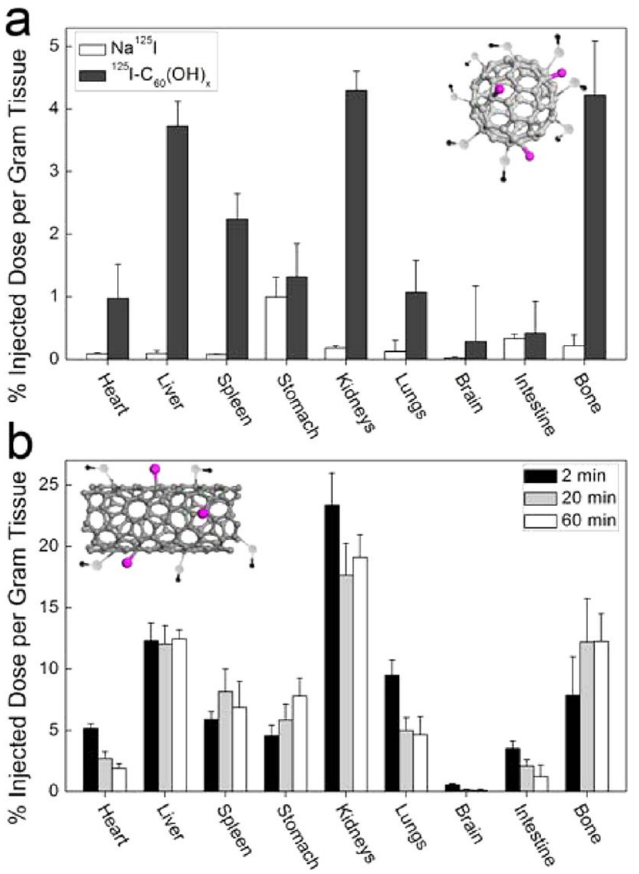


FIGURE 7. Biodistribution of (a) ^{125}I-fullerenols and (b) ^{131}I-SWCNTols in mice after iv injection. The same surface chemistry endows different carbon NMs with similar distribution properties. Adapted from refs 17 and 29 with permission. Copyright 2006 Springer and Copyright 2008 Informa.

4.1. Bioavailability. In the safety assessment, hazard depends on two factors, namely, toxicity and bioavailability.[49] The determination of bioavailability is crucial for the safety assessment. For iv administration, NMs come directly into the bloodstream making the bioavailability 100%. However, for oral dosing, inhalation, and ip injection, the bioavailability is limited and varies upon the properties of carbon NMs. For example, tau-MWCNTs are hardly absorbed through the gastrointestinal track (Figure 3),[14] resulting in minor risk to other organs except the gastrointestinal track.

4.2. Accumulating Organs. The most obvious information acquired from isotopic labeling is the accumulating organs. The toxicological evaluations should first focus on the accumulating organs and their related systems.

Currently, the toxicity studies focus on the liver, spleen, and lungs, which are the major accumulating organs after carbon NMs enter the blood circulation. We have performed a systematic evaluation of tween 80 dispersed SWCNTs in mice.[15,42] According to the ^{13}C skeleton labeling, SWCNTs accumulated in liver, lungs, and spleen.[15] Thereafter, toxicity to liver and lungs was observed.[42] For those excreted via the renal pathway, renal toxicity should be considered.

The majority of carbon NMs deposit in lungs after pulmonary exposure.[7,8,38,39] Therefore, the evaluation of the damage toward the respiratory and cardiovascular systems is the most imperative.

4.3. Toxicity Evaluation Period. The accumulation and excretion of carbon NMs also provide suggestions on designing the evaluation period. Both acute and chronic toxicity of carbon NMs should be taken into account. The toxicity evaluation period is selected based on the ADME properties.

For example, amino-CNTs are retained in the body for only several hours; therefore they are more probable to arouse acute toxicity.[27] For those cases accumulating shortly and excreting rapidly, a short evaluation period is required. On the other hand, for carbon NMs with a long-term retention inducing chronic toxicity, more attention should be paid to the long-term toxicity.

5. Proposed Themes for Future Research

To develop *in vivo* quantification methods for carbon NMs, selection of a suitable labeling strategy is crucial. Referring to Scheme 2, the incorporation (skeleton) labeling methods are very stable and suitable for the long-term studies. On the other hand, some radioactive labeling methods are more convenient and cheaper and hence satisfactorily suitable in the short term studies.

We therefore suggest a combination of labeling with different isotopes. For instance, CNTs were labeled with both stable ^{13}C and radioactive ^{125}I for long-term and short-term studies, respectively.[9,15,29,32,48] By using ^{125}I labeling, the short-term information can be achieved quickly. The convenience of ^{125}I labeling allows us to screen samples in a high throughput method with satisfactory sensitivity. After that, carbon NMs of special interests can be subject to ^{13}C labeling for a long-term evaluation.

Also, carbon NMs and the attached functional groups could be double labeled with different isotopes for simultaneously tracing the distribution and metabolism of NM derivatives. Such combination of the double labeling would largely enhance the quantification of carbon NMs *in vivo*.

Previous ADME studies mainly focus on the absorption, distribution, and excretion of carbon NMs. *In vivo* metabolism is much less studied, and only very few papers have been published in this respect. The major difficulty is the lack of suitable quantification methods. The metabolism of carbon NMs includes two aspects, the breakdown of the carbon

skeleton and the detachment/attachment of the functionalities. To study the latter aspect, other techniques could also be employed beyond the radioactive labeling, such as studying the detachment of PEG from PEG-SWCNTs in mice by Raman spectrometry.[44]

The last but not least issue is long-term exposure at low dosages, for example, cases occurring in a workplace. Obviously, such investigations require particularly adequate labeling methods. Seemingly, the only available isotope labeling meeting the requirements is the ^{14}C skeleton labeling. However, the whole process of synthesis, handling, and detection of ^{14}C is quite difficult and complicated. Therefore, in future, developing new routes for such evaluations is highly encouraged. Besides, the development and adoption of various standards, including carbon NMs, quantitative analysis methods, and protocols, are highly promising in the future.

6. Conclusions

In summary, we have reviewed the isotopic labeling and quantification of carbon NMs *in vivo*, in view of their advantages and disadvantages. The quantitative information of carbon NMs *in vivo* is summarized, and the implications to the NanoEHS evaluation are discussed accordingly. For future studies, the comparison and calibration of the analytical data between different laboratories have been proposed. At this point, the development and adoption of standards, including carbon NMs, analytical methods, and protocols, are highly demanded. In the line of the basic developing context of the contemporary analytical chemistry, more efforts should be paid to pursue high sensitivity and accuracy, high throughput, real time, and *in situ* analysis. Developing new *in vivo* quantitative analytical methods are encouraged, especially for the *in vivo* metabolism of carbon NMs and the long-term exposure to carbon NMs at low dosage.

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FOOTNOTES

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