

カーボンナノチューブのバイオ

1. 細胞との相互作用・細胞毒性・細胞内消化・細胞外排出
2. 動物体内での挙動(蓄積、排出)
3. 動物毒性試験
4. バイオ応用

細胞内部への取り込み機構

- エンドサイトーシス

巨大分子を取り込む経路はクラスリン被覆ピットと小胞による“受容体を介したエンドサイトーシス”細胞膜上のカベオラという窪み(直径~50nm)を介して取り込まれるカベオラ依存性エンドサイトーシス

- ファゴサイトーシス

多核白血球、単球、マクロファージなどによる細菌や細胞の死骸などの取り込み。小胞はそのままリソソームと合体し、物質を加水分解酵素により消化する。

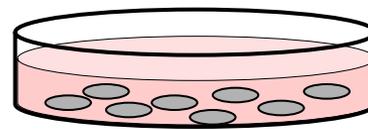
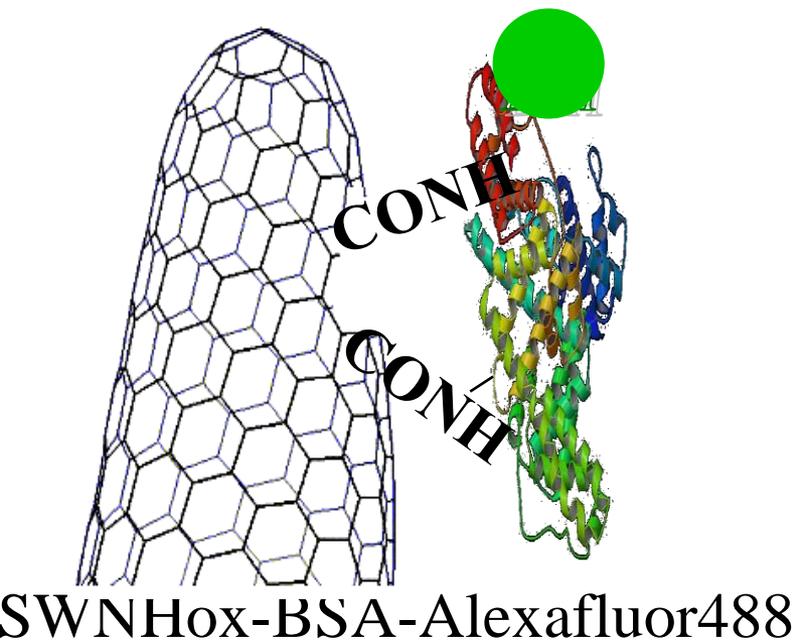
- ピノサイトーシス

食細胞以外の細胞でも発達した機構の一つであり、細胞外液を細胞質に取り込むことで、細胞に必要な水溶性の栄養分を取り込む

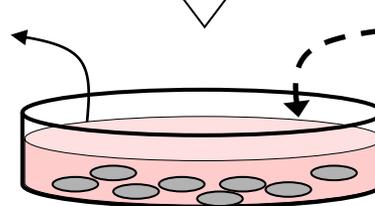
- ピアシング

MWNTで見つかった現象。細胞膜中を拡散して中へ入る。

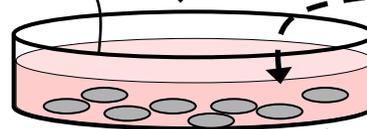
細胞内への取り込み



Cell $2 \times 10^4/ml$
Transformed Rat cell
5RP7 (HSRRB, Japan).



SWNHox-BSA*
& Culture medium



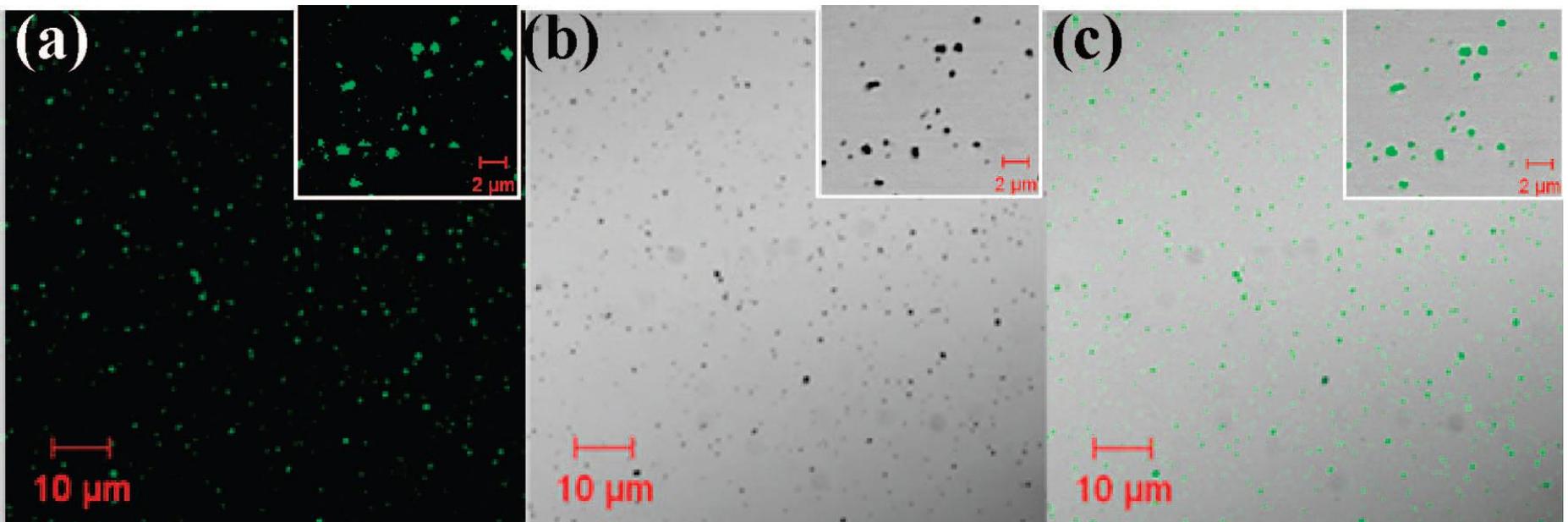
Culture medium

Confocal microscopy

Flow cytometry

Incubation condition: 37°C, 5 % CO₂,

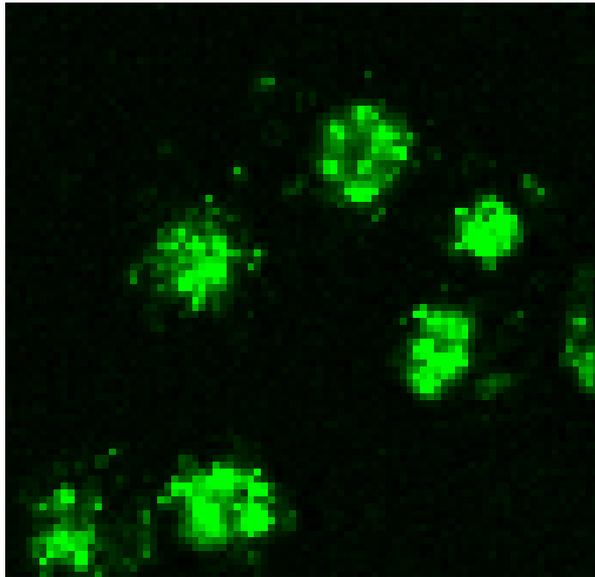
Confocal microscope images of SWNHox-BSA-AF (excitation 488 nm, emission detected at 510 nm)



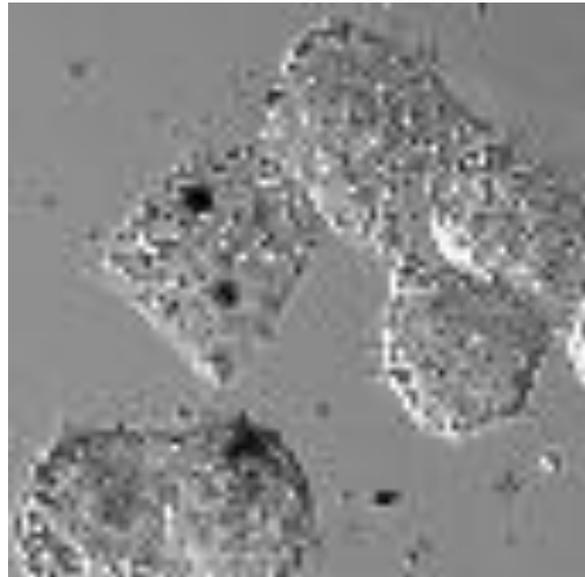
SWNHoxから遊離しているBSA-AFがないことを確認。

細胞内へ取り込まれたSWNHox-BSA-Fluoro488。 レーザー共焦点顕微鏡による観察

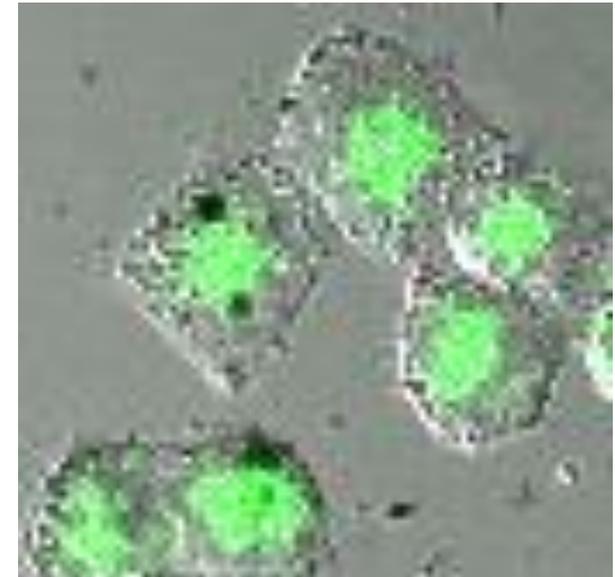
Confocal microscope images.



fluorescence image



DIC image



superposed image

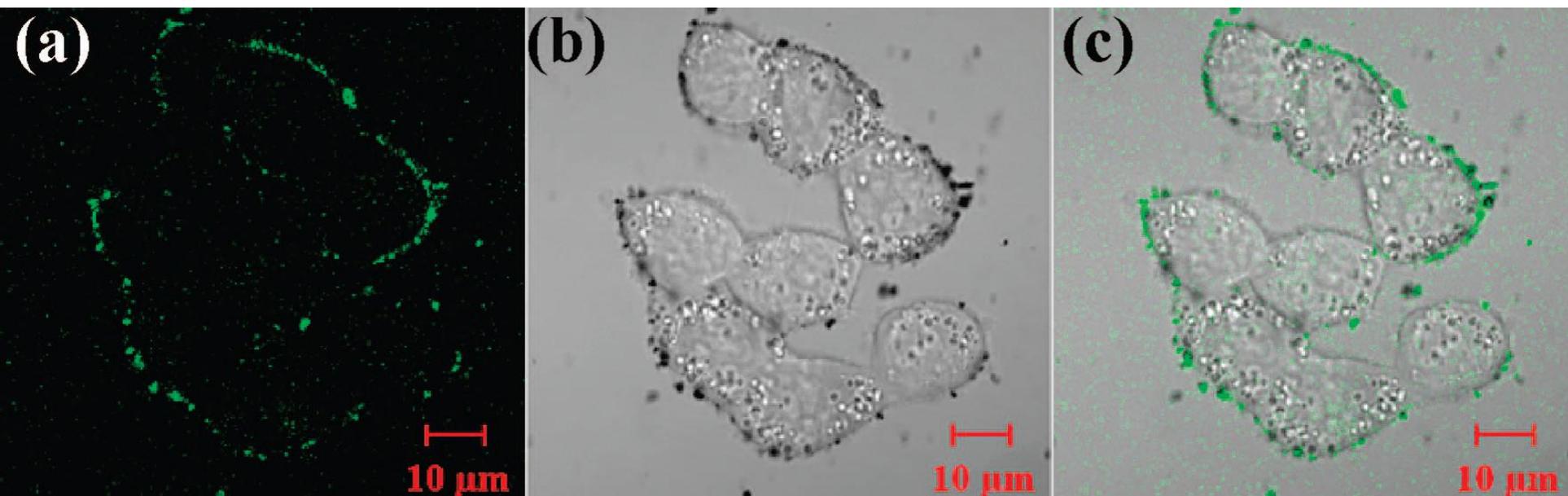
Zhang et al. ACSNano 2007

Cell: HR460(Human lung cancer cell)

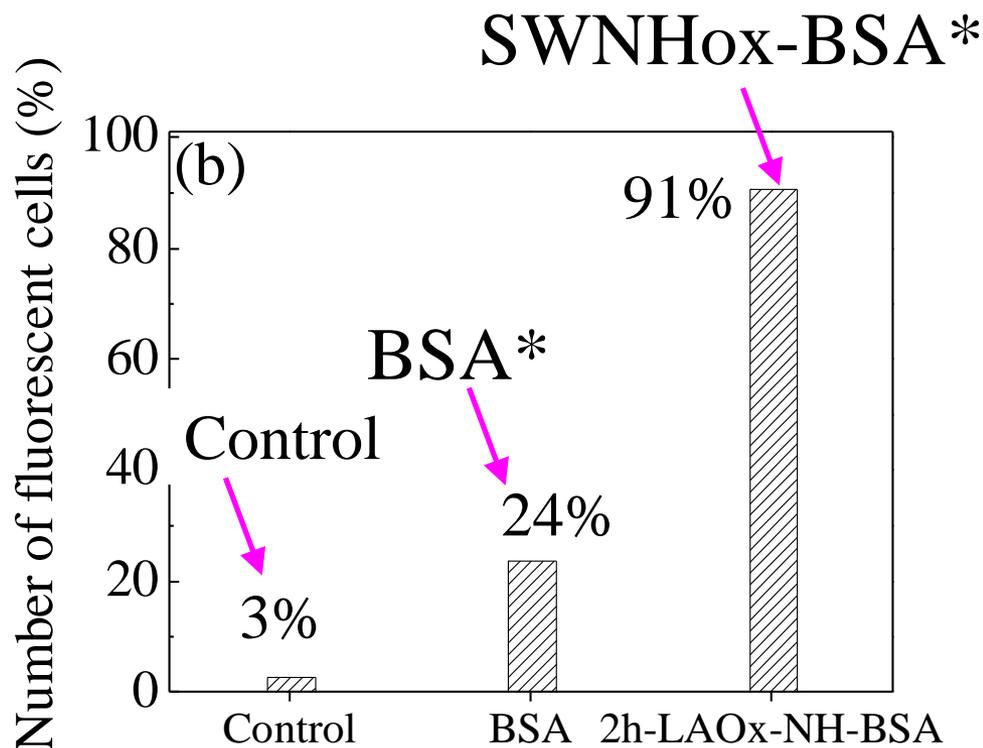
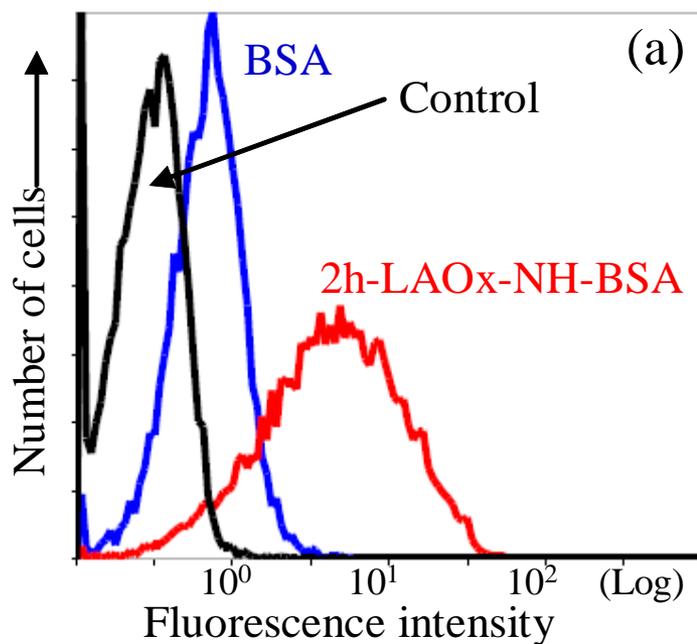
SWNH-Alexa Fluor 488

Differential interference contrast

4°Cでのインキュベーションした時には、SWNHox-BSAは細胞内へ取り込まれなかった。
従って、H460細胞のSWNHox取り込み機構は、エンドサイトーシスである。



SWNHox-BSA-AFを取り込んだHR460細胞の数をFlow cytometryを用いて数える。



生データ

解析後

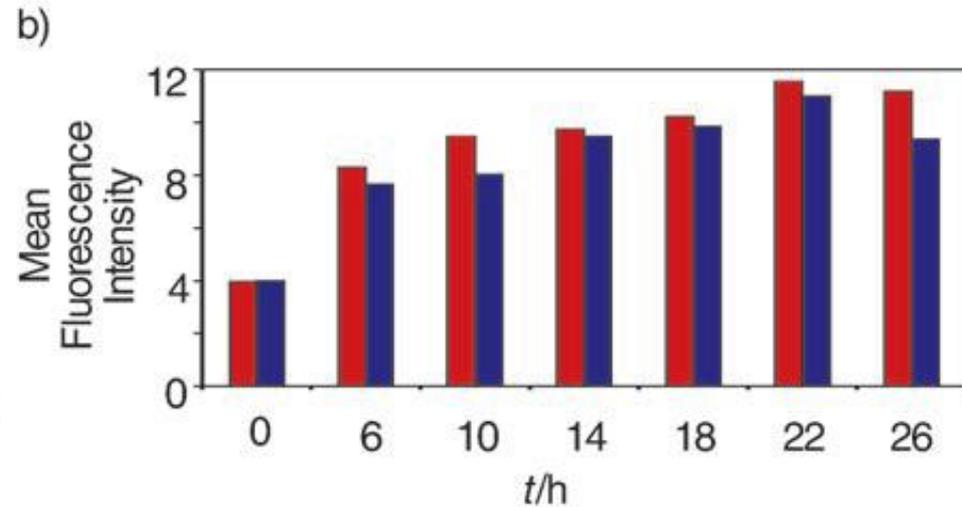
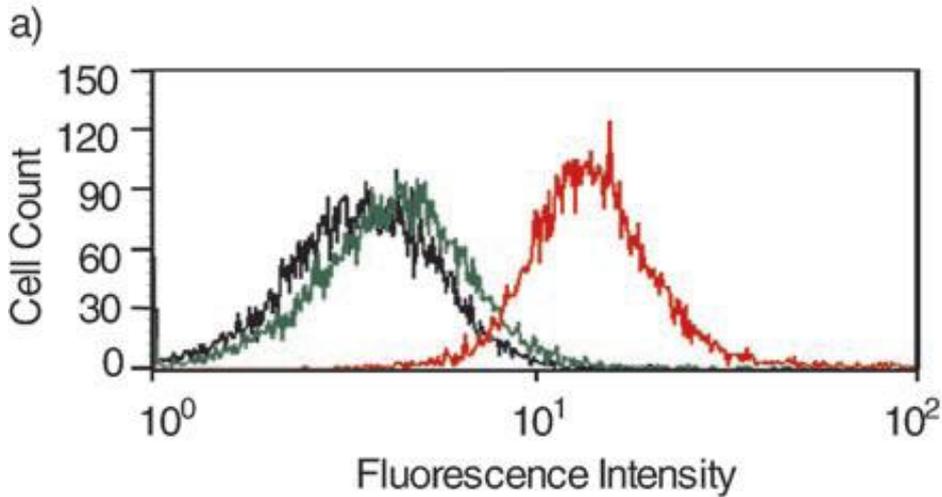
BSA*は取り込まれにくい。

SWNHoxは取り込まれやすい。

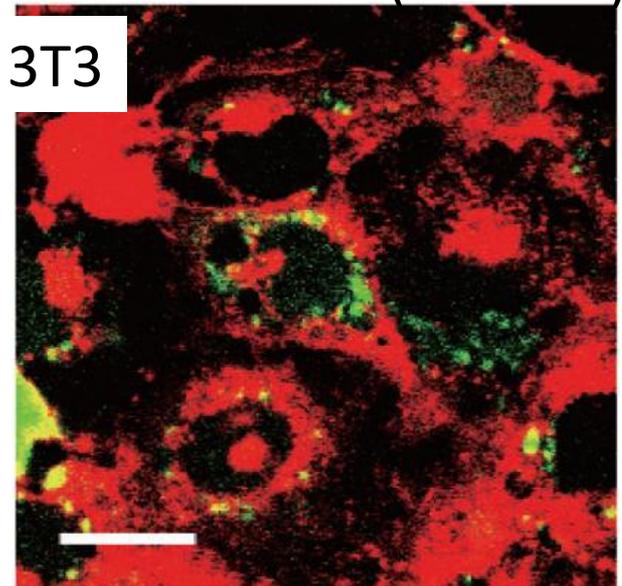
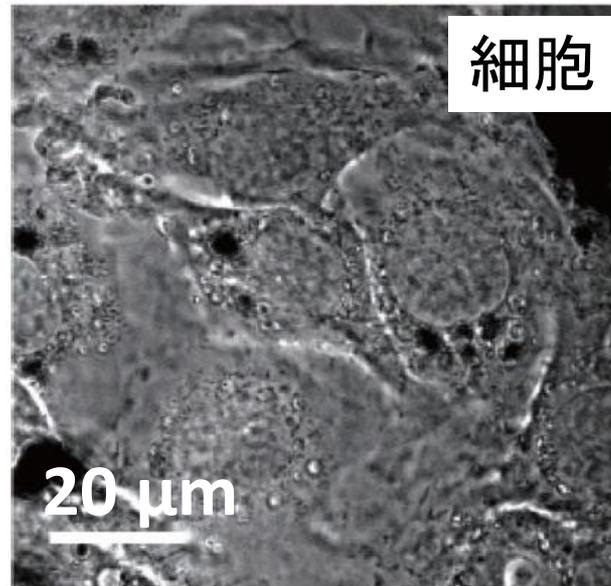
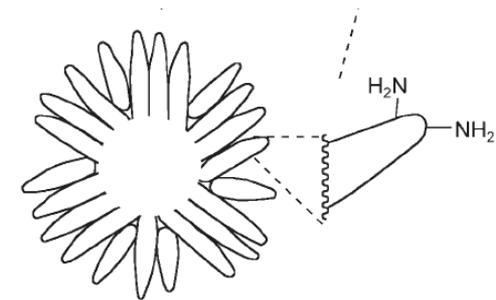
Zhang et al. ACSNano 2007

Amino nanohorns: Preparation, dispersion, and internalization into cells.

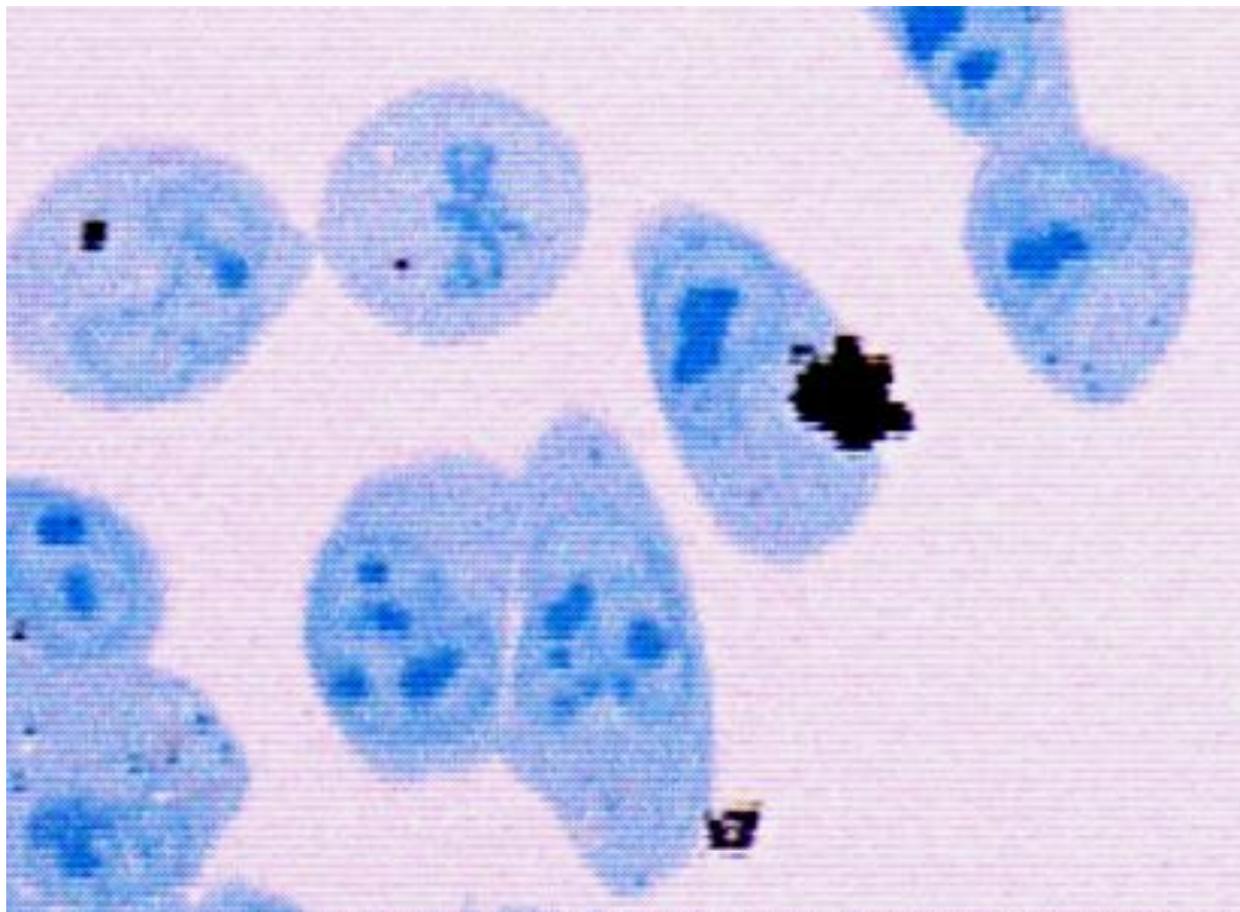
Isobe et al. *Angew. Chem. Int. Ed.* **45**,6676 (2006).



green: amino-CNH-Oregon Green
red: cell membrane (FM4-64)

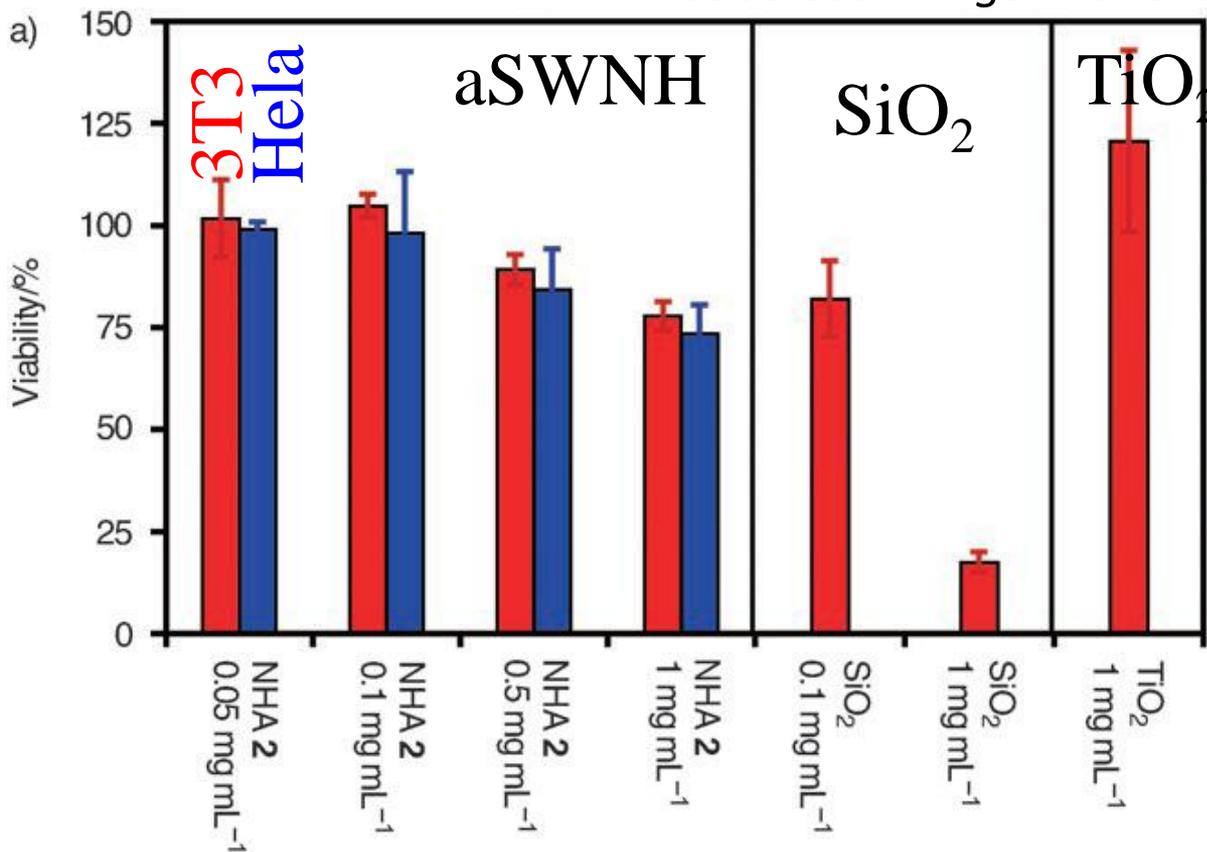


SWNHoxに分散剤コーティングを行わないと、PBS中で均一分散しない。細胞の内部には、ほとんど入らない。外壁に付着する傾向がある。



CDDP@SWNHox

細胞毒性



Bradford protein assay

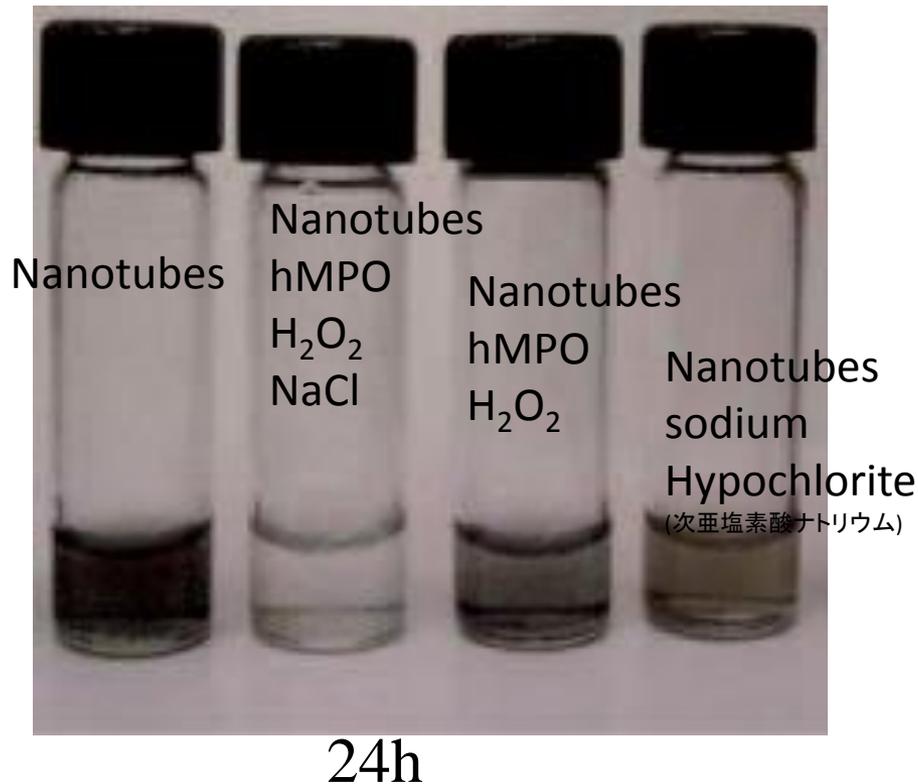
Coomassie Brilliant Blue G-250 (クーマシーブルー)は酸性溶液中でタンパク質と結合するため、吸光度が465 nmから595 nmへシフト。



培地 0.1 mg/mL 1 mg/mL

Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation

Kagan et al. Nature Nanotech. 2010

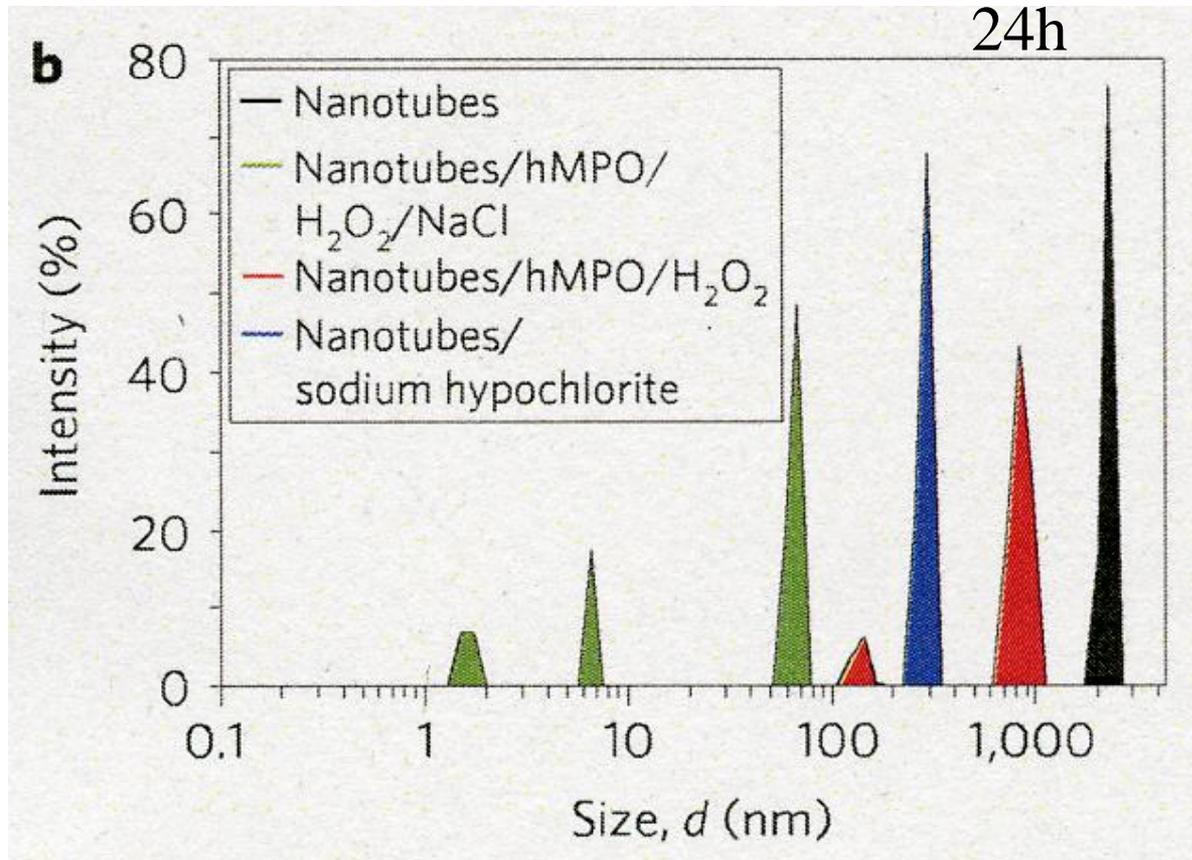


Myeloperoxidase: 好中球のみに存在する酵素。過酸化水素(H₂O₂)と塩素イオン(Cl⁻)から次亜塩素酸(HOCl)を産生。酵素反応によって生じたHOClは、高い殺菌効果をもつ。

好中球: 白血球のひとつで、食細胞。細胞外寄生生物を退治。

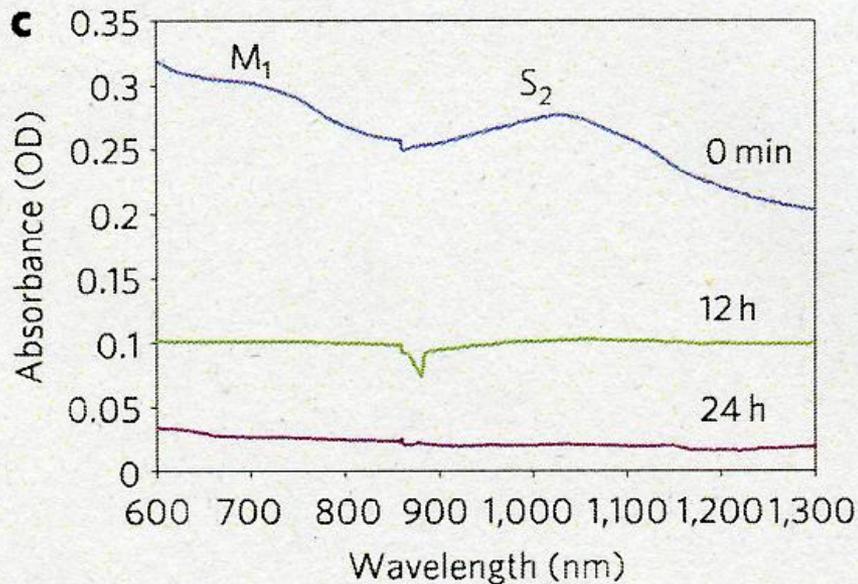
hMP: human myeloperoxidase

Dynamic light scattering showing multiple smaller peaks corresponding to biodegraded nanotubes.

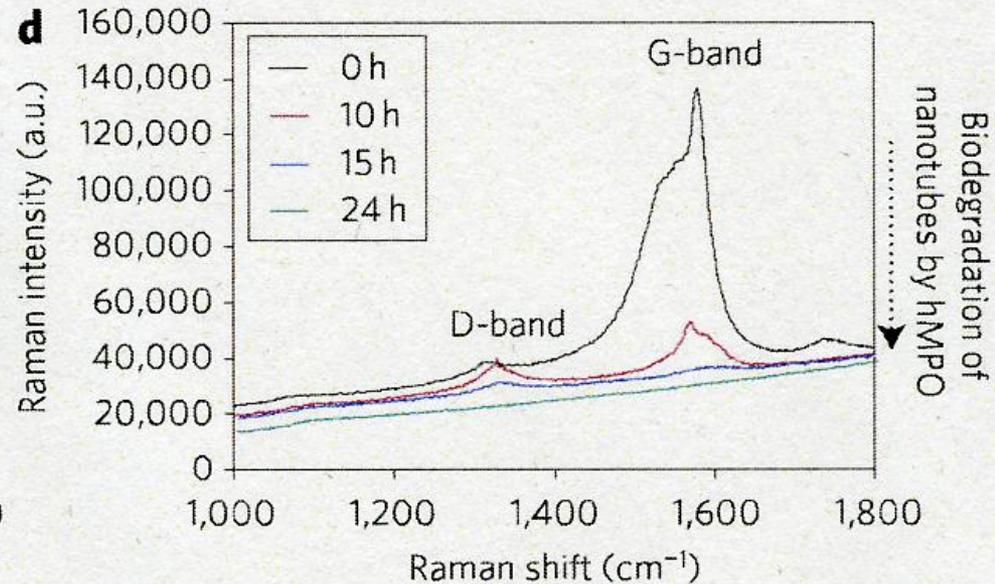


In hMPO and H₂O₂ after 12h incubation: (GC-MS measurements)
short-chain tri-carboxylated alkanes, alkenes, molecular ions of di-carboxylated short-chain, mono-carboxylated products

Vis-NIR spectra showing loss of M1 and S2 bands as nanotubes are degraded in the presence of hMPO and H₂O₂.

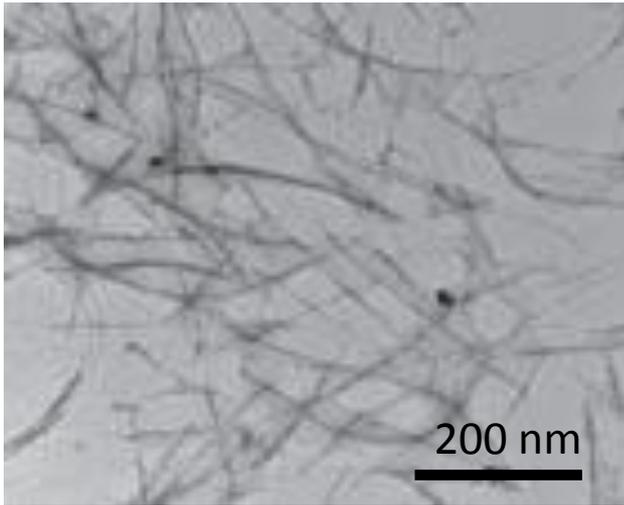


Raman spectra (excitation, 633 nm) of ethanol-dried nanotubes (black) and (hMPO and H₂O₂)-treated nanotubes, showing loss of the characteristic G-band, followed by appearance and decay of the D-band over time



TEM observation: SWNTs were degraded.

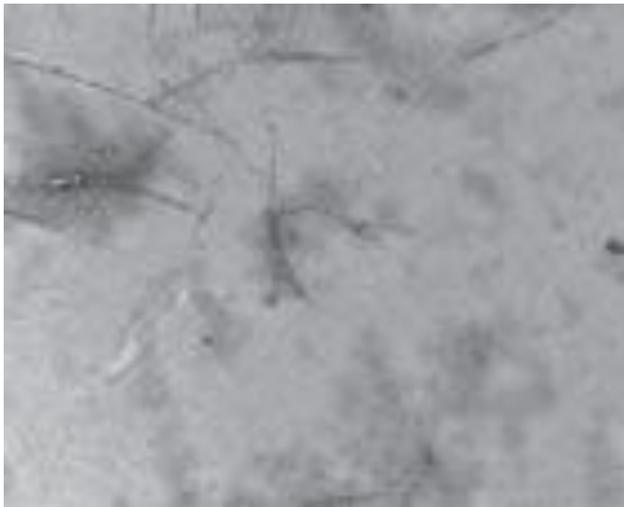
24h



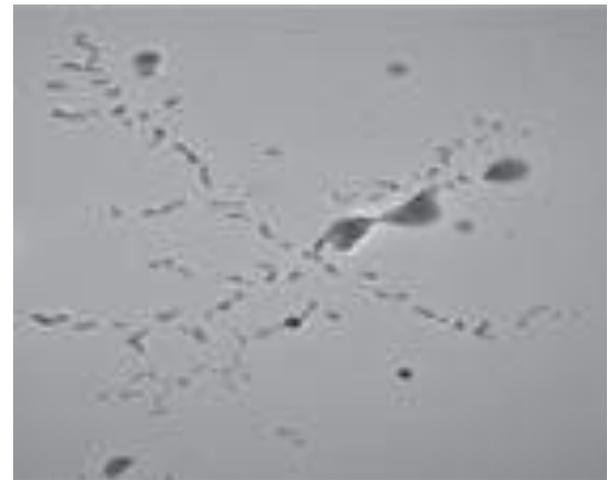
Nanotubes



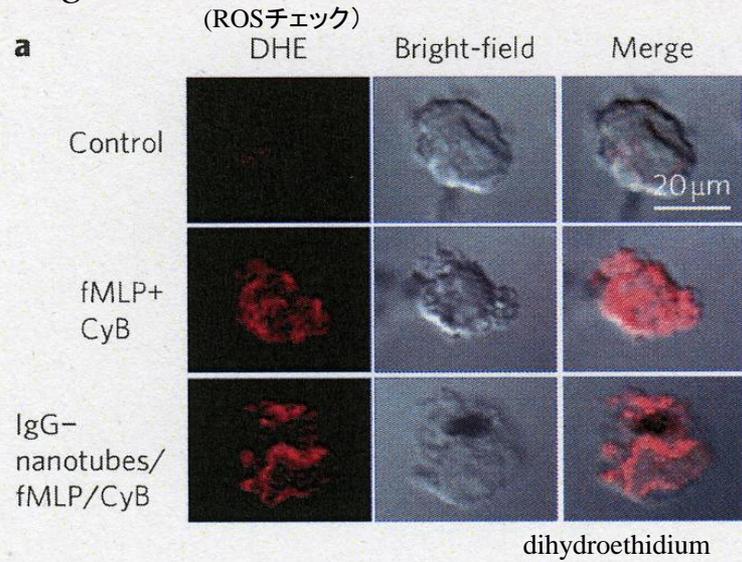
Nanotubes
hMPO
H₂O₂
NaCl



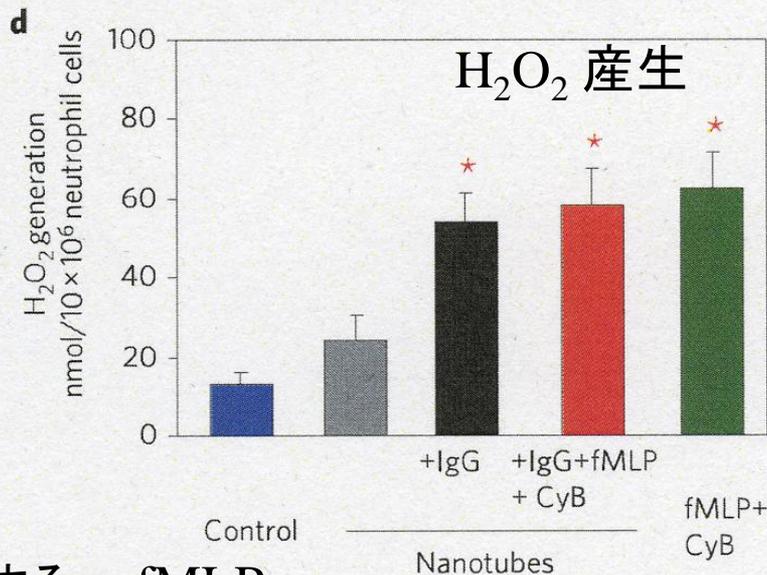
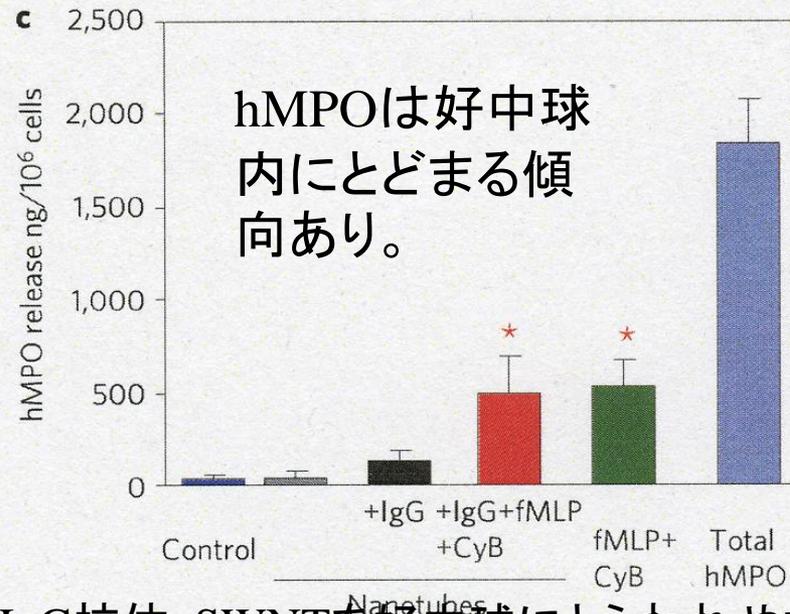
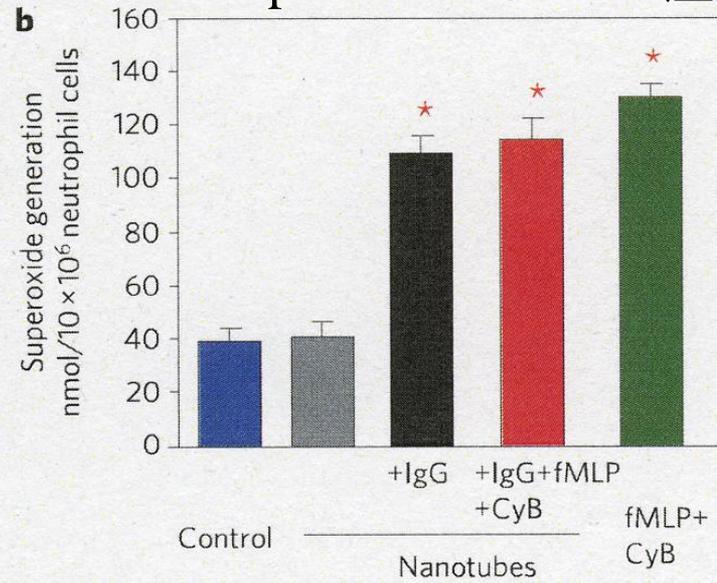
Nanotubes
hMPO
H₂O₂



Nanotubes
sodium
Hypochlorite
(次亜塩素酸ナトリウム)

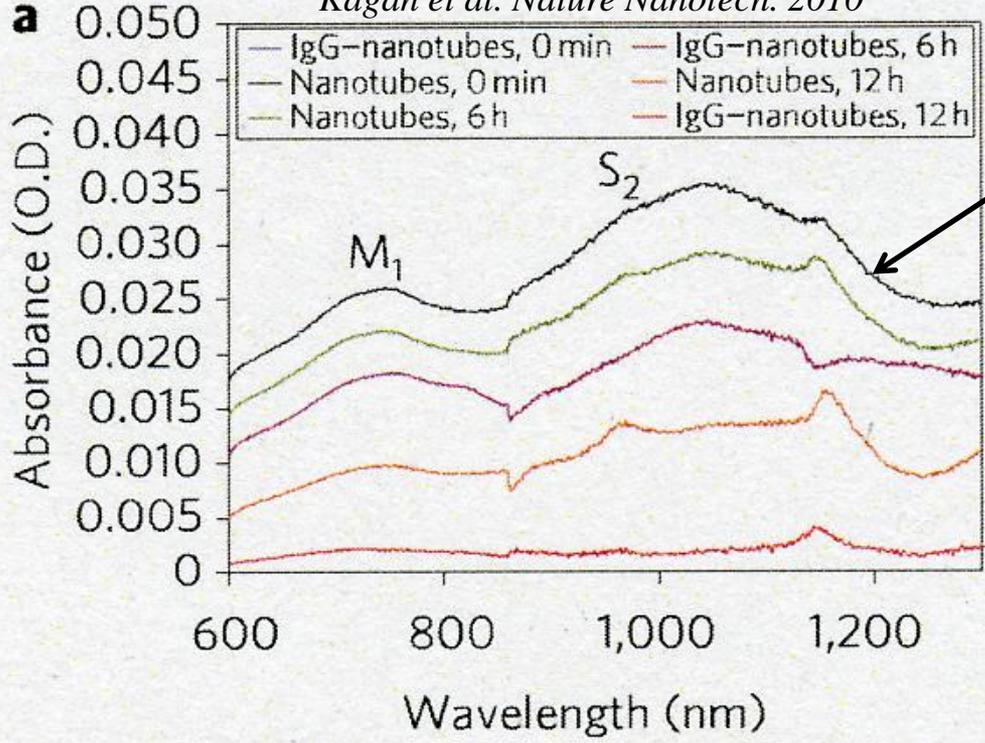


Super OxideはHOCl産生に必要



IgG抗体: SWNTを好中球にとらわれやすくする。 fMLP (N-formyl-methionyl-leucyl-phenylalanine): 好中球の活性化。 CyB (cytochalacyn B): 好中球の顆粒から、酸をださせる。 HMPOの活性化とリリースを促す。

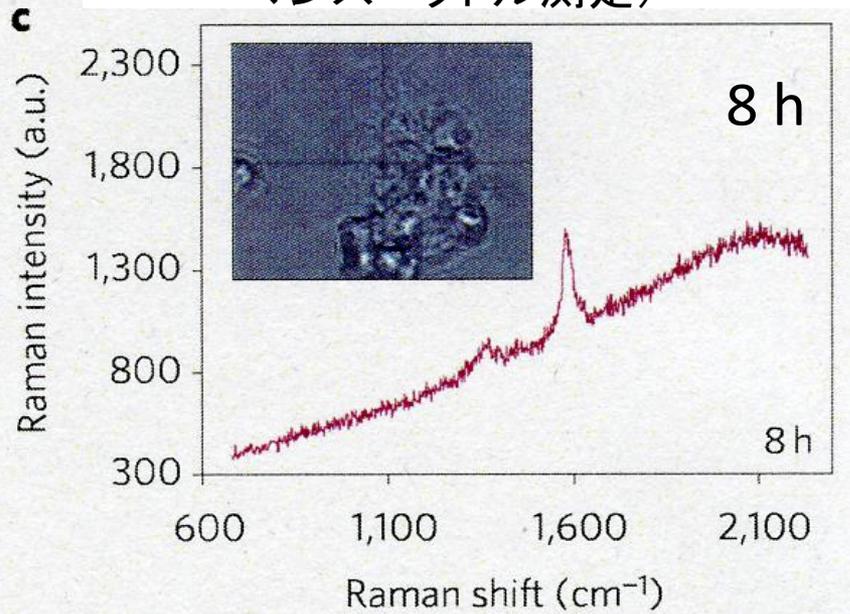
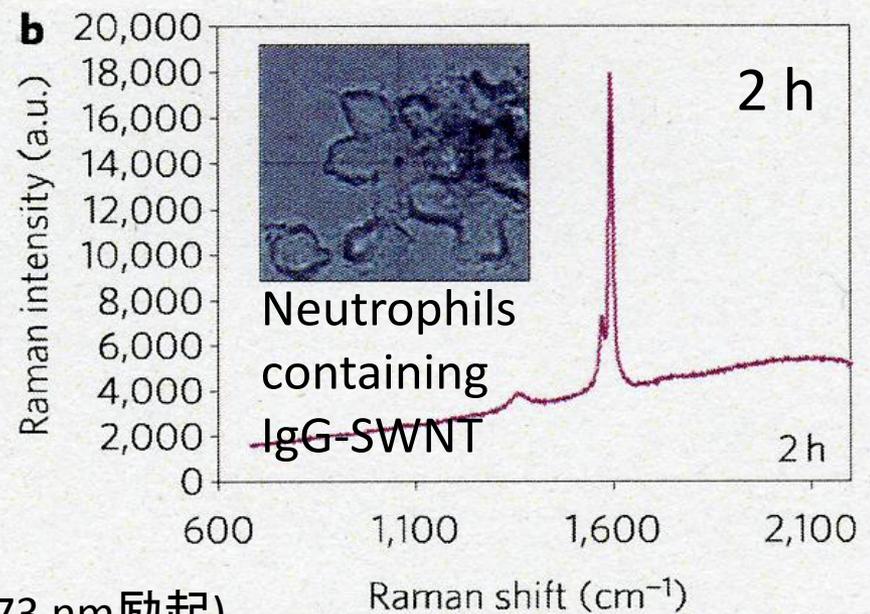
ヒト好中球によるSWNTの分解



IgG-SWNT, 0h
SWNT, 0h
IgG-SWNT, 6h
SWNT, 6h
IgG-SWNT, 6h
SWNT, 6h

(Cellは超音波で分解して、そのままvis-NIR吸収スペクトル測定)

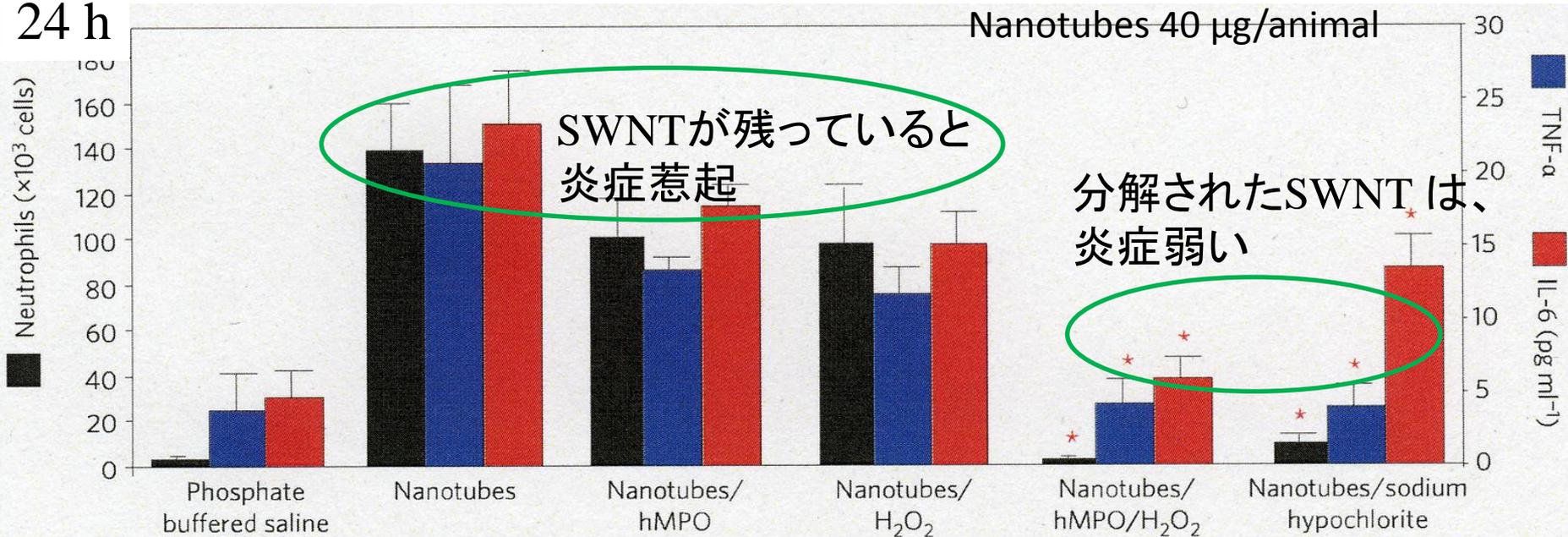
(パラホルムアルデヒド固定してラマンスペクトル測定)



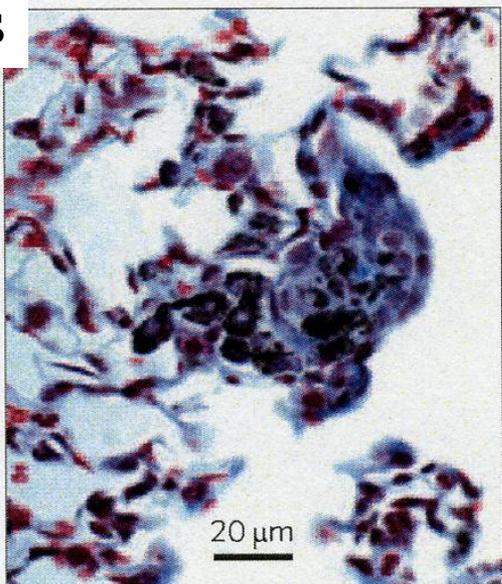
(473 nm励起)

ラット気管投与、肺の組織検査

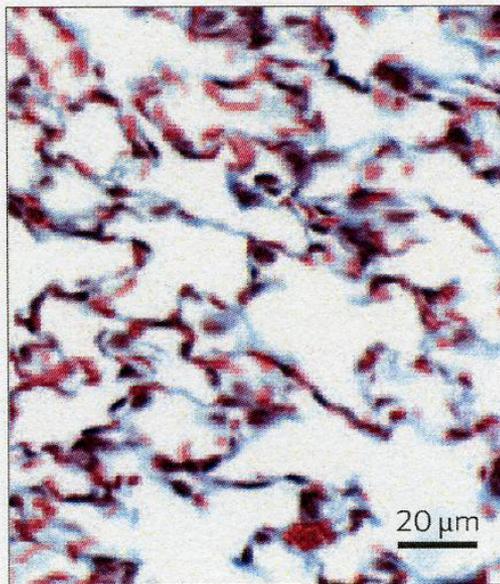
24 h



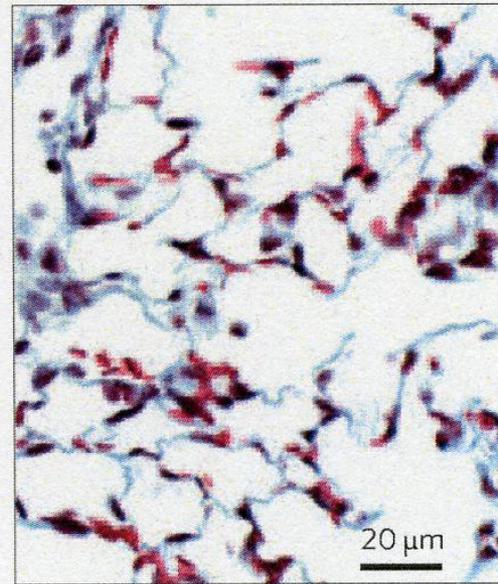
7 days



Nanotubes



Nanotubes/hMPO/H₂O₂



Phosphate buffered saline

KaganらによるSWNTの生体内分解実験

条件付き(好中球を使用 あるいは IgG使用)でのSWNT分解。
分解は細胞実験でしか確認していない。

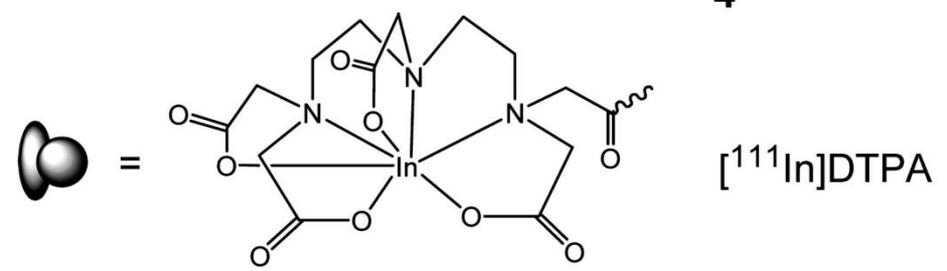
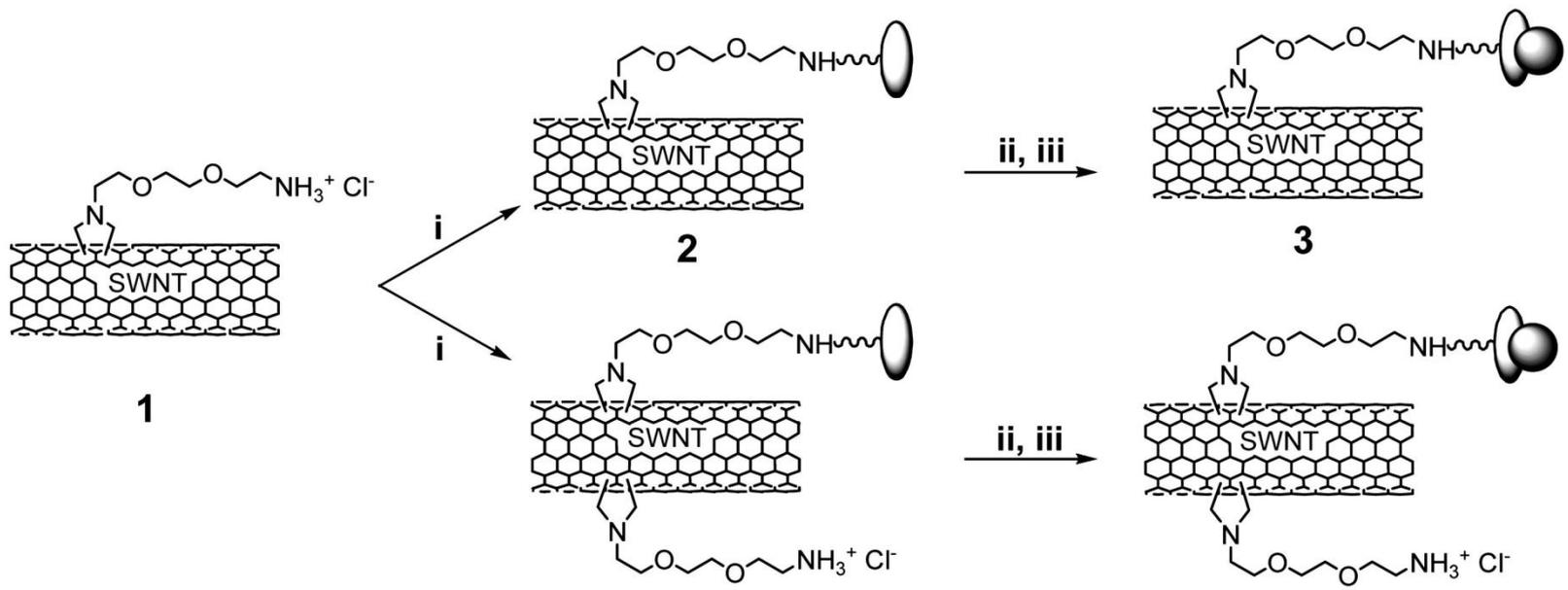
生体内では、マクロファージにより貪食される。
マクロファージのリソソームでは H_2O_2 による分解が主流。
 H_2O_2 は、 $HOCl$ ほど強い作用はない。
よって、実際に生体内で分解するかどうかは、確認必要。

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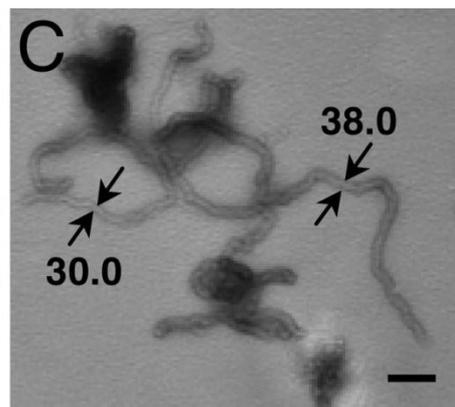
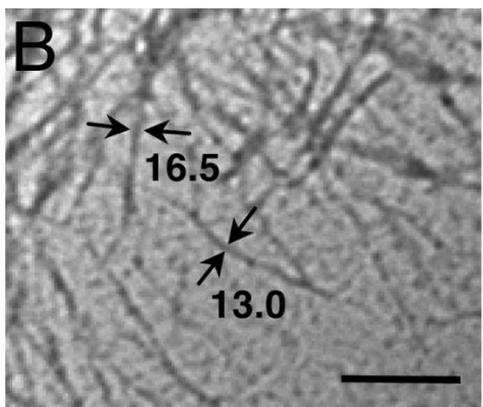
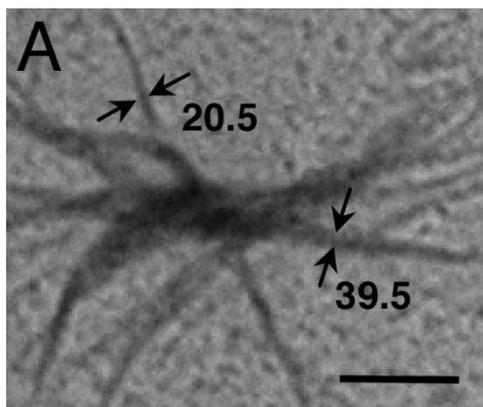
生体内のCNT分布をどのように計測するか？

- ラベルを付加
 - 放射性元素
 - ^{13}C
 - 蛍光物質
 - MRI
- SWNTの場合
 - SWNTの蛍光
 - SWNTのラマン散乱光
- チューブ内にラベルを内蔵



Tissue biodistribution and blood clearance rates of intravenously administered carbon nanotube radiotracers

Singh et al. PNAS 2006



A

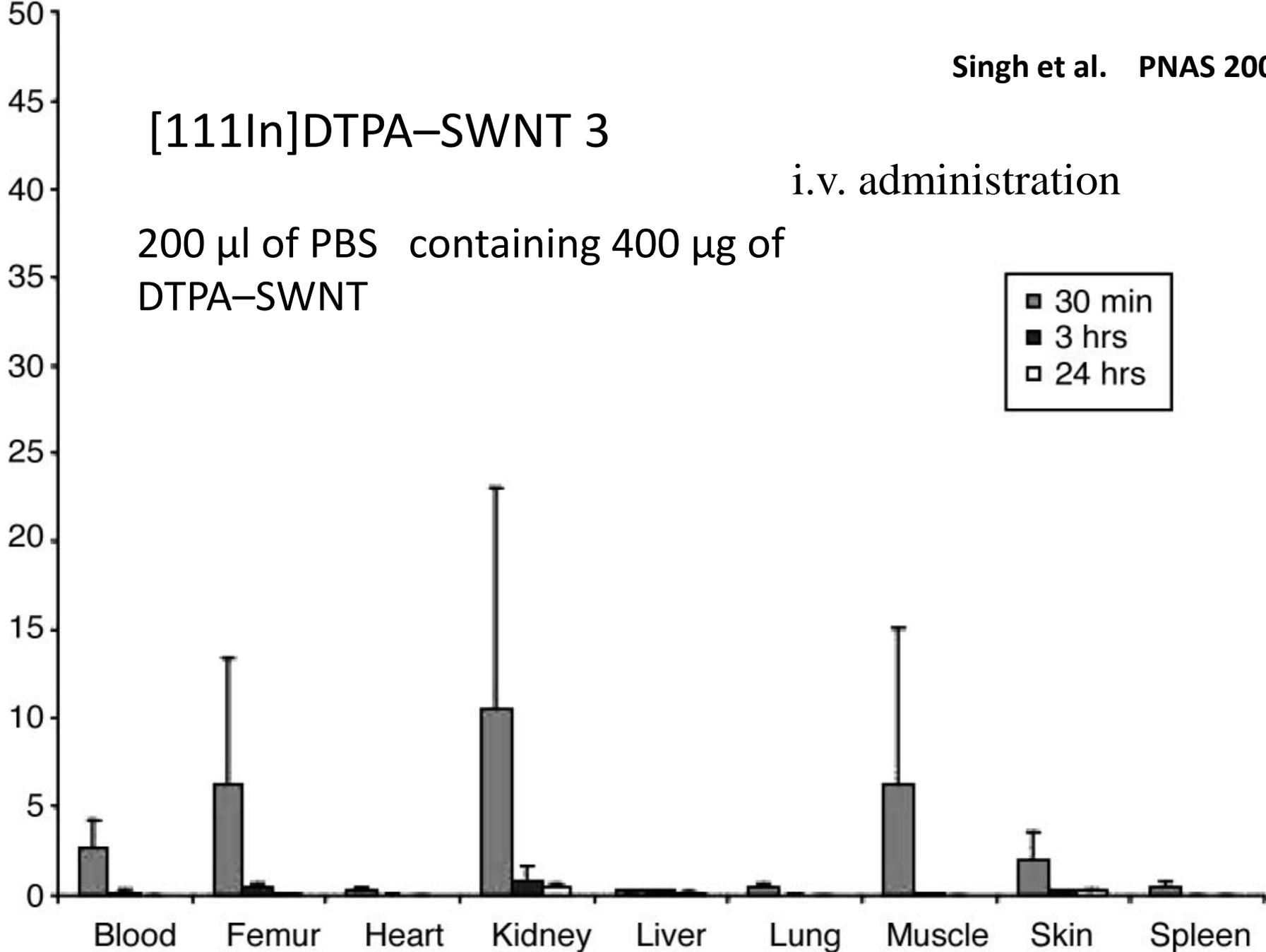
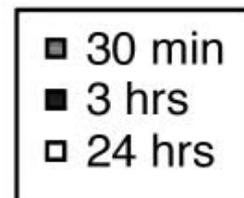
Singh et al. PNAS 2006

[¹¹¹In]DTPA–SWNT 3

i.v. administration

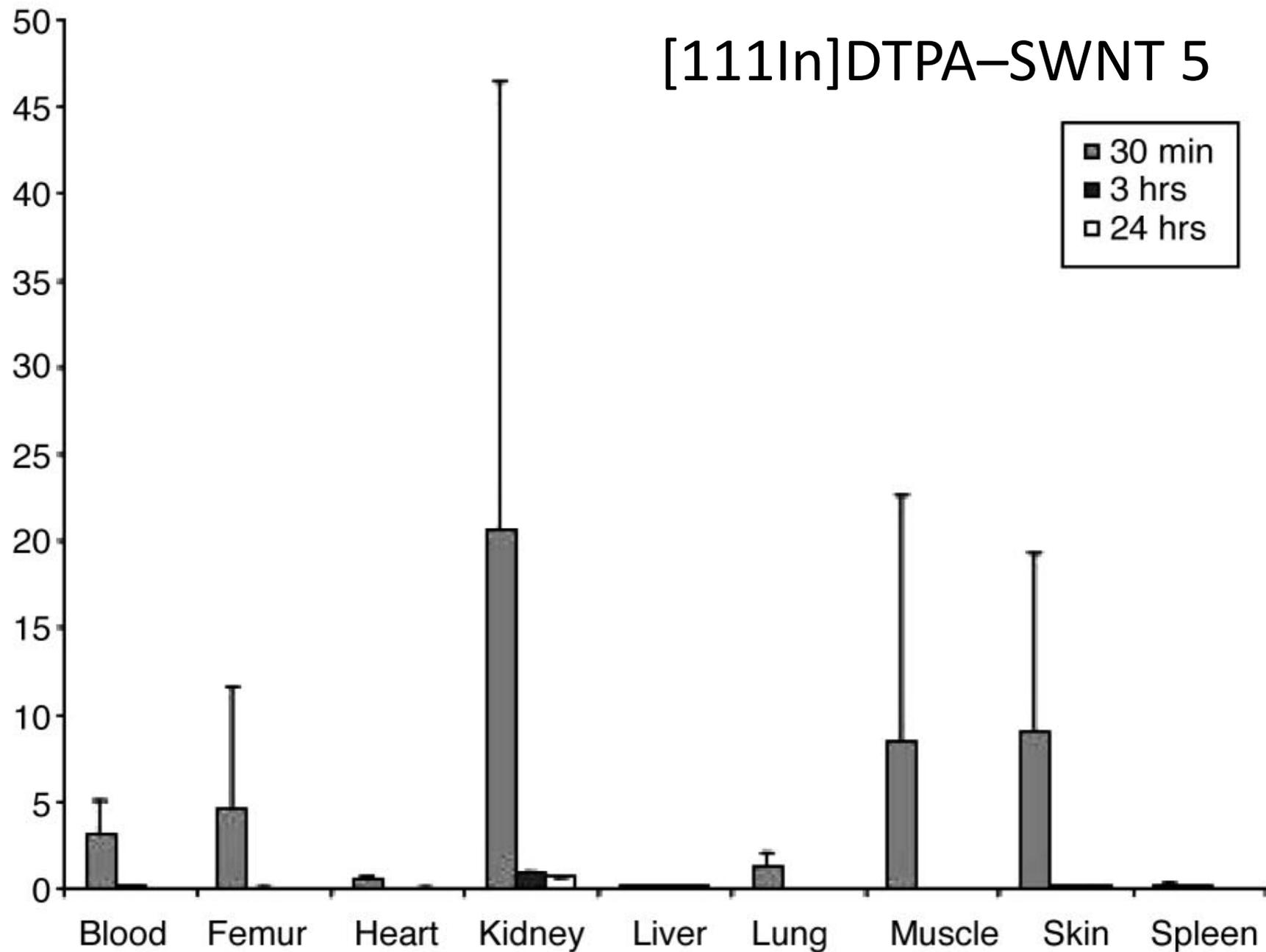
200 μ l of PBS containing 400 μ g of
DTPA–SWNT

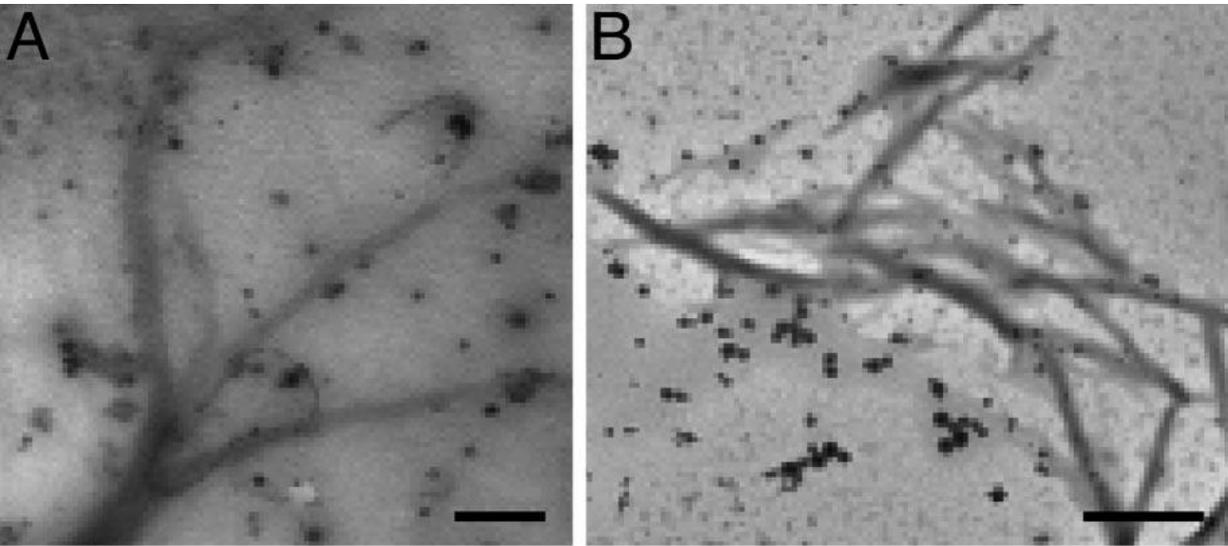
Percent Injected Dose per Gram Tissue



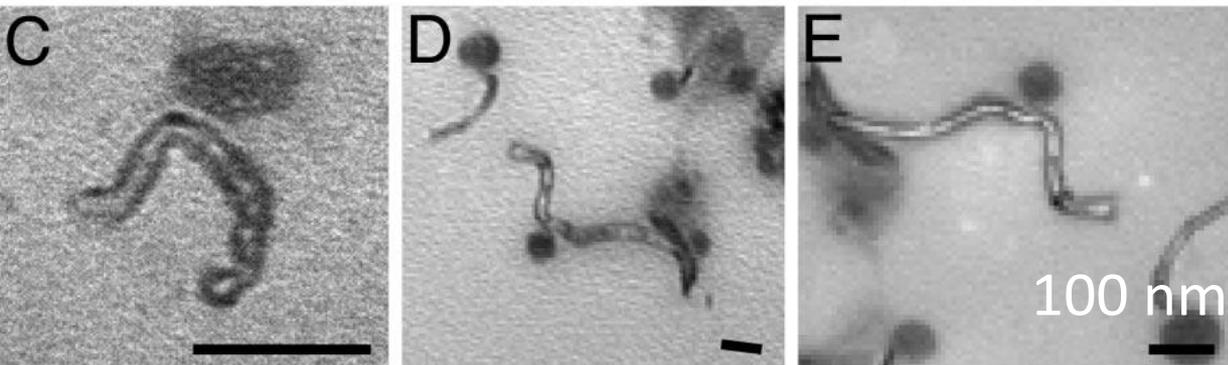
B**[¹¹¹In]DTPA-SWNT 5**

Percent Injected Dose per Gram Tissue

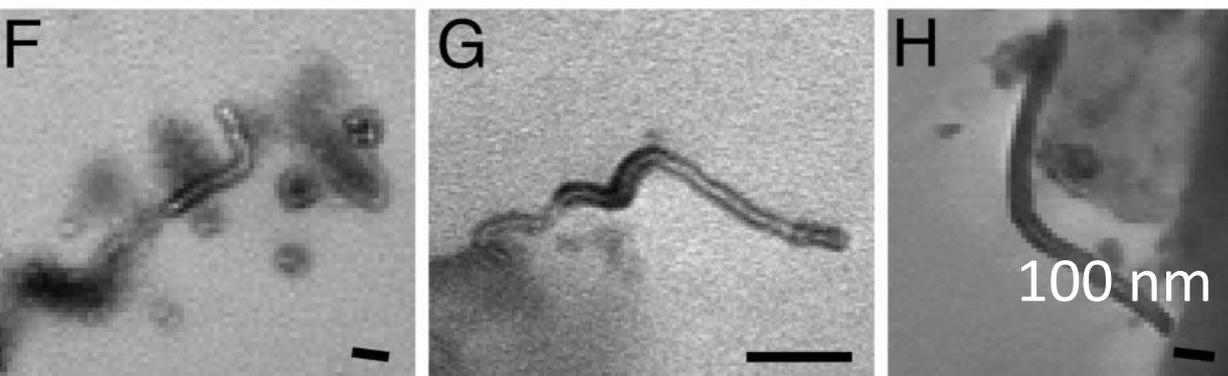




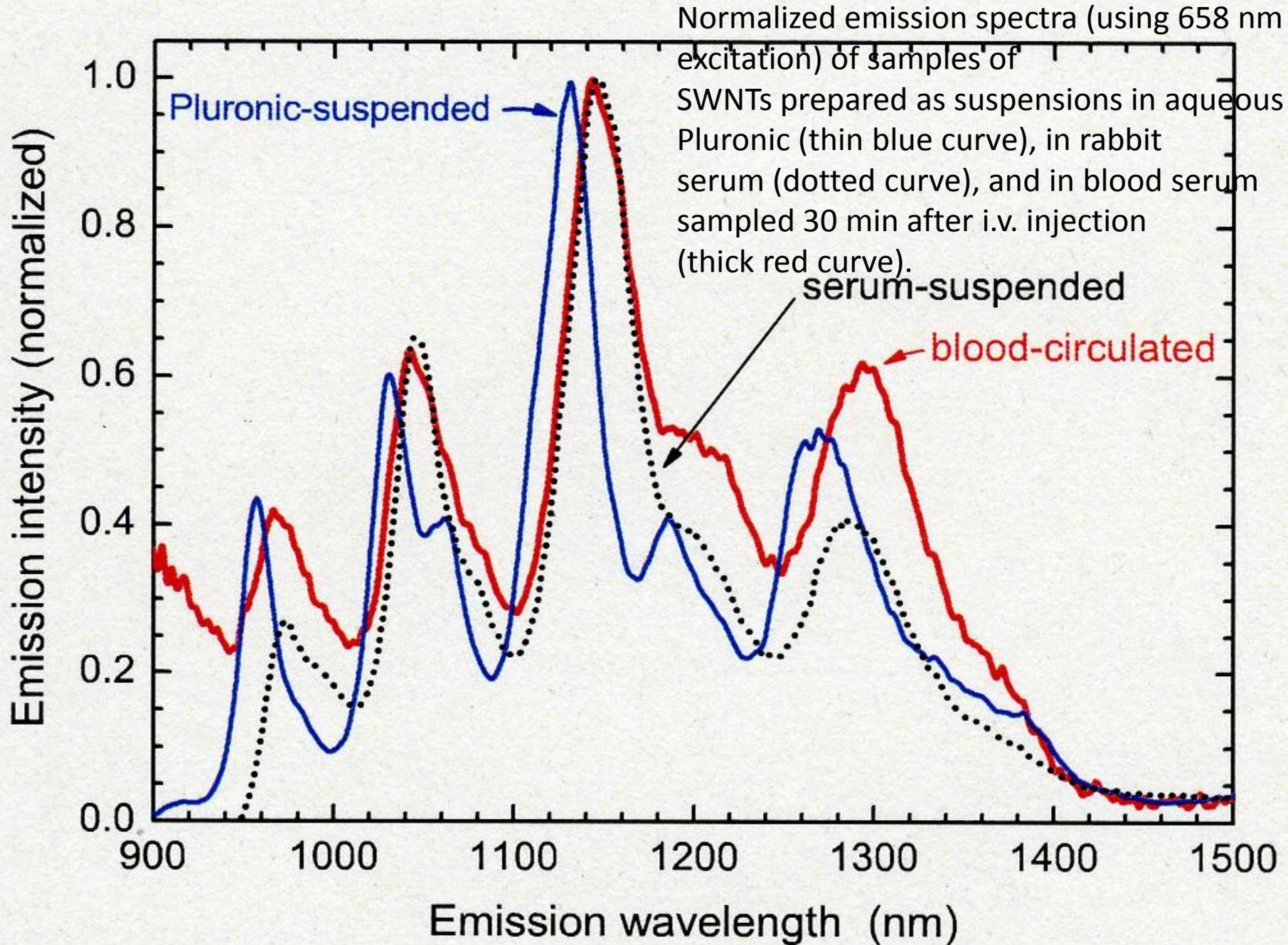
DTPA-SWNT from supernatant of urine

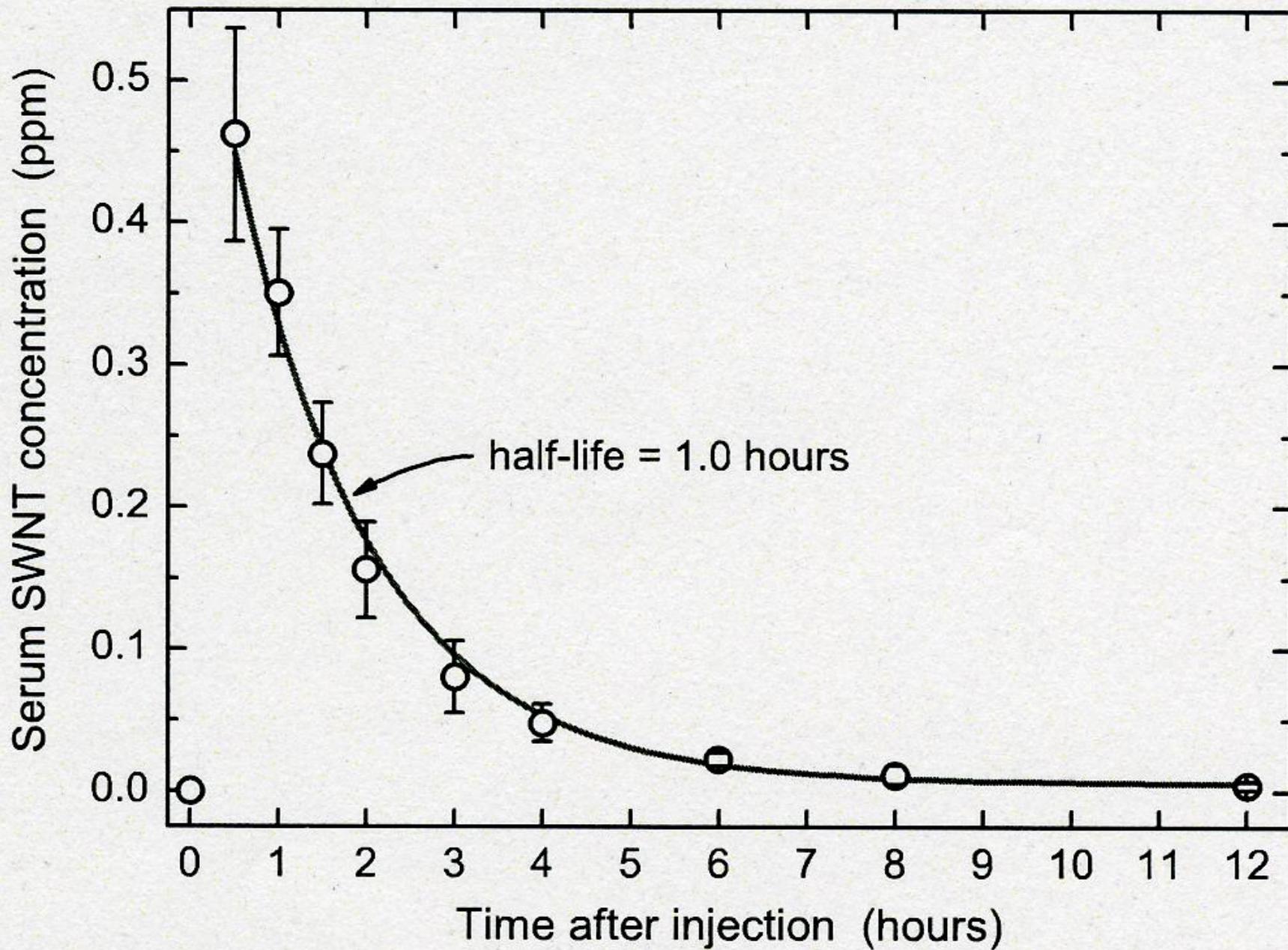


DTPA-MWNT in the supernatant

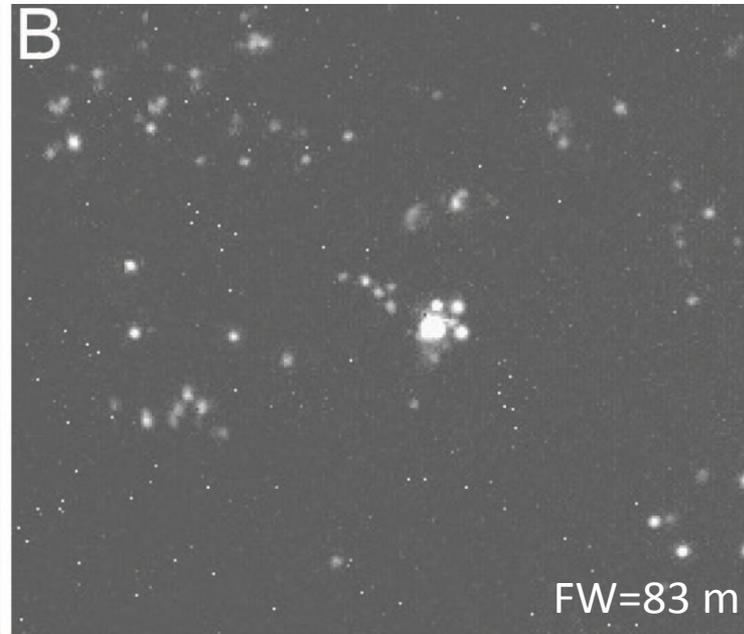
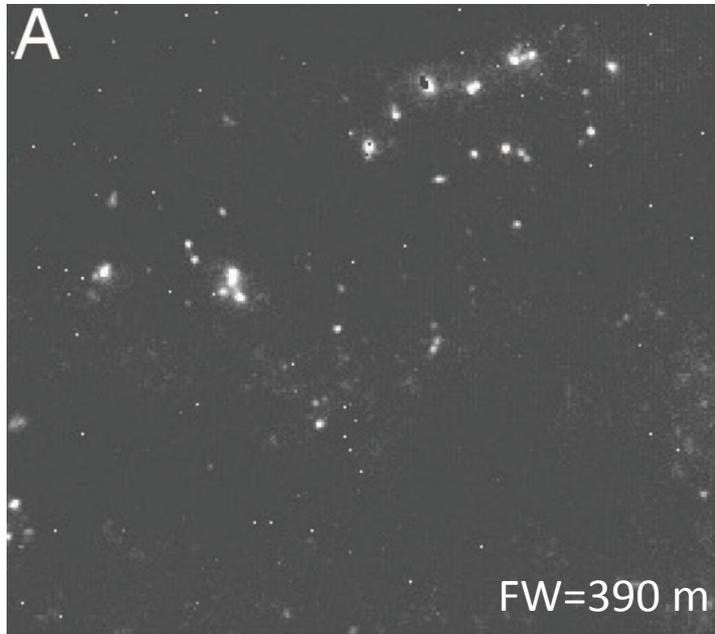


DTPA-MWNT in the precipitate

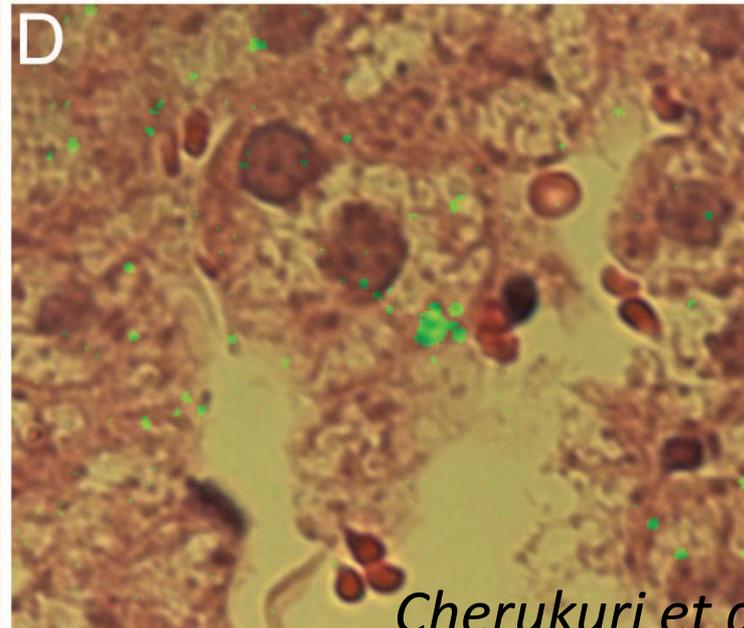
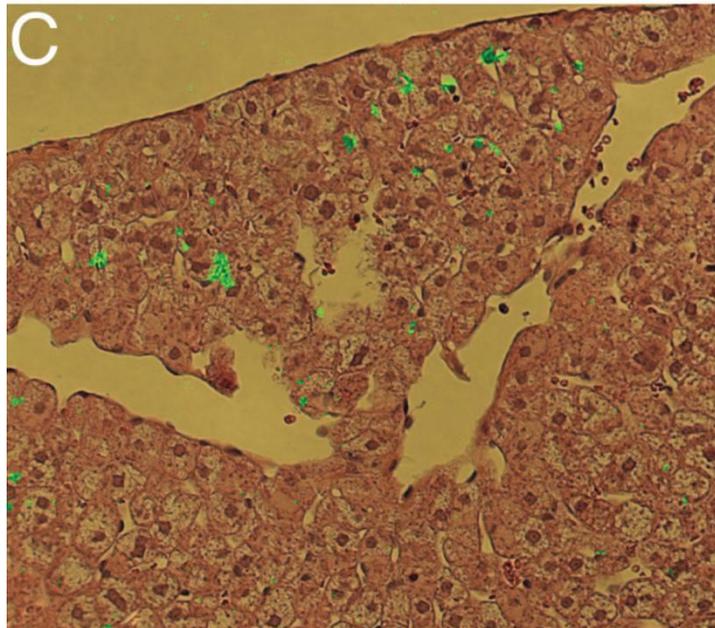




Near-IR SWNT fluorescence images in liver tissue



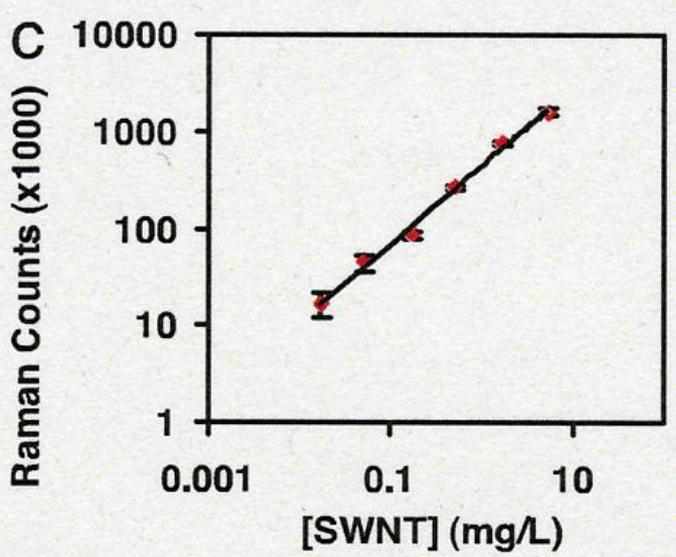
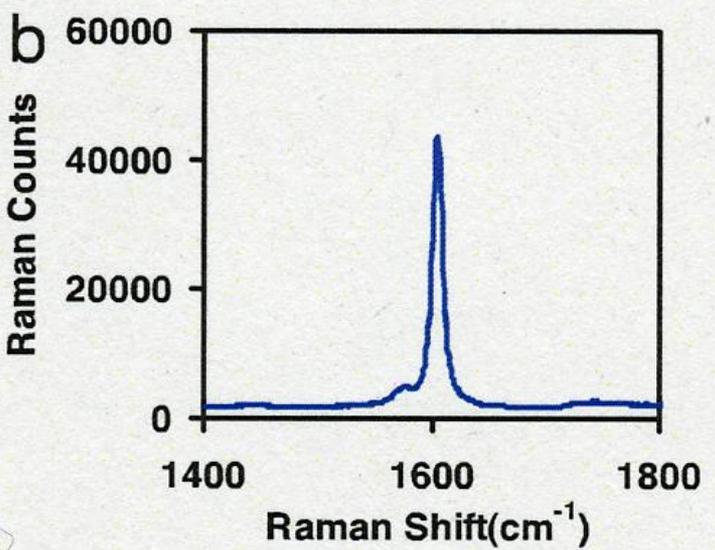
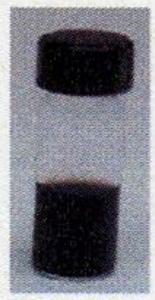
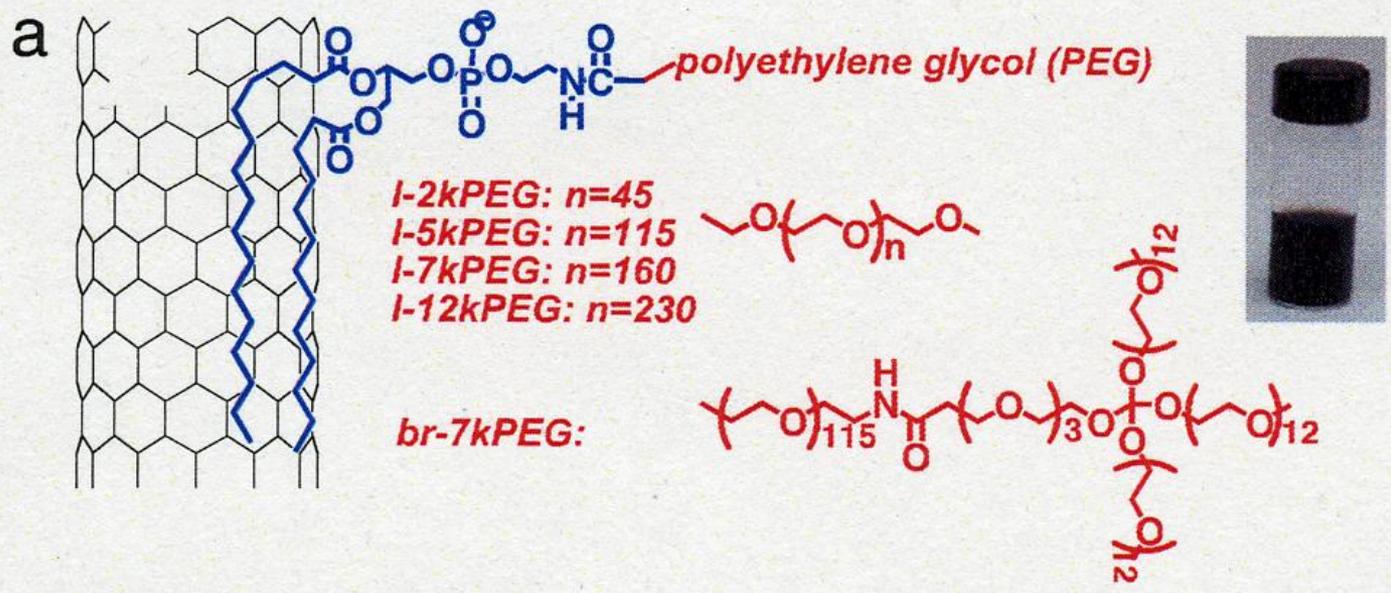
rabbits
24 h after i.v.



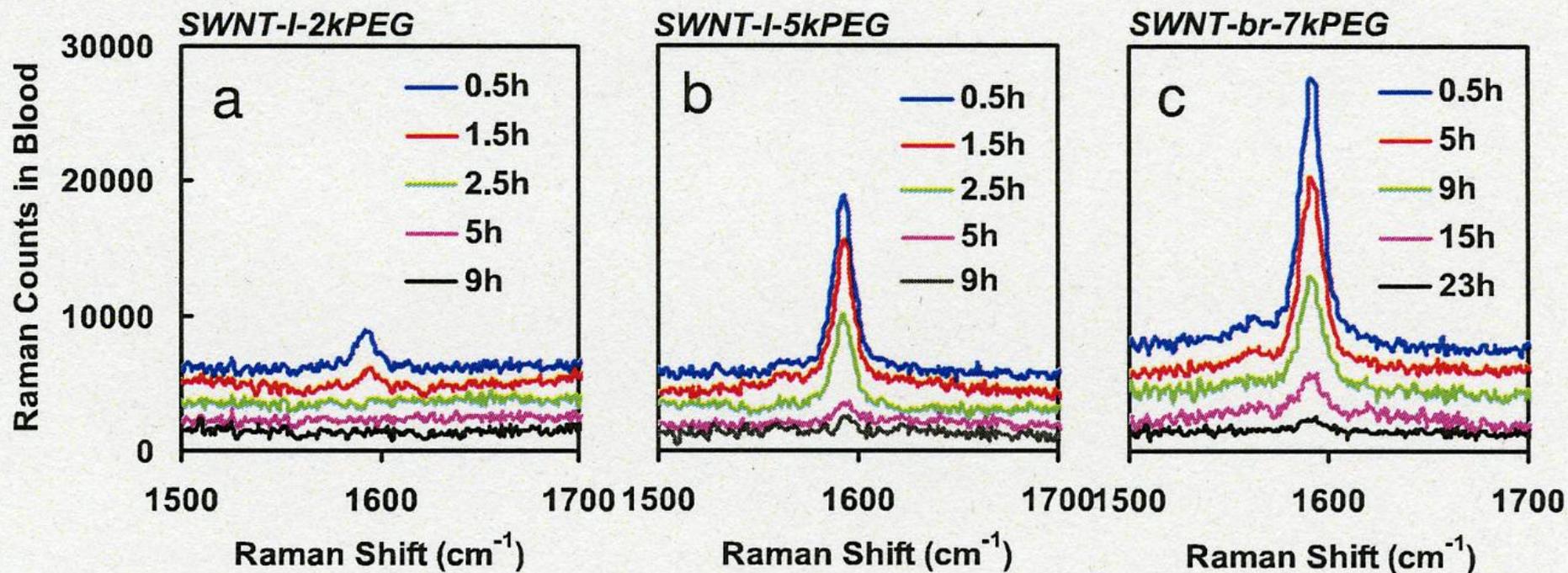
Cherukuri et al PNAS 2006

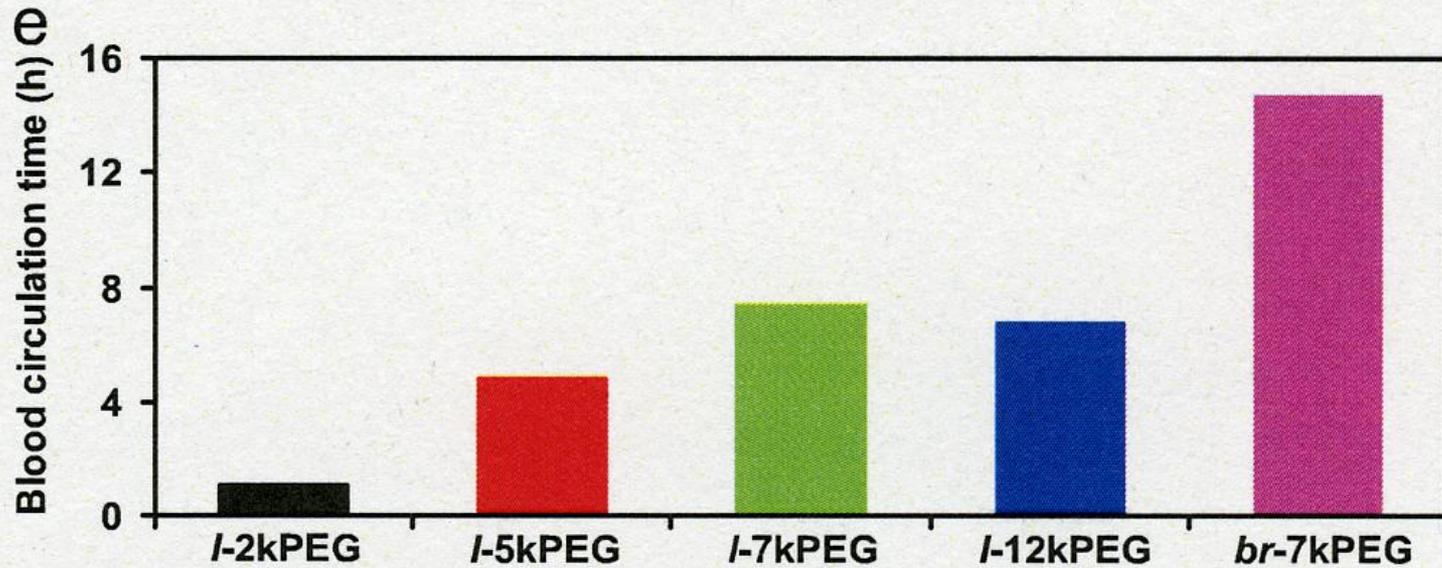
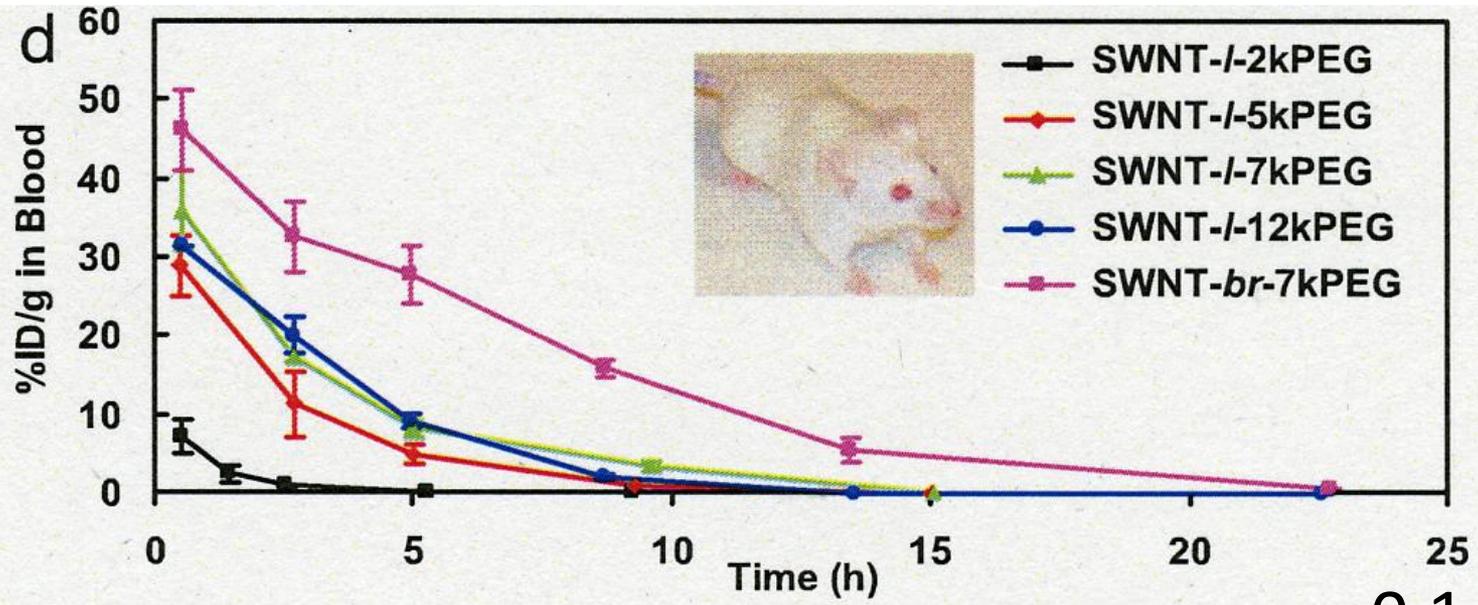
Circulation and long-term fate of functionalized, biocompatible single-walled carbon nanotubes in mice probed by Raman spectroscopy

Zhuang Liu* PNAS 2008



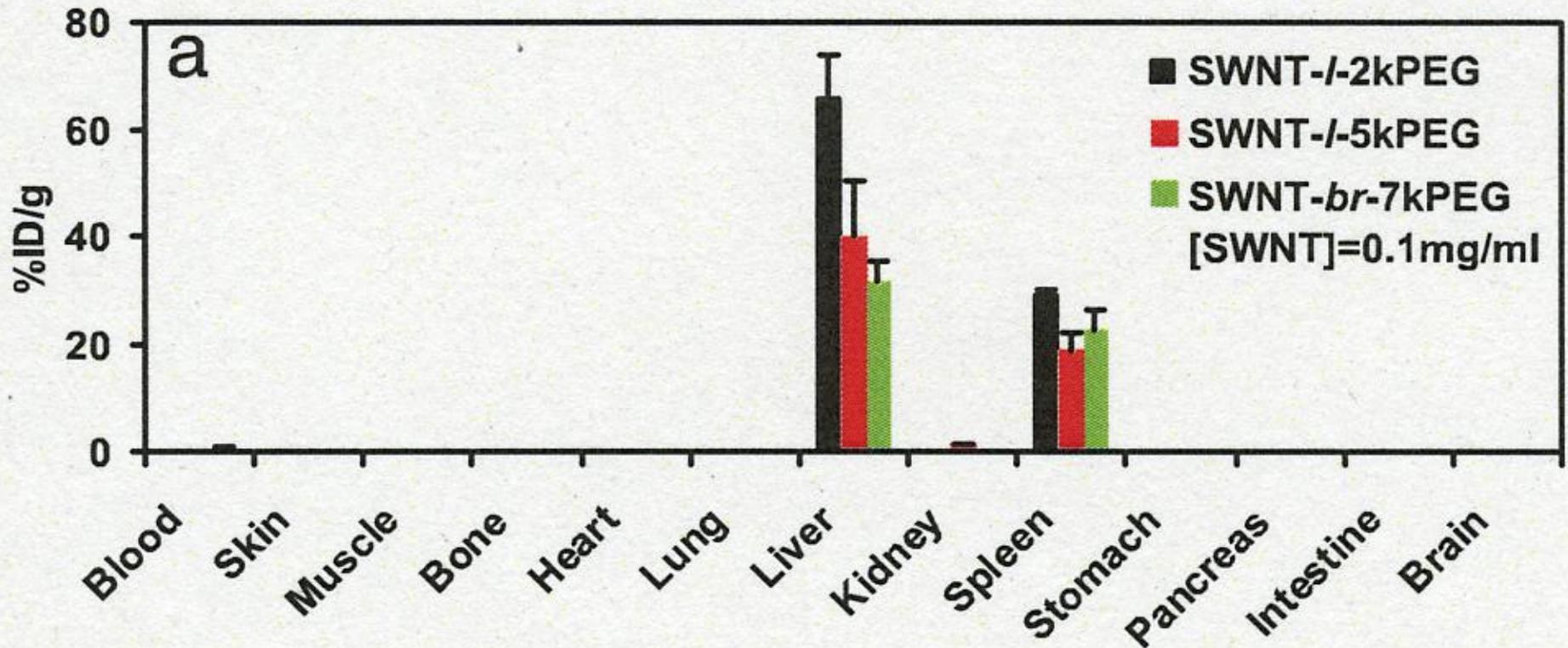
0.1 mg/ml





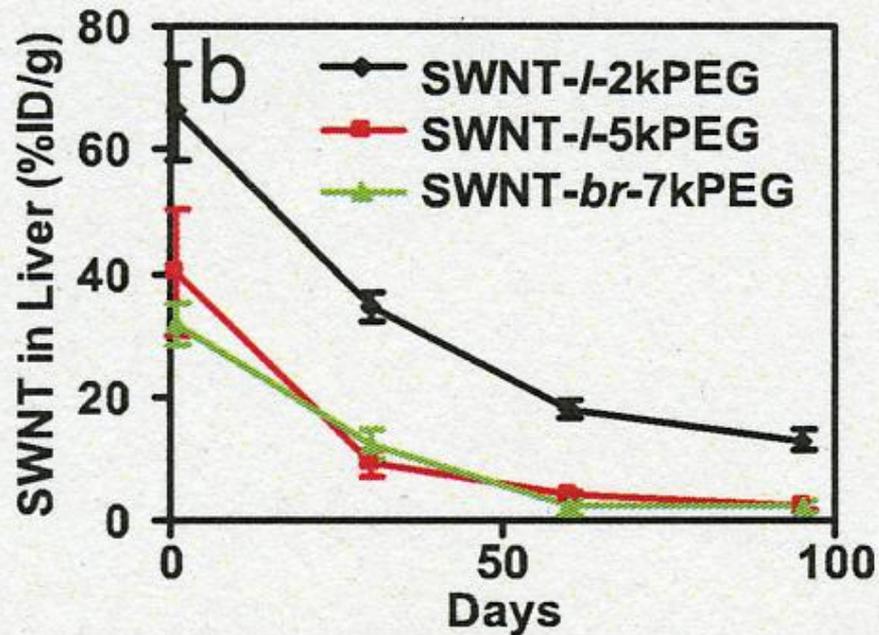
After 1 day

0.1 mg/ml

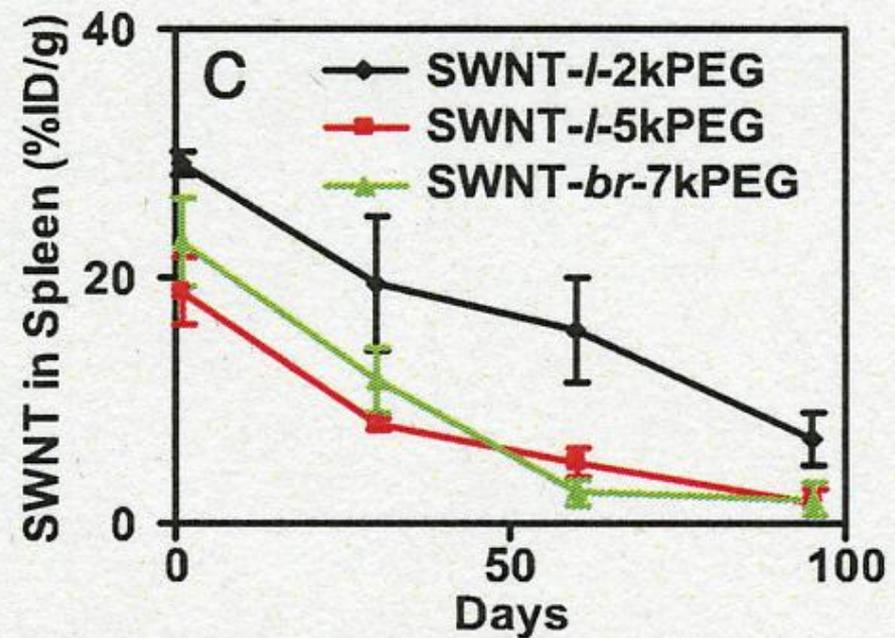


0.1 mg/ml

Liver



Spleen

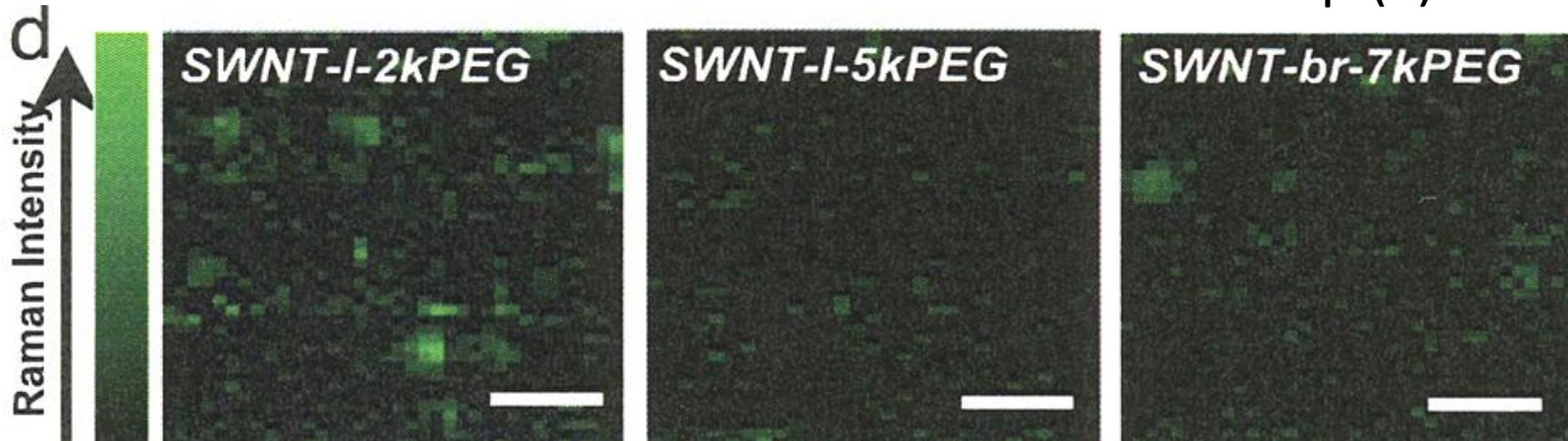


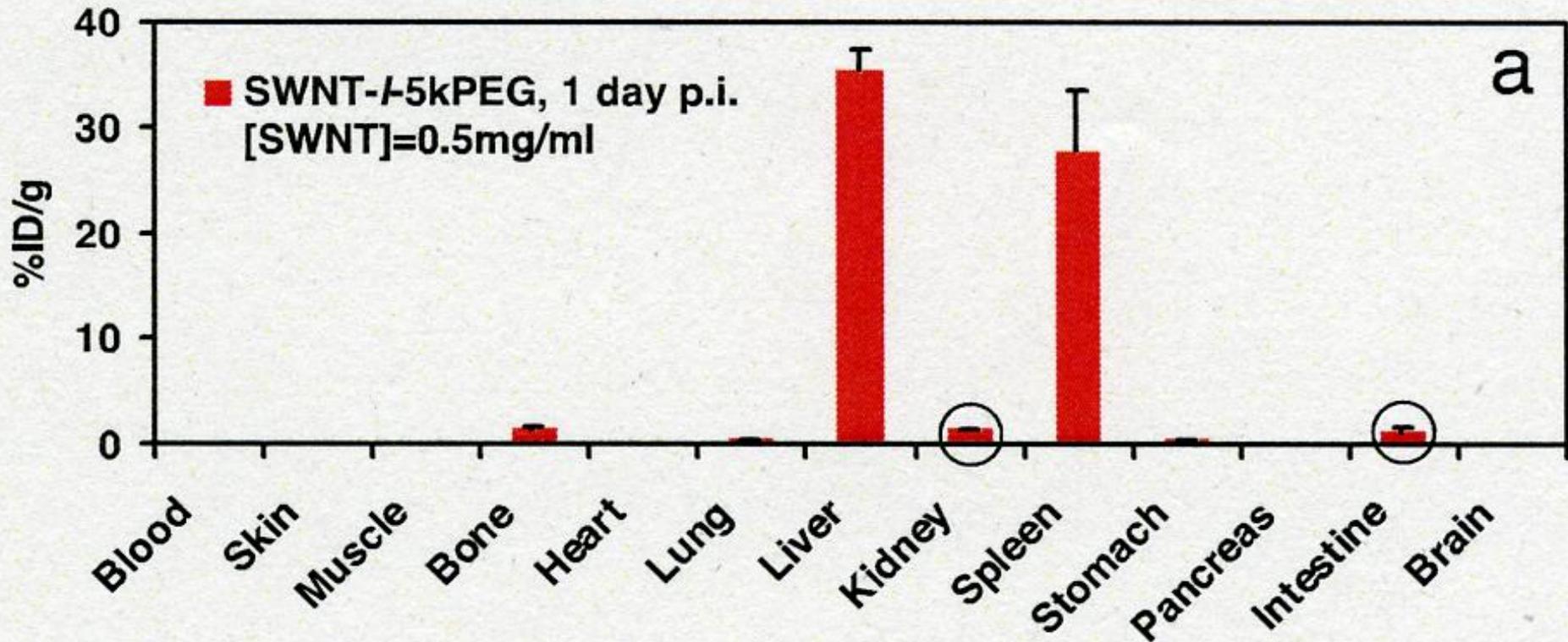
The SWNT concentrations in most organs are below detection limit.

three to four mice per group

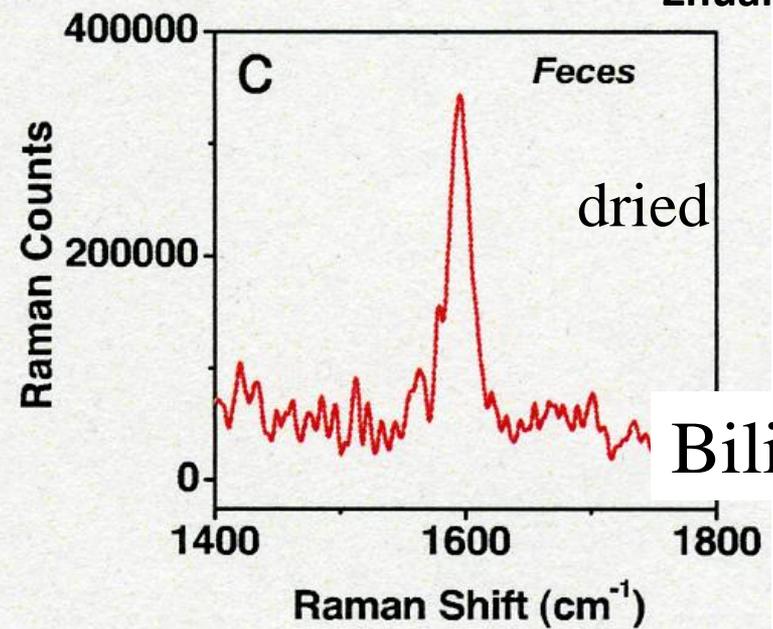
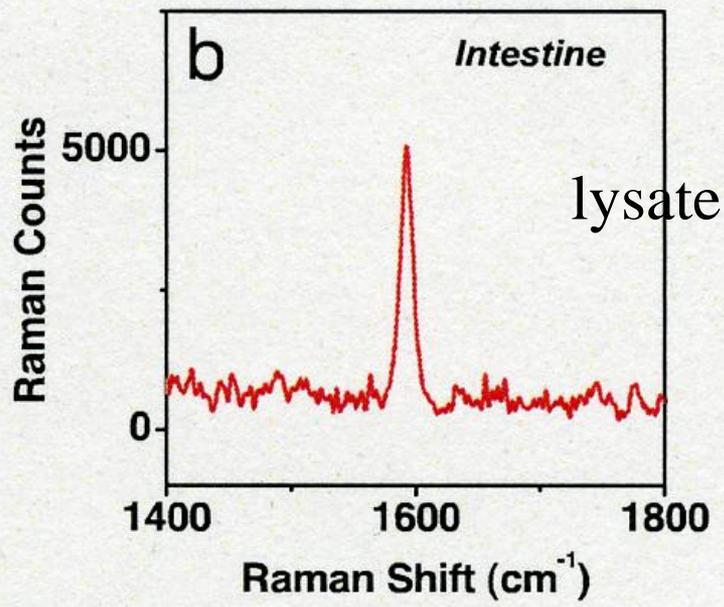
Raman mapping images of liver slices

3 months
0.1 mg/ml
200 μ l(?)



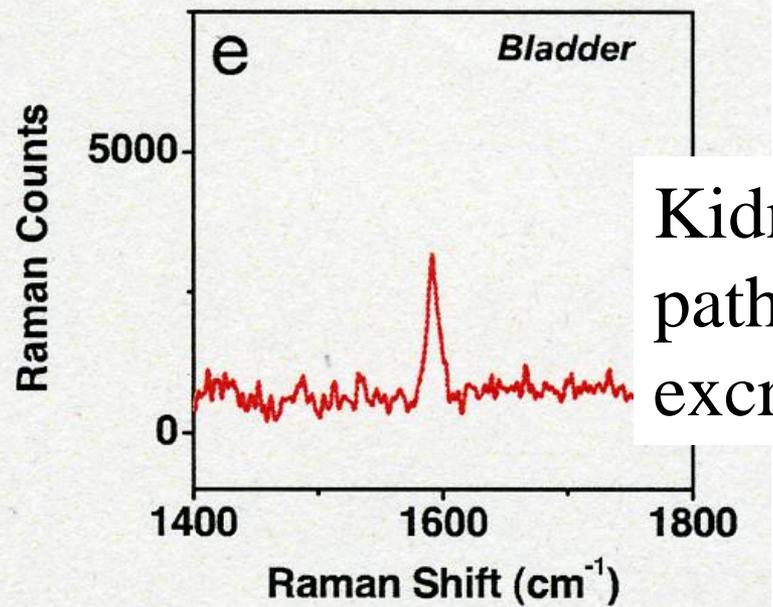
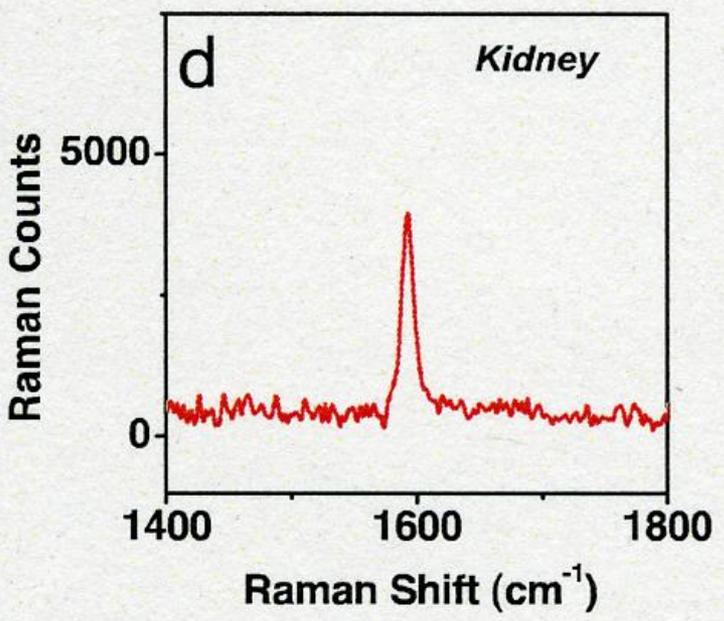
0.5 mg/ml 200 μ l 3 mice

24h
Dose:
0.5 mg/ml
200 μ l



Biliary excretion

胆囊

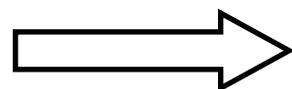
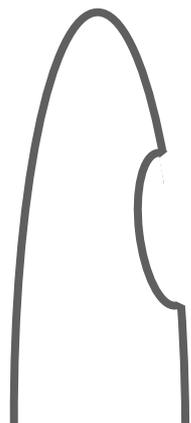


Kidney-urine –
pathway
excretion

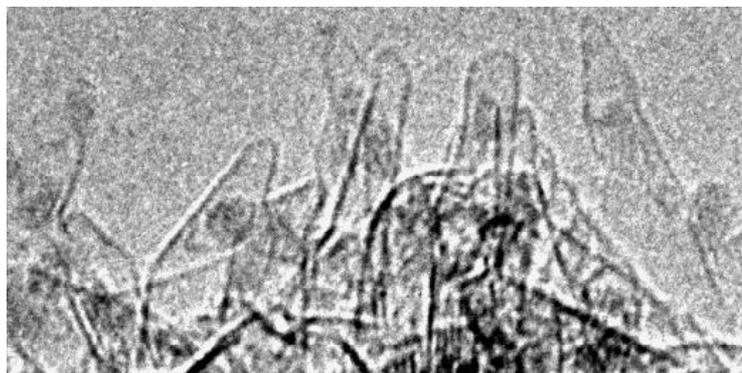
MRI: Deposition of Fe_3O_4 in SWNHox

SWNHox

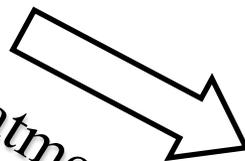
$\text{Fe}(\text{OAc})_3@ \text{NHox}$



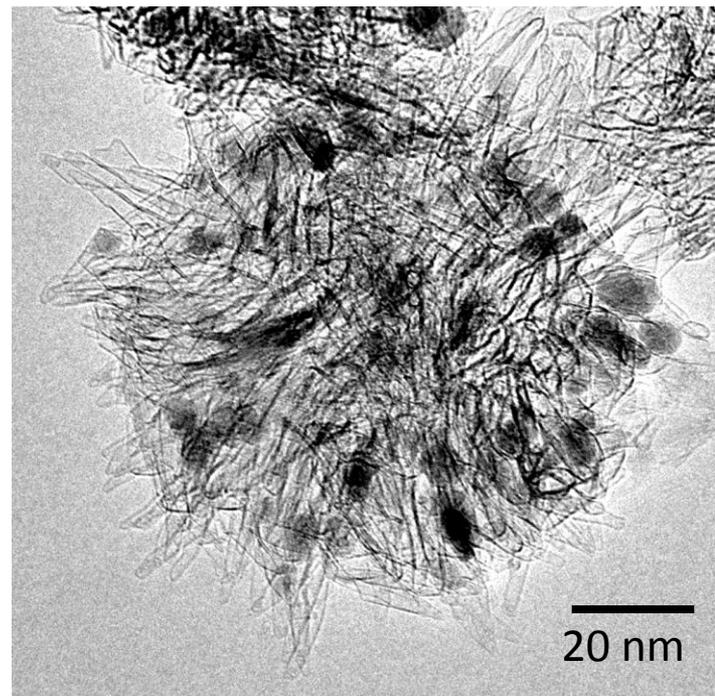
Gd acetate
deposition
in
Ethanol



Heat-treatment in Ar

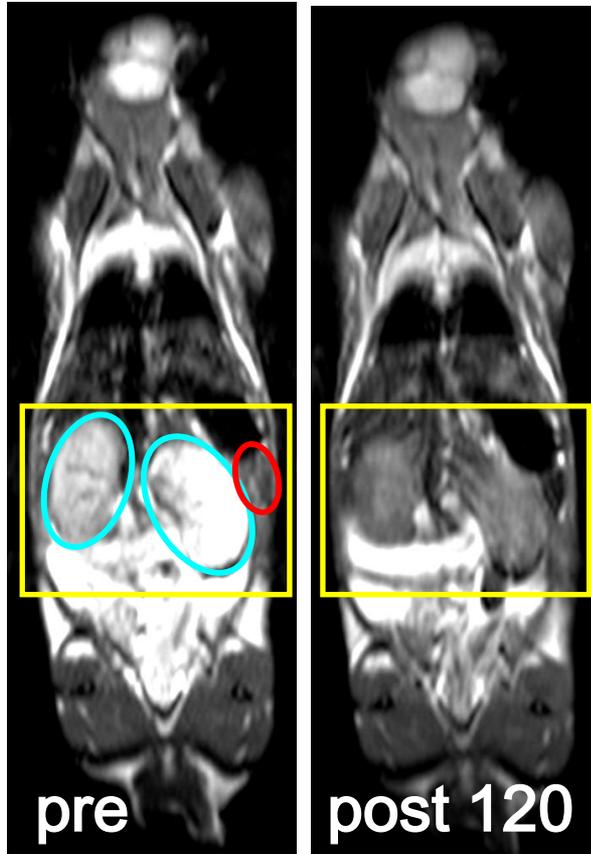


Fe_3O_4 -SWNHox



Miyawaki et al. Adv. Mater. 2006.

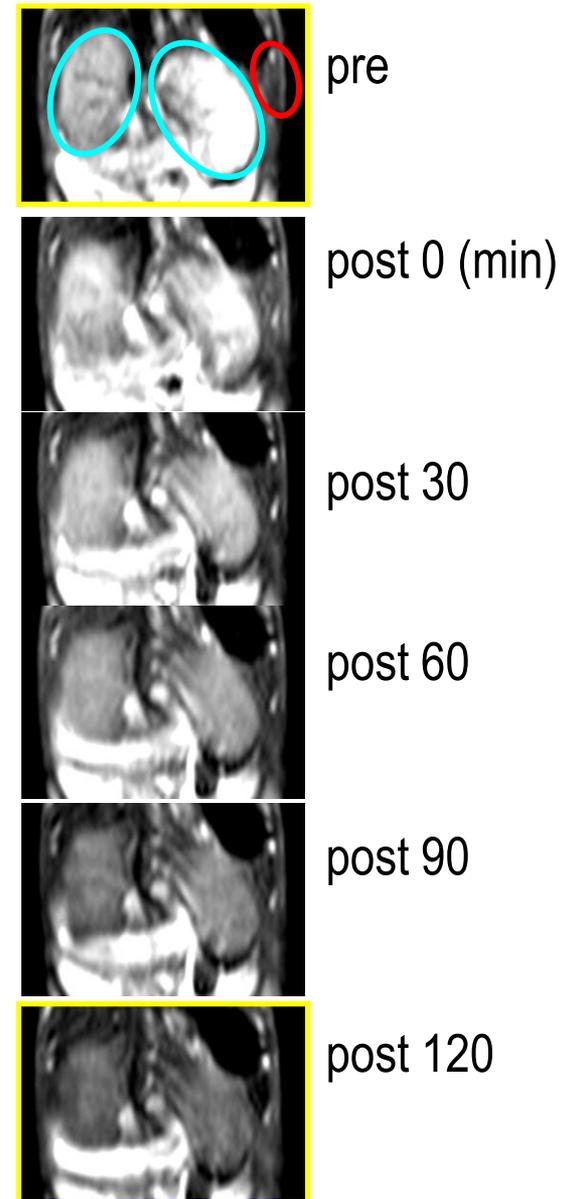
In vivo MRI imaging of magnetite-attached SWNHox



T_2^* -weighted images

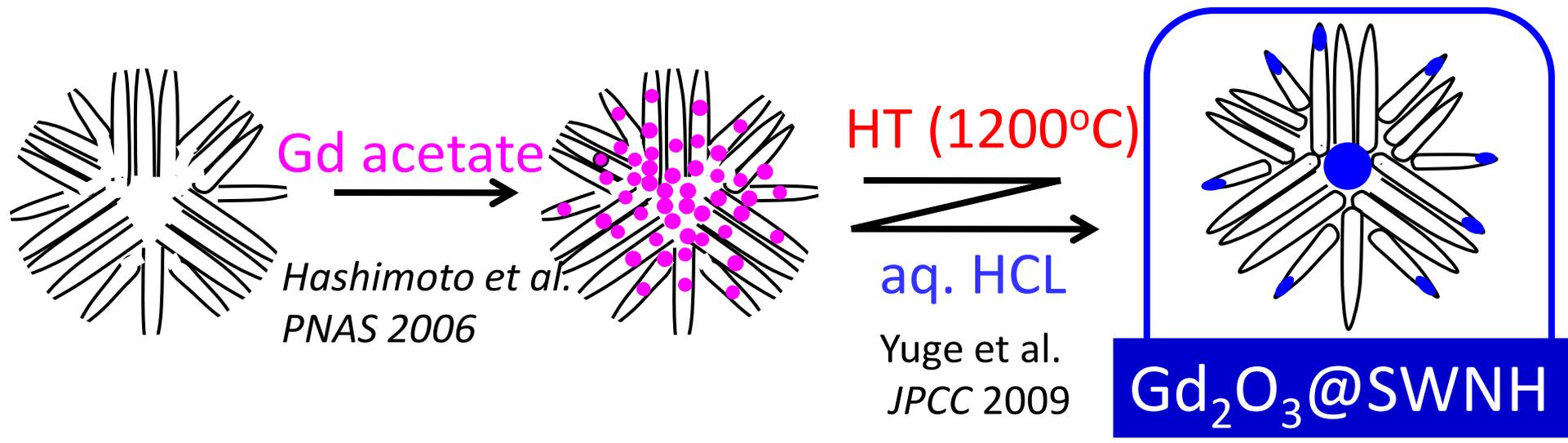
Conc. = 0.5 mg cm^{-3} in PBS

Dose amount = 0.5 cm^3



Miyawaki et al. *Adv. Mater.* 2006.

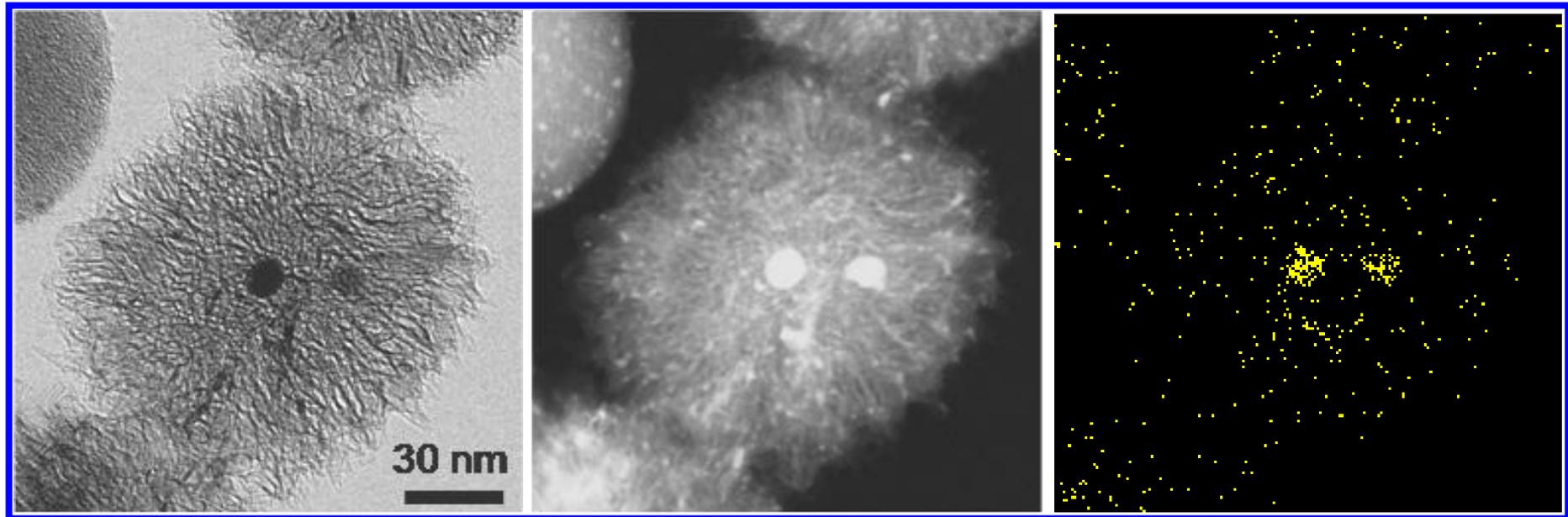
New Label: Gd_2O_3 Nanoparticles Embedded in SWNHs



TEM

Z-Contrast

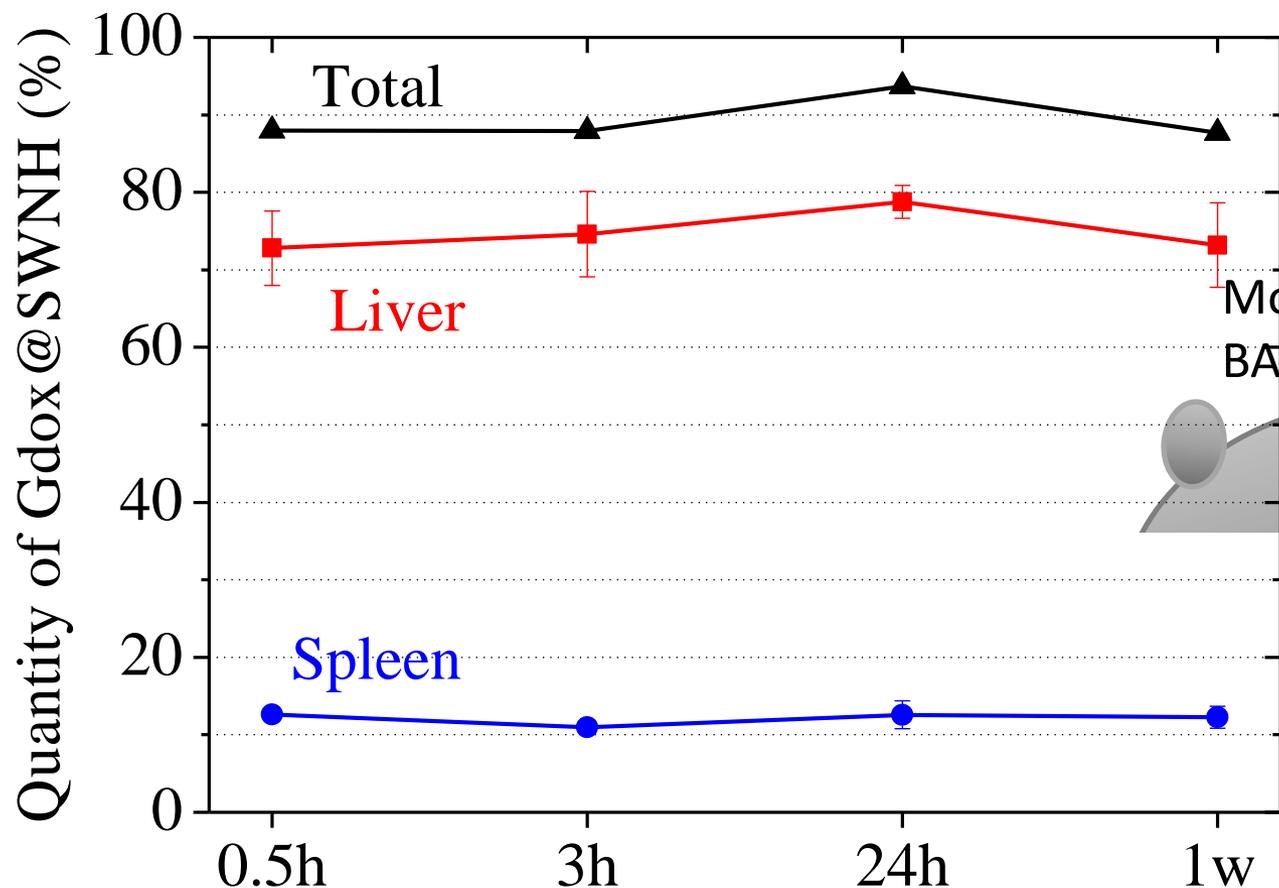
Gd-mapping



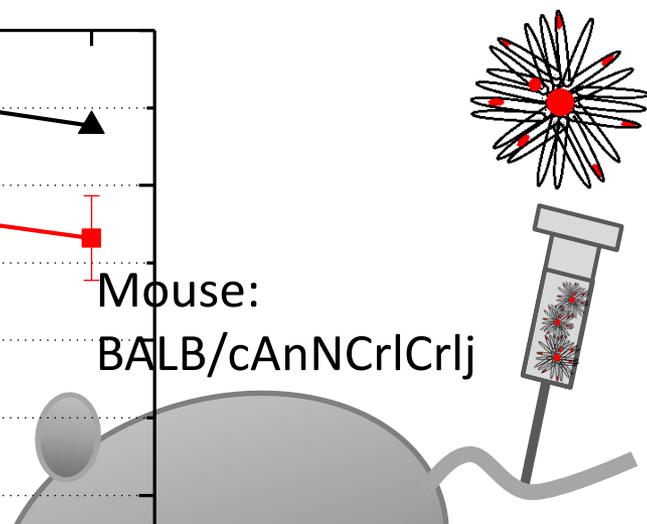
Bio distribution of $Gd_2O_3@SWNH$

Method: Internal organs \rightarrow Dry \rightarrow Combustion

\rightarrow Ash in HCl \rightarrow ICP-AES elemental analysis



Periods after injection of $GdOx@SWNH$



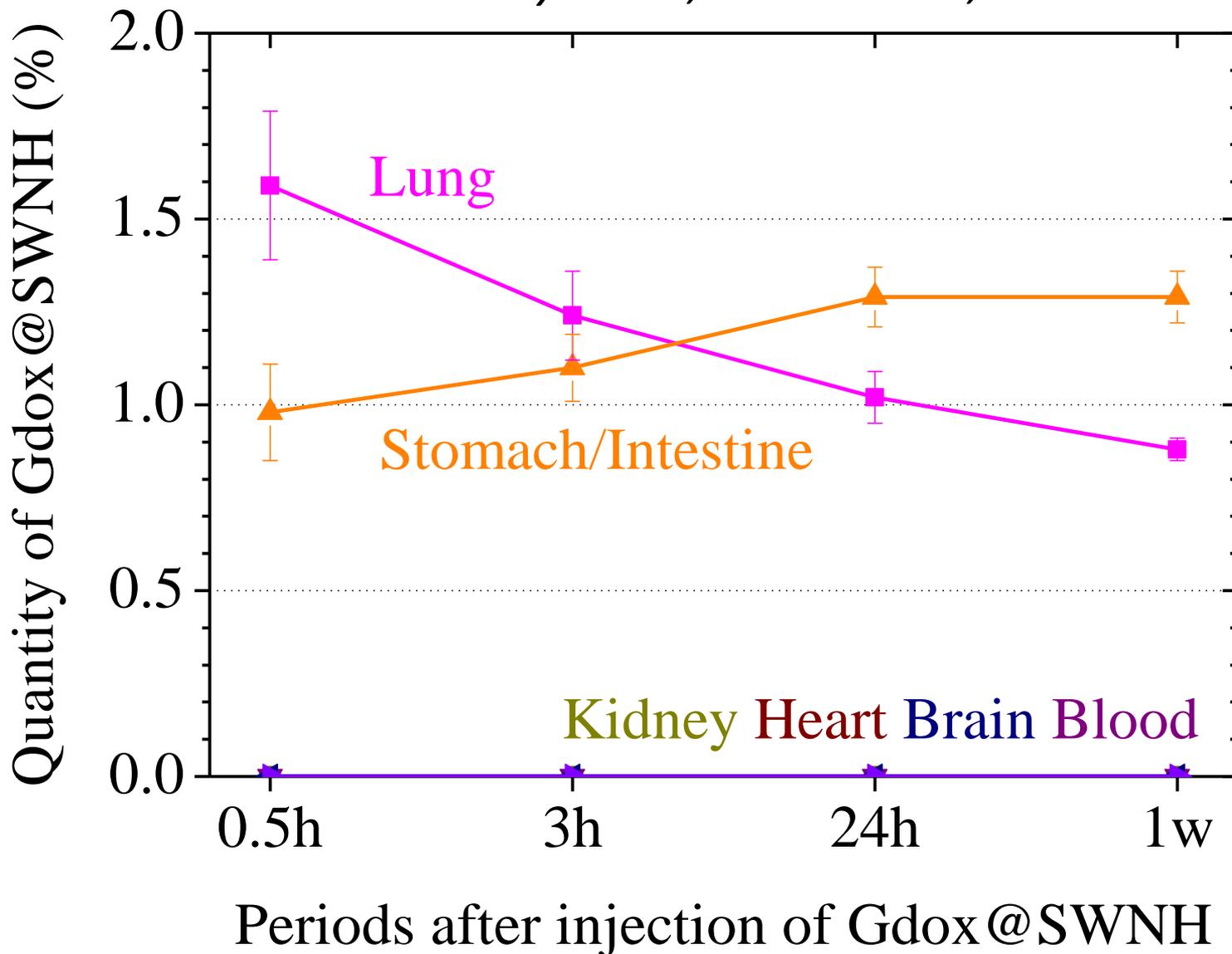
Mouse:
BALB/cAnNCrIcrlj

Dose: $GdOx@SWNH$
 ~ 0.4 mg/mouse
(~ 20 mg/kg)

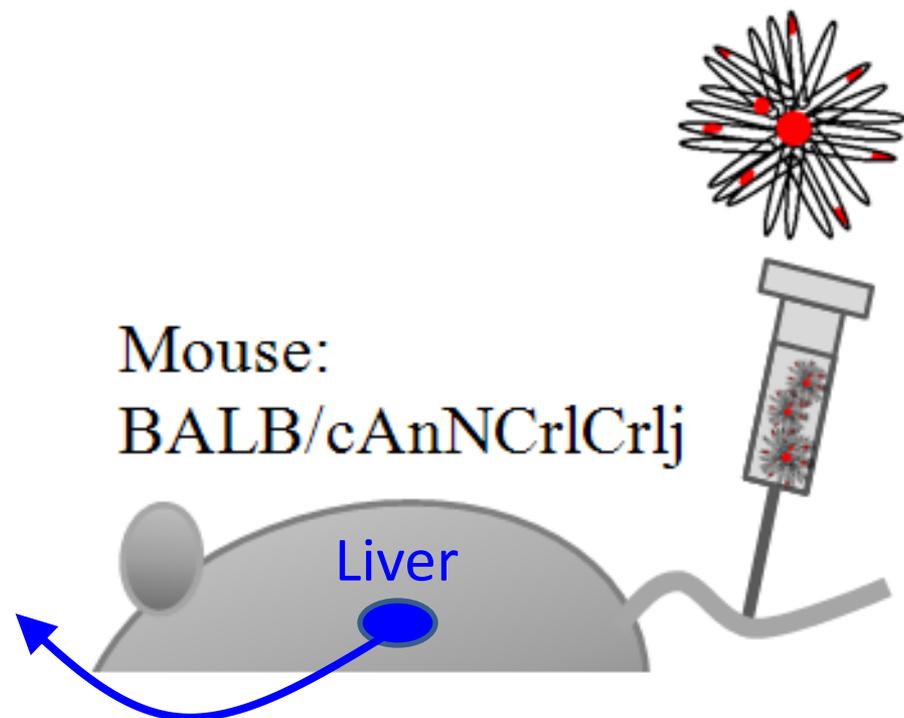
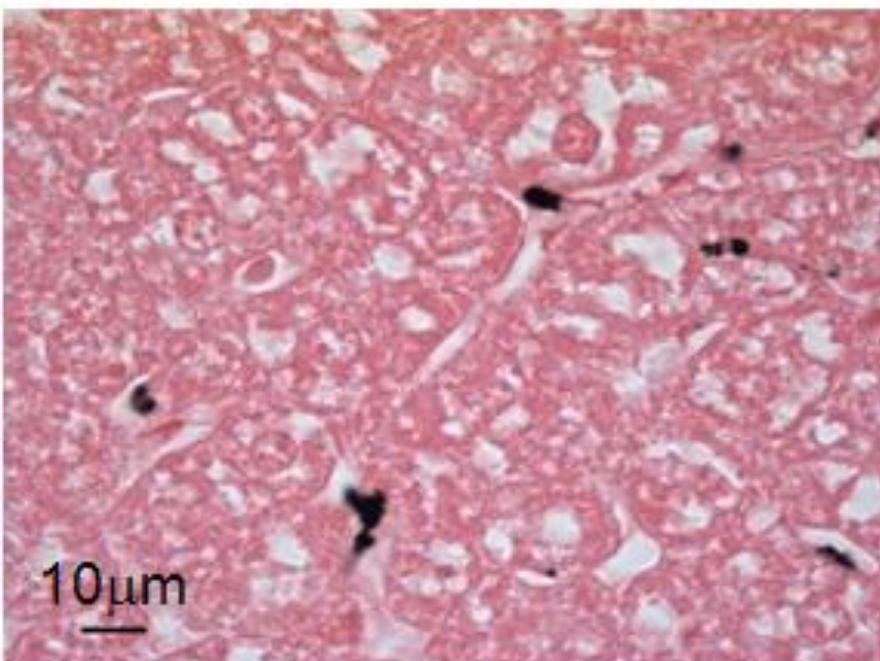
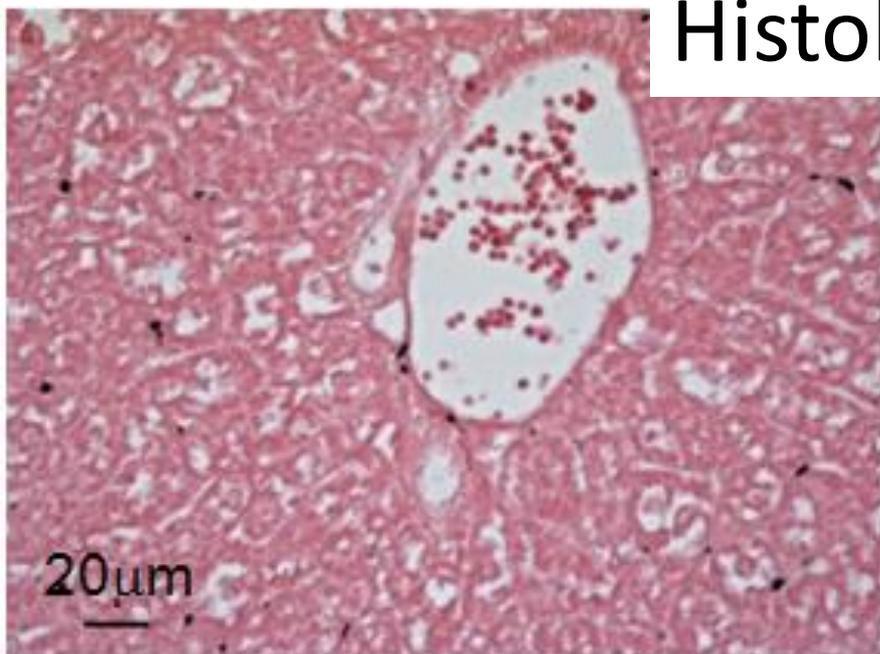
Miyawaki et al.
ACS Nano 2009

Bio distribution of $Gd_2O_3@SWNH$

Miyawaki, Matsumura, et al. ACS Nano 2009



Histological observation of liver

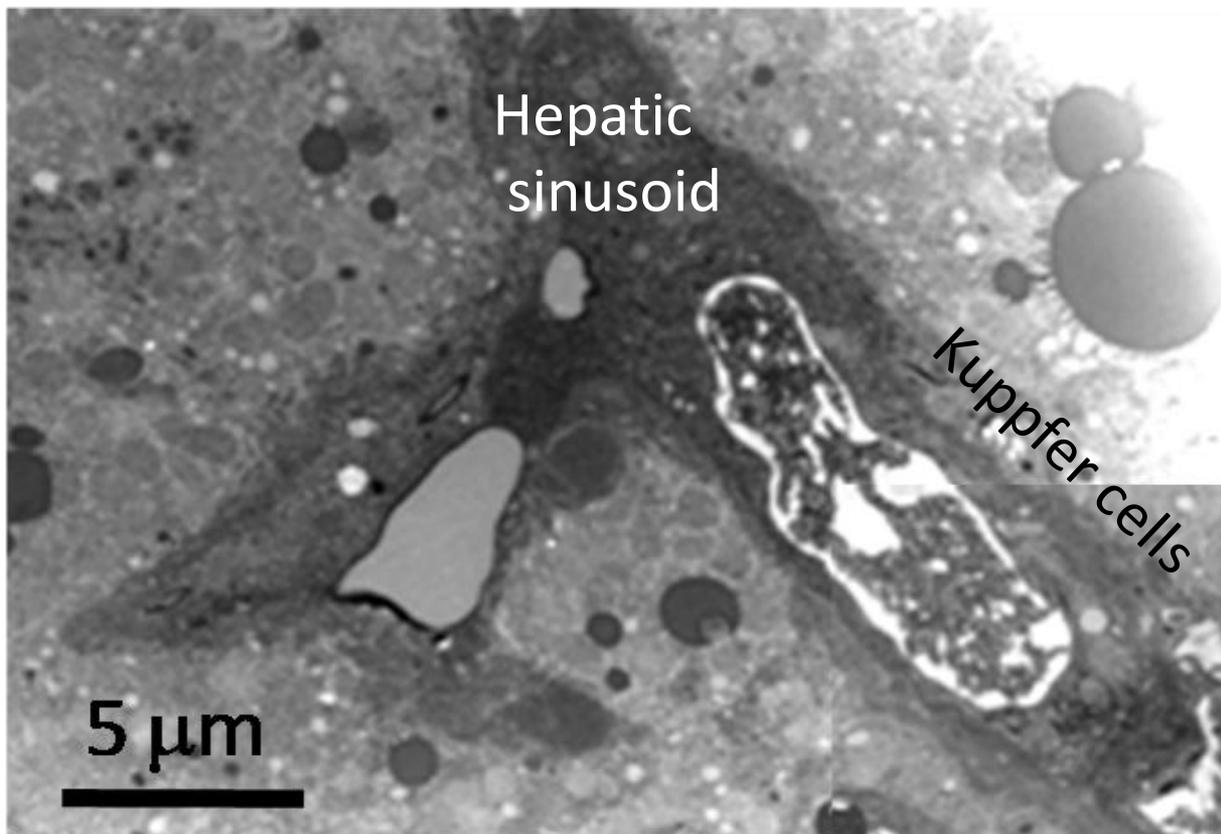


Dose: Gdox@SWNH
~ 0.2 mg/mouse
(~ 10 mg/kg)

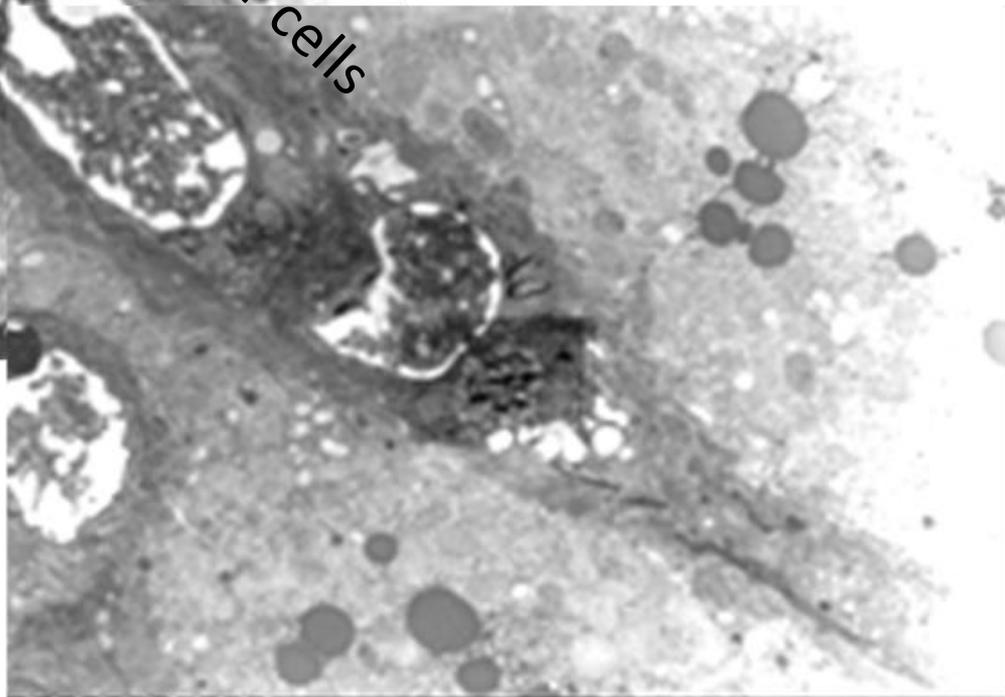
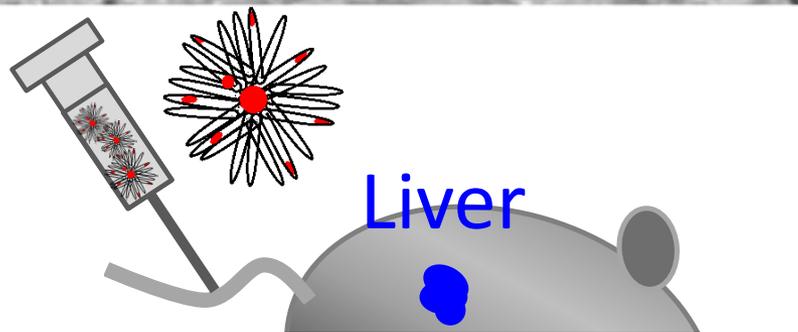
Solvent: Glucose solution

ACS Nano 2009

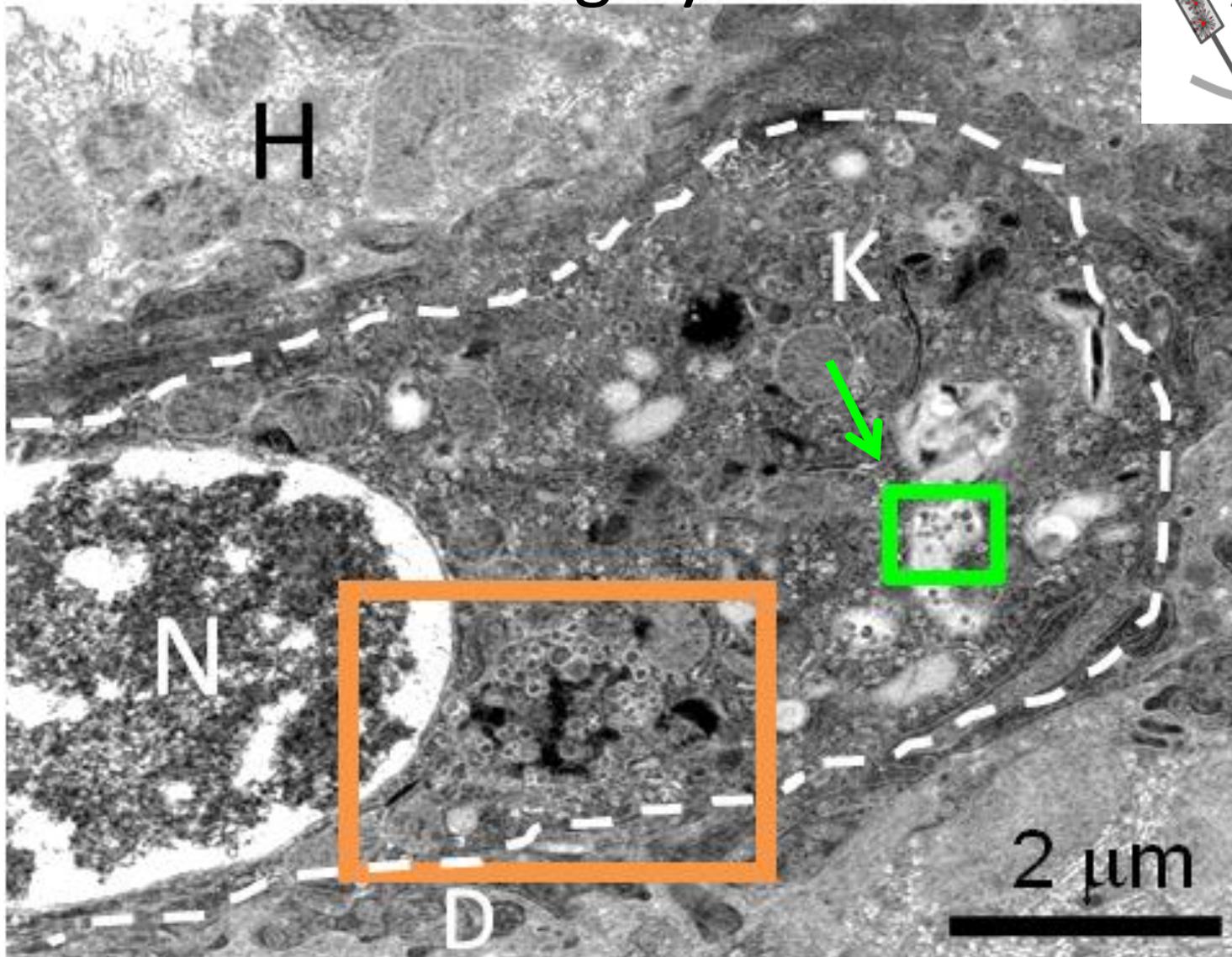
Ultrastructural observation: $Gd_2O_3@SWNH$ in liver



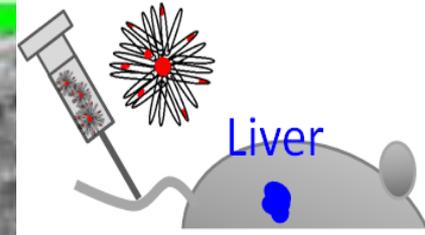
Miyawaki et al.
ACS Nano 2009



SWNHs in Phagolysosome



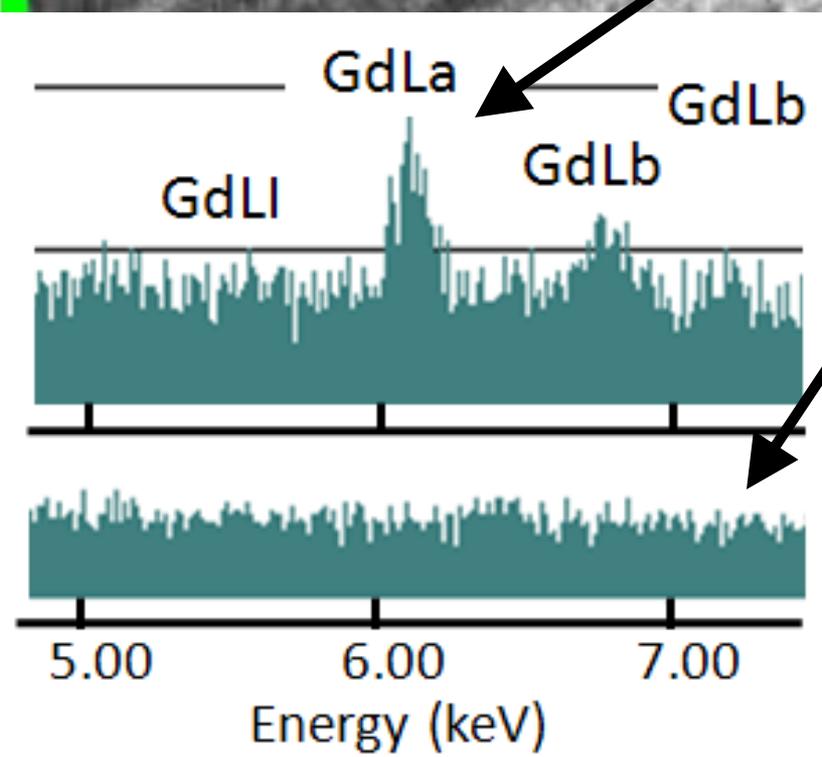
H, Hepatocytes; N, Nucleus; D, Perisinusoidal space of Disse
En, endothelial cell; Kupffer cell *Iyawaki et al. ACS Nano 2009*



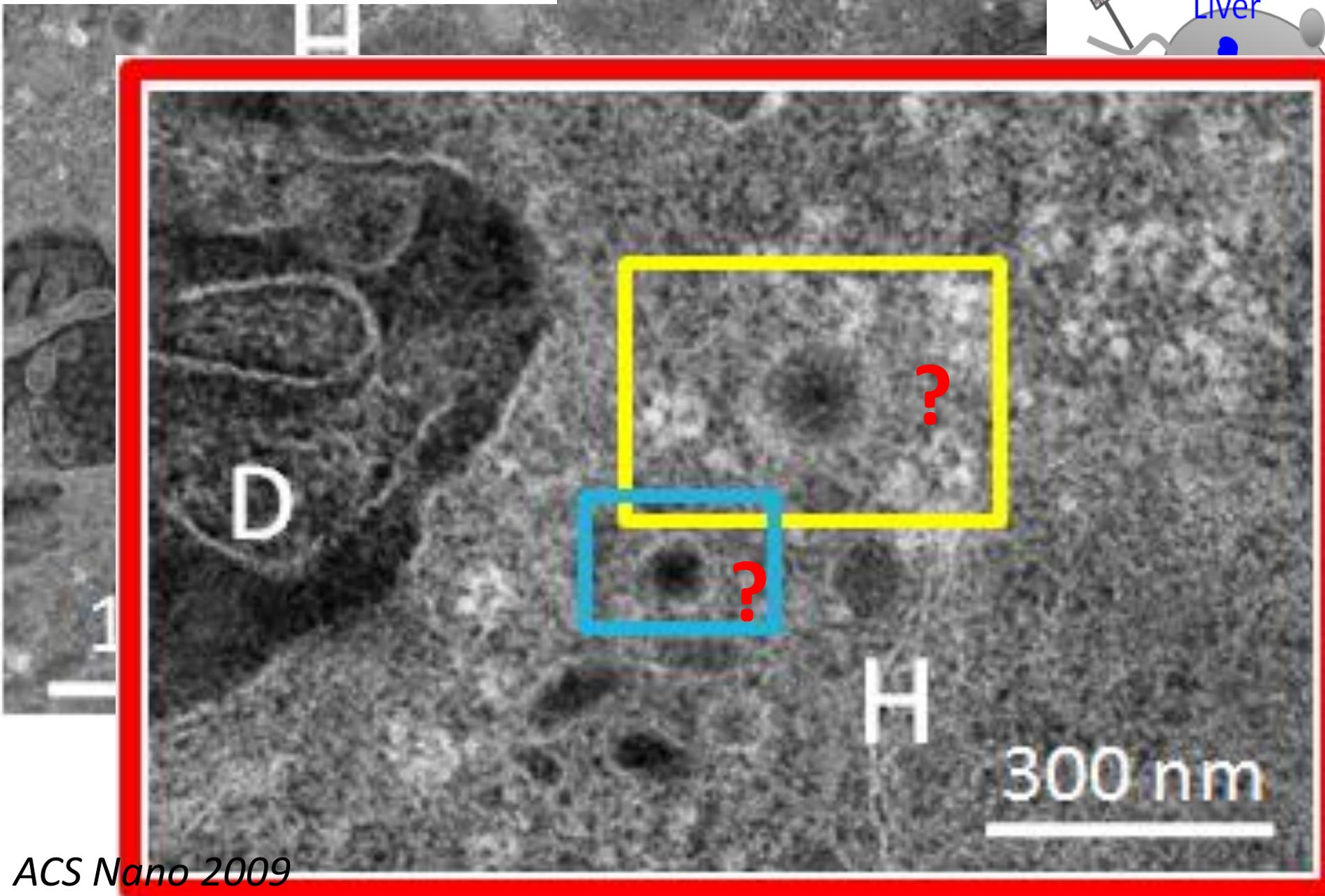
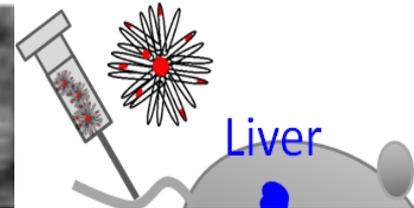
ZC

Phagolysosome

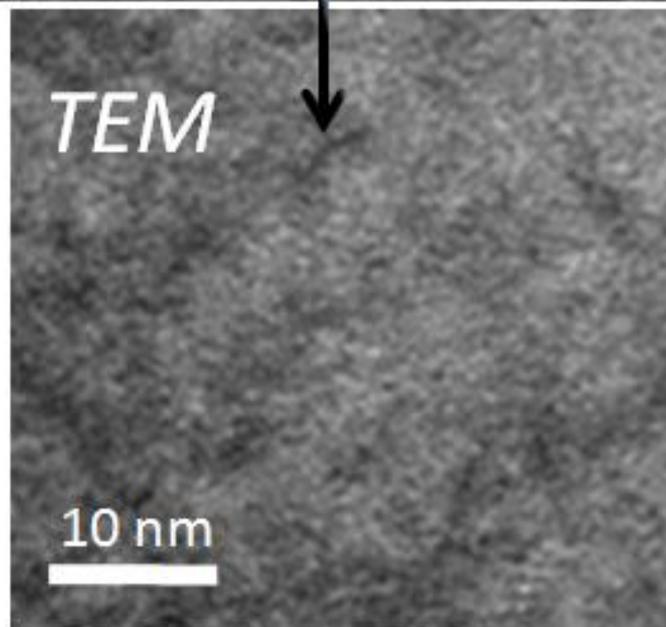
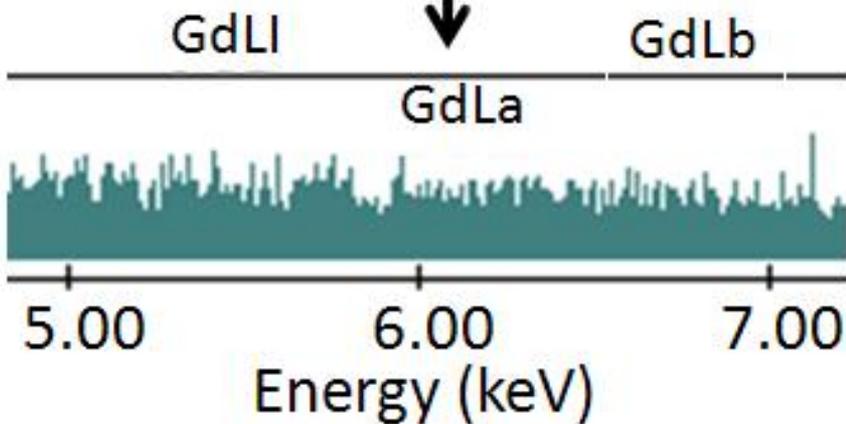
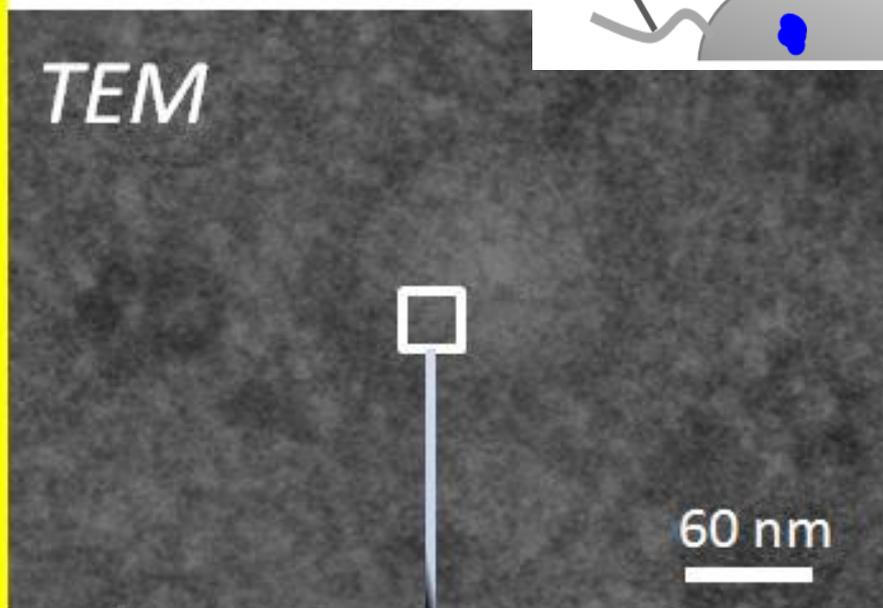
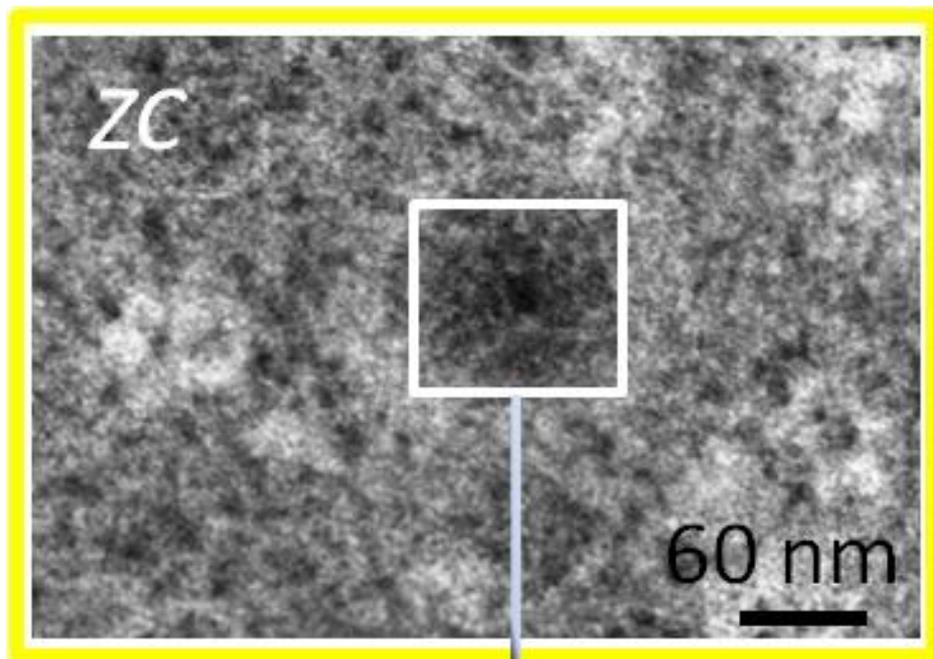
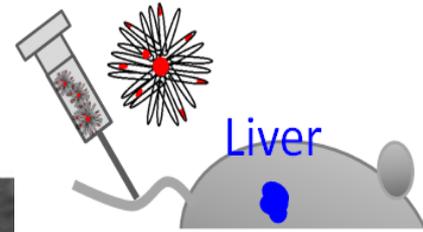
200 nm



Are these SWNHs ?



No Gd was found → No SWNHs



カーボンナノチューブのバイオ

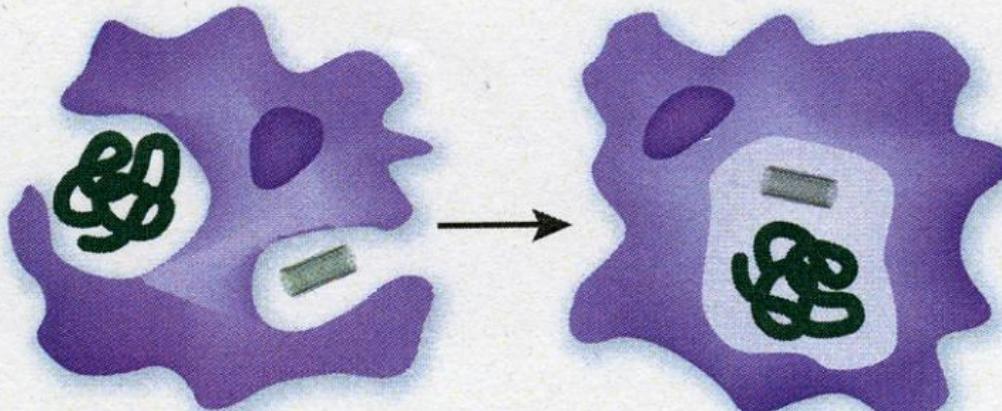
1. 細胞との相互作用・細胞毒性・細胞内消化・細胞外排出
2. 動物体内での挙動(蓄積、排出)
3. 動物毒性試験
4. バイオ応用

カーボンナノチューブの毒性

Kostarelos ,Nature Bio 2008

a

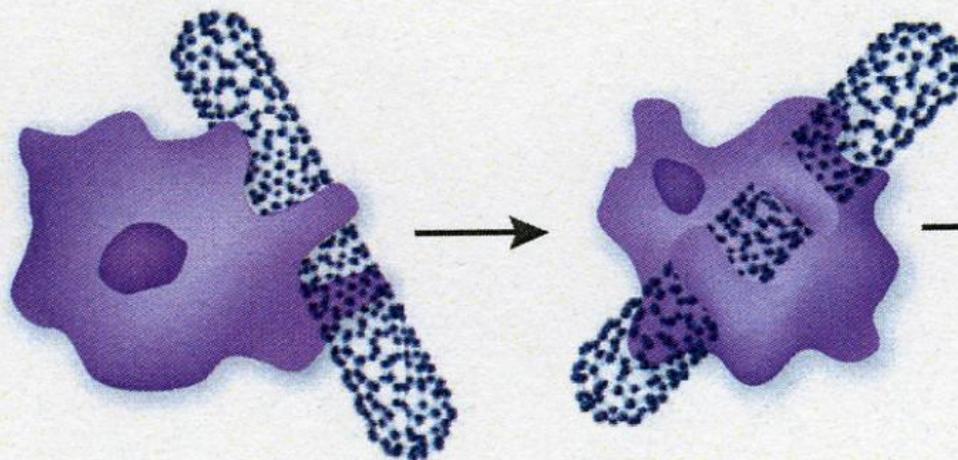
Short or tangled MWNT



Effective phagocytosis

Clearance through lymphatic system

Long and rigid MWNT



Incomplete phagocytosis

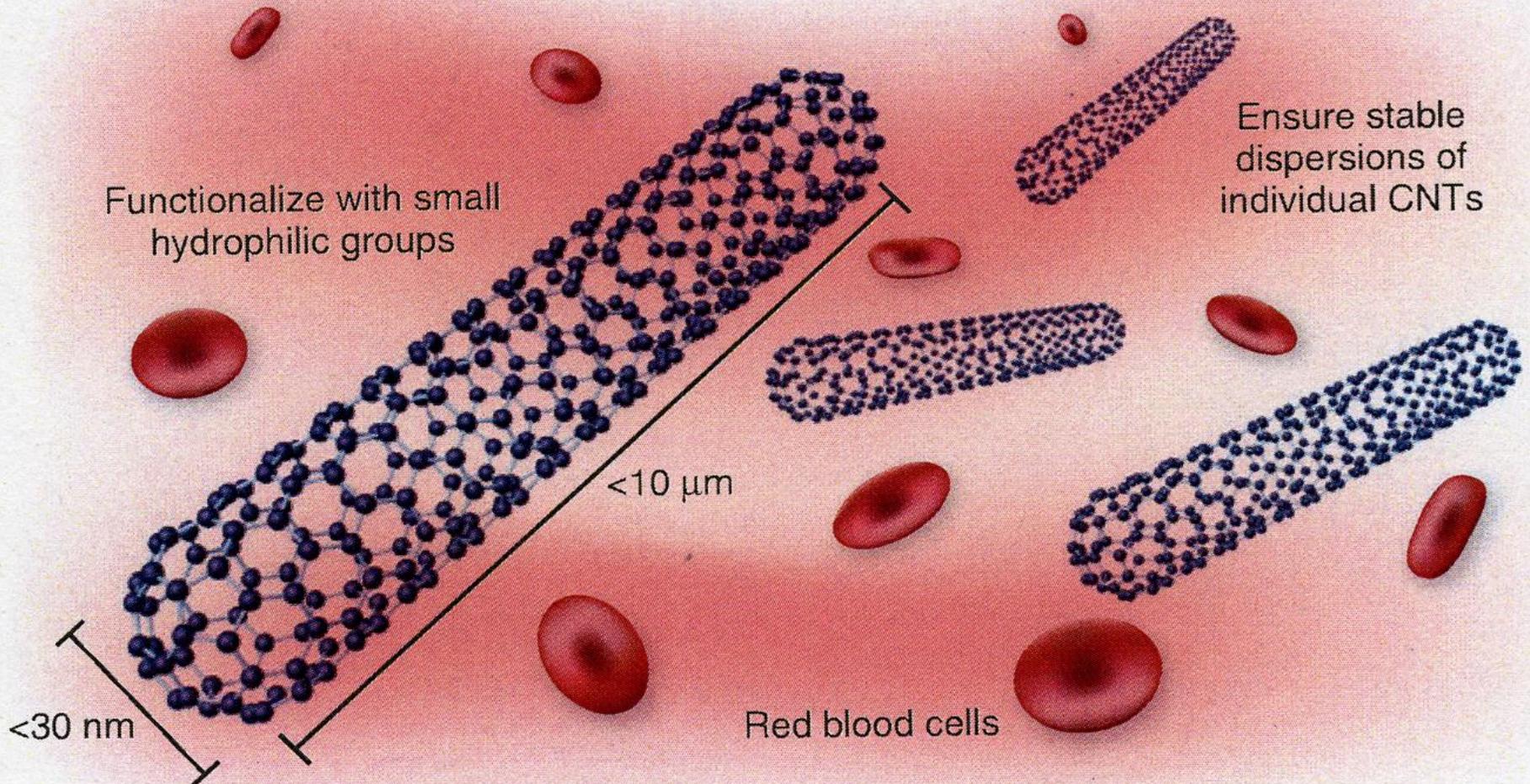
Mutagenesis caused by MWNT accumulation may cause cancer

Functionalized MWNTs

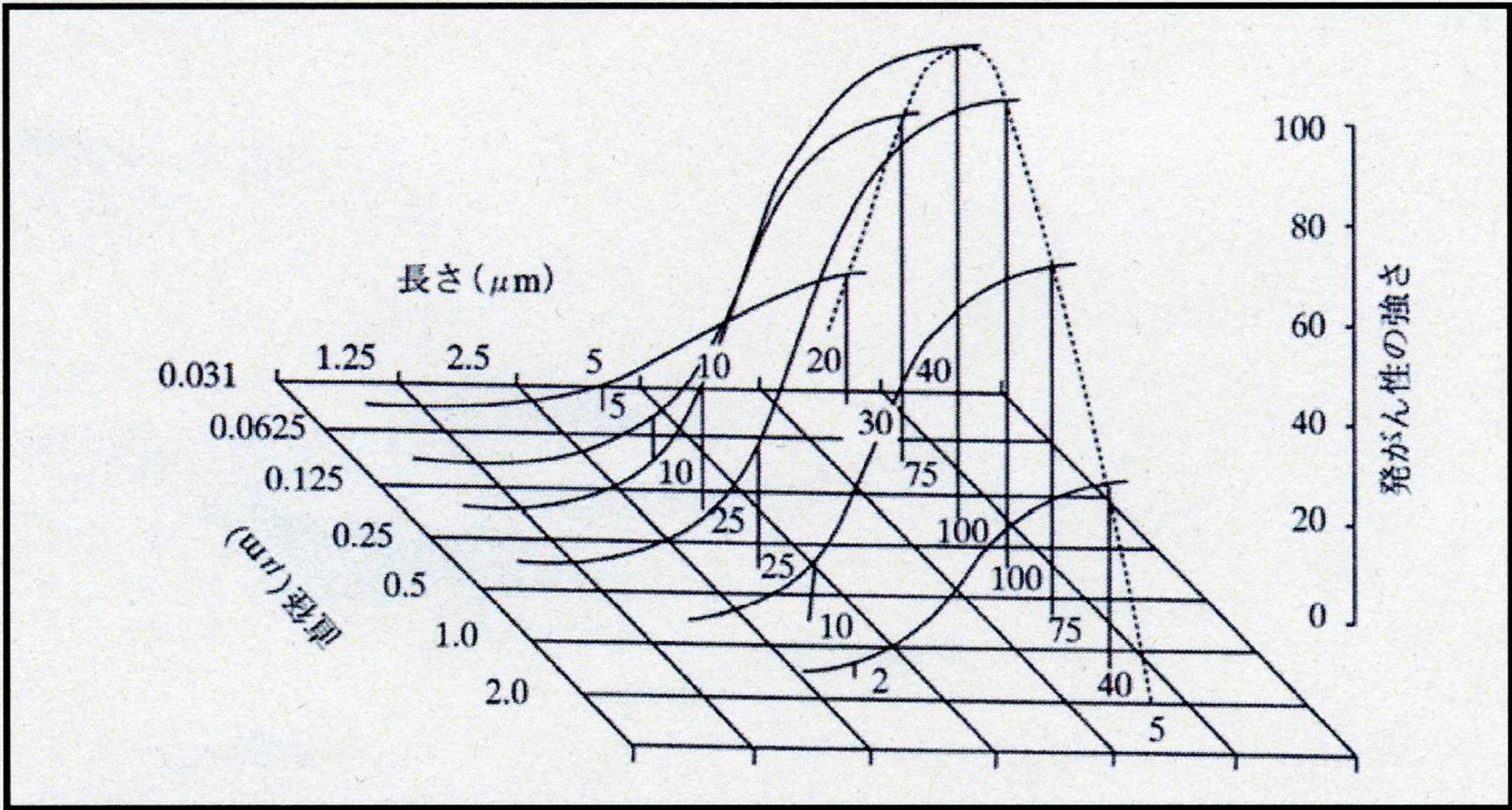
- ◆ $L < 5 \mu\text{m}$, $D: 20\text{-}30 \text{ nm}$, Dose: $50\text{-}400 \mu\text{g}$, i.v.
- ◆ 炎症など起こさずに速やかに尿排出

Kostarelos Nature Bio 2008

b

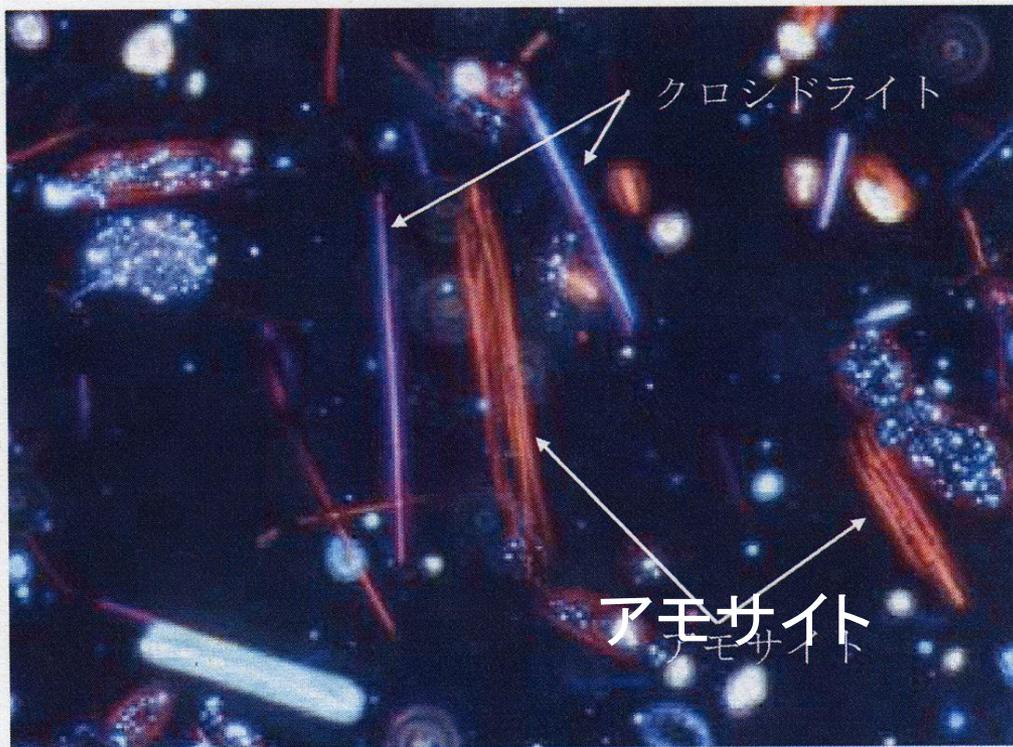


Long Fiberに対する中皮細胞の応答
“Peritoneal Macrophages”



(出典：石綿・ゼオライトのすべて)

図 2. 2-1 スタントナーポッツの仮説



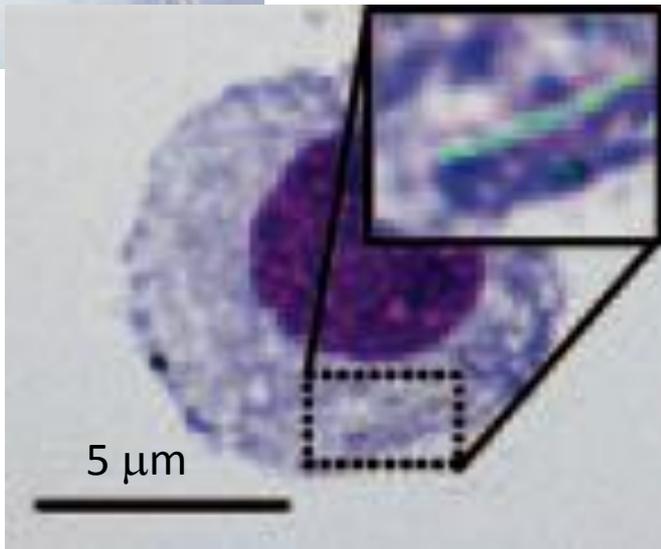
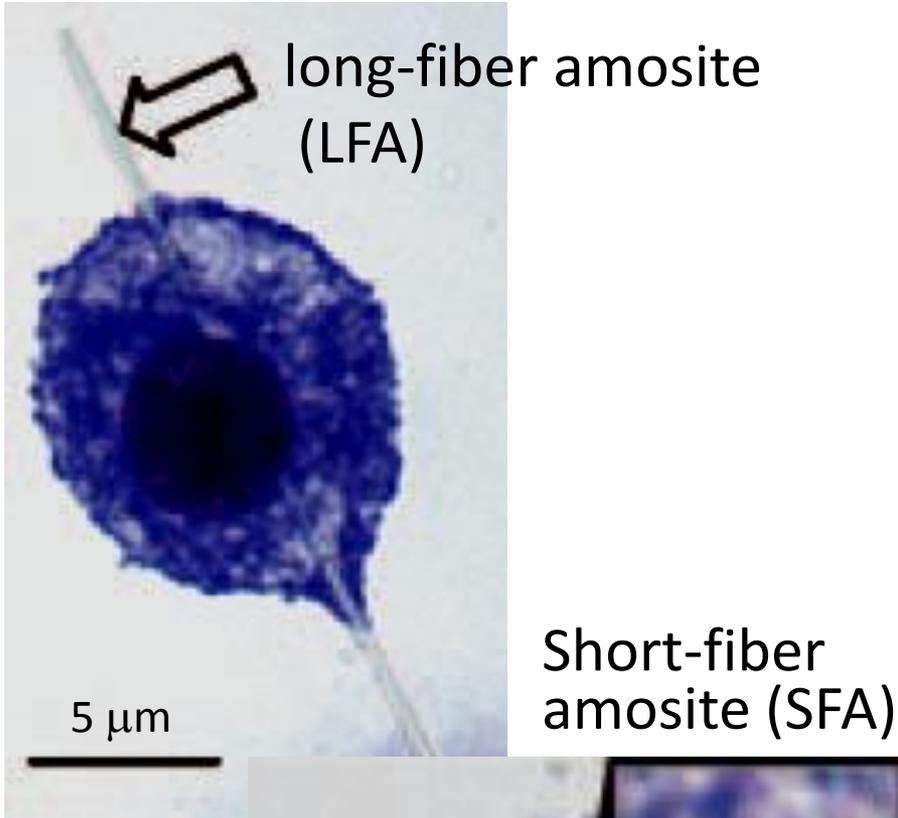
屈折率：1.680 倍率：×100

(写真提供：中外テクノス株式会社)

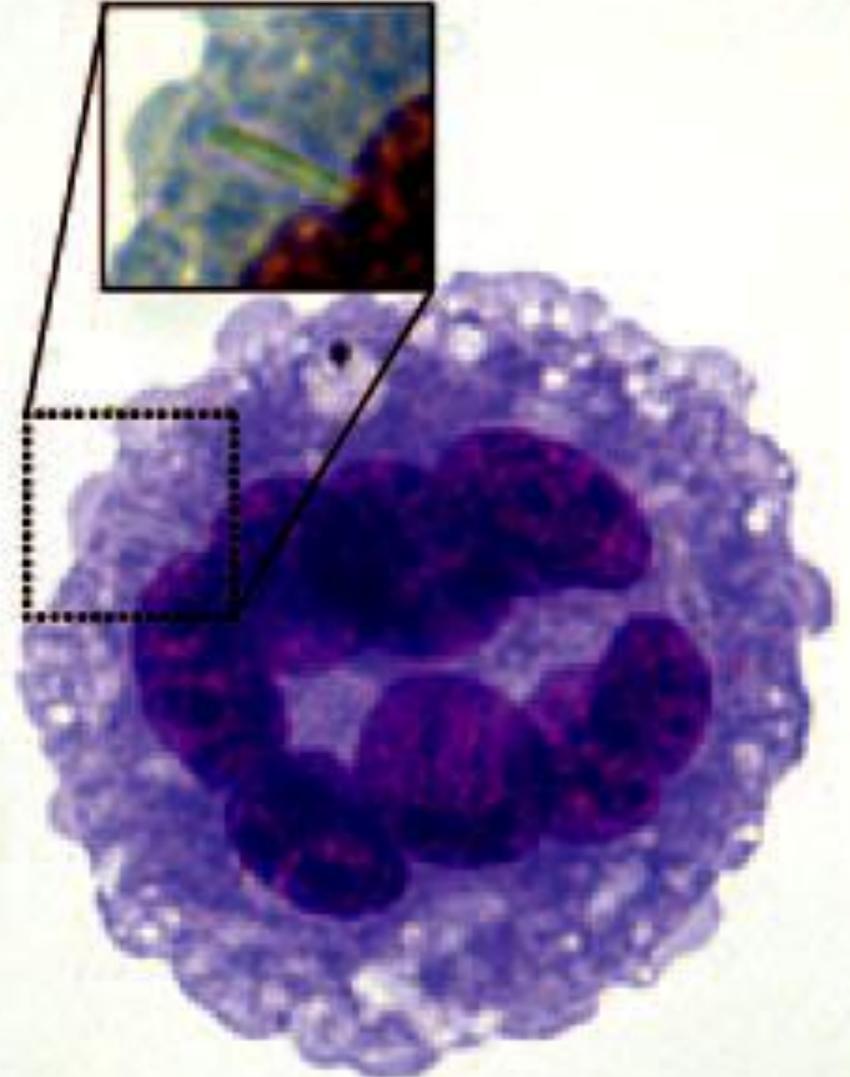
(2) 繊維状物質の有害性*1
スタントナーポッツの仮説(図2.2-1参照)に示すとおり、アスベストに限らず、
細長い繊維は発がん力があり、特に繊維径 $0.25\mu\text{m}$ で、長さ $20\mu\text{m}$ 程度が最大の発がん性に寄与するということが認識されるようになった。なお、繊維径が $4.5\mu\text{m}$ 以上の繊維状物質については、一過性であるが皮膚障害があることに留意しておく必要もある。
(出典：石綿・ゼオライトのすべて)

*1: :繊維状物質測定マニュアル、
(社)日本作業環境測定協会

Peritoneal Macrophages



SFA, foreign body giant cells (FBGCs)



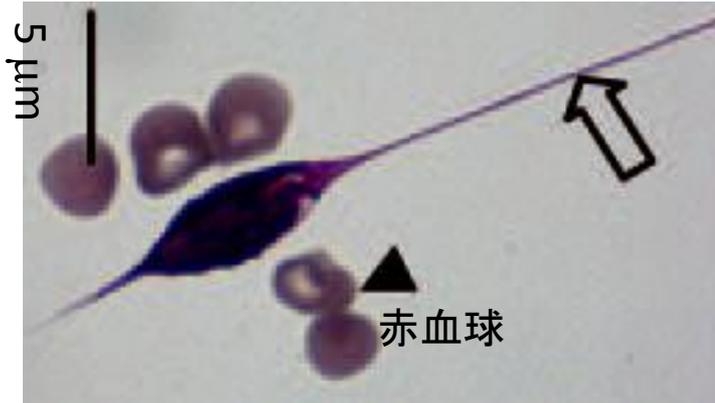
FBGC:マクロファージが炎症環境で融合してできる。

*Poland, „Donaldson
(Nature Nanotech 2008)*

Peritoneal Macrophages

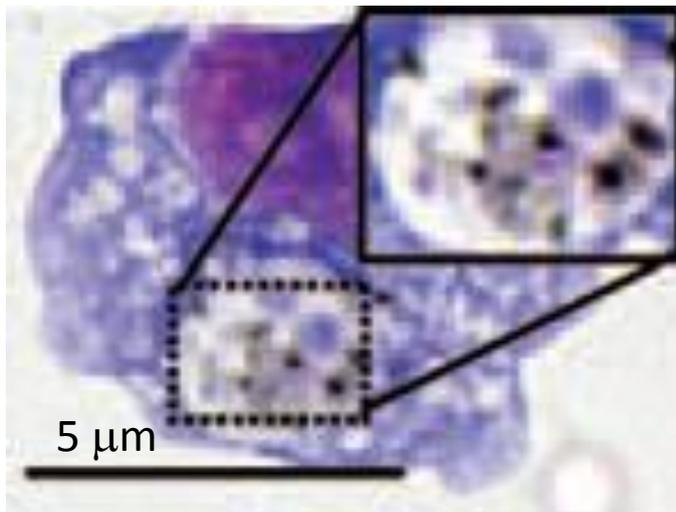
NTlong2

(diameter 165 nm, length $r < 56 \mu\text{m}$)

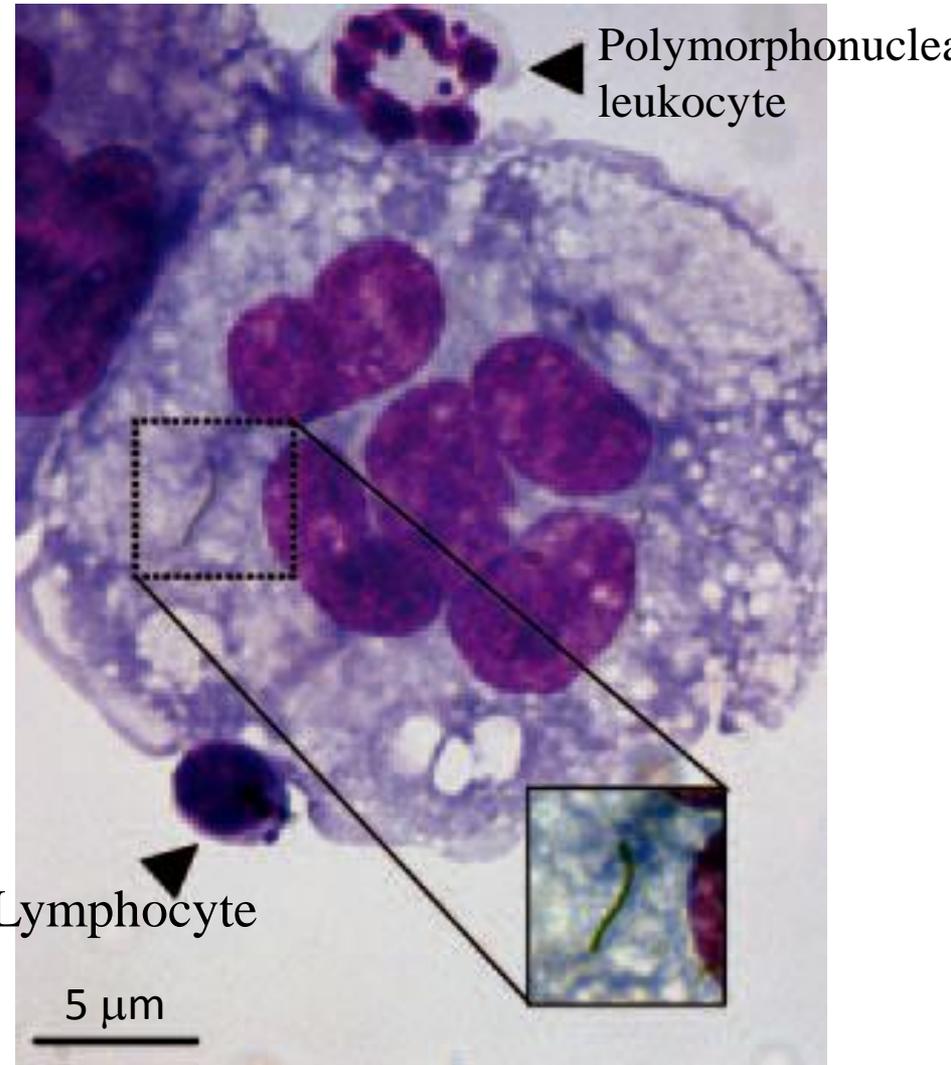


NTtang1

(diameter 15 nm, length 1-5 μm)

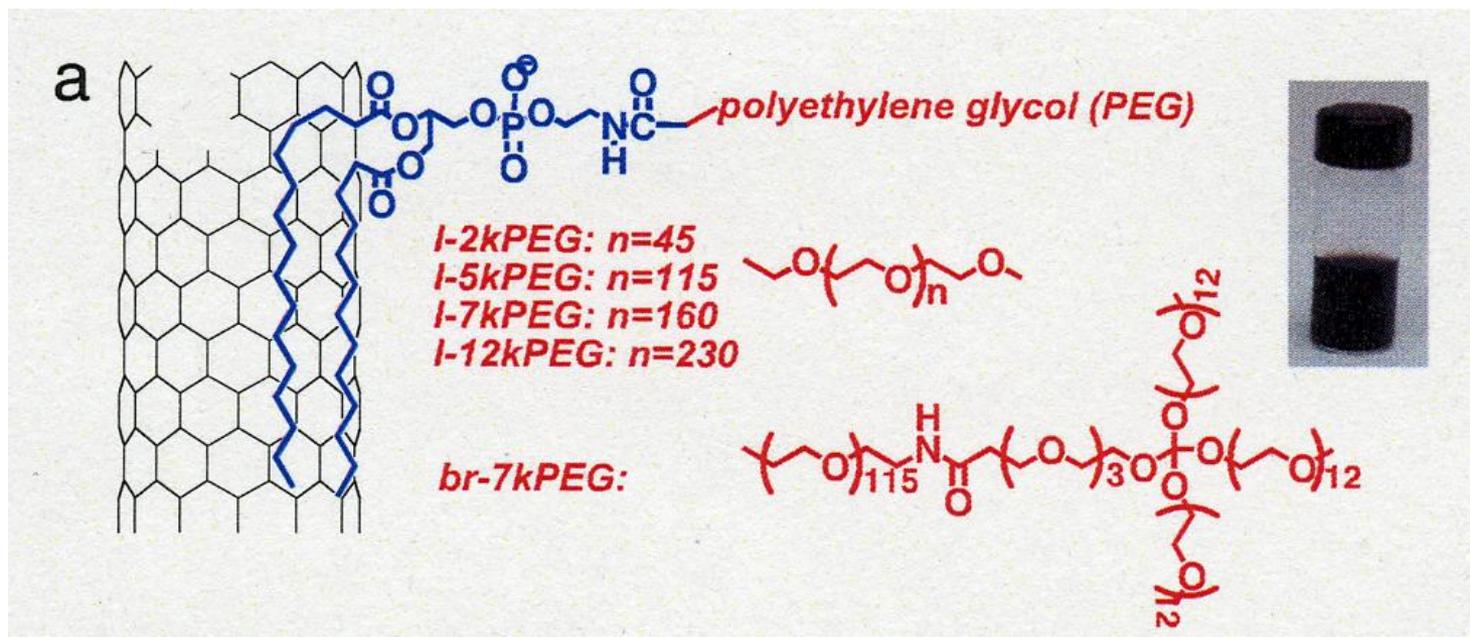


NTlong2 in FBGC



SWNTの毒性

Zhuang Liu* PNAS 2008



i.v., Dose: 0.1 mg/ml (200 μ l, \sim 0.1 mg/kg), 3 months, 30 mice

No toxic side effects were found:

- Necropsy (解剖検査)
- Histology (組織学的検査)
- Blood chemistry measurements (血液化学検査—肝臓の酵素)

カーボンナノホーンの毒性

修飾カーボンナノホーン: 動物実験から毒性は見つかっていない

- ◆ マウス、尾静脈投与、投与量6 mg/kg、期間2, 4, 26週。
- ◆ 炎症を惹起しなかった:
 1. 組織学的検査
 2. 血球検査 (単球、好中球、好酸球、リンパ球、その他)
 3. サイトカイン測定 (IL6, TNF- α 、など)

カーボンナノチューブのバイオ

1. 細胞との相互作用・細胞毒性・細胞内消化・細胞外排出
2. 動物体内での挙動(蓄積、排出)
3. 動物毒性試験
4. バイオ応用

Drug Delivery with Carbon Nanotubes for In vivo Cancer Treatment

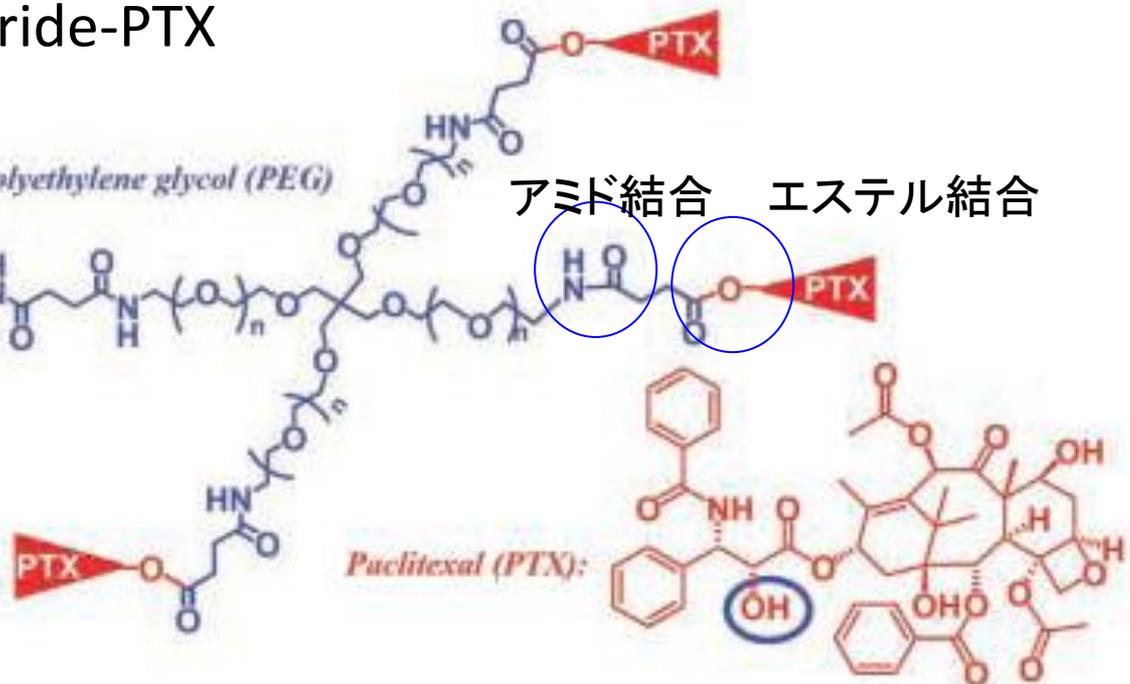
Zhuang Liu, *Cancer Research* 2008

SWNT-PEG-succinic anhydride-PTX

20-300 nm



Polyethylene glycol (PEG)

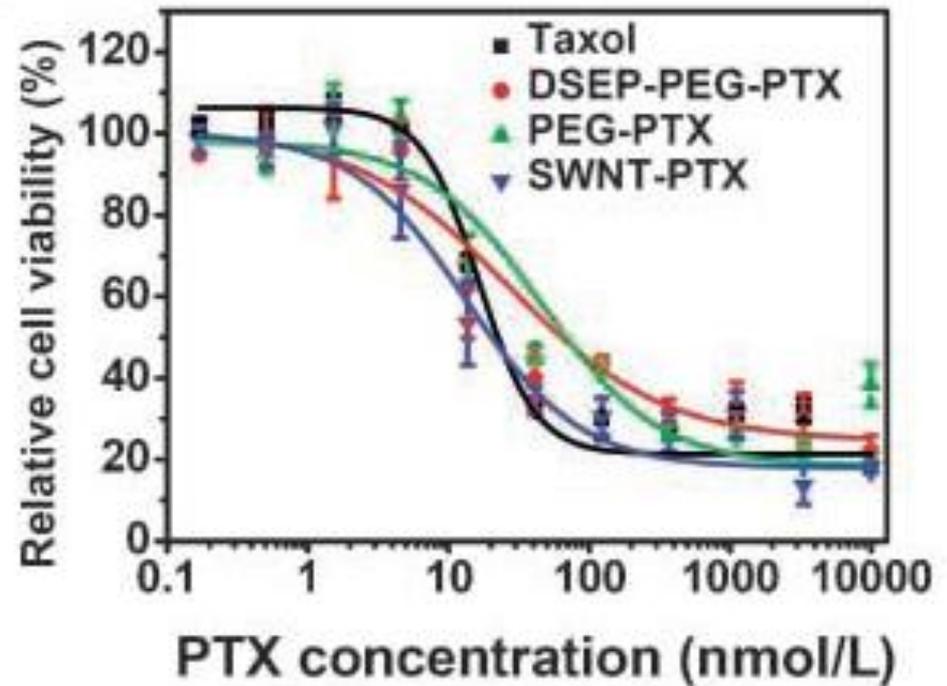
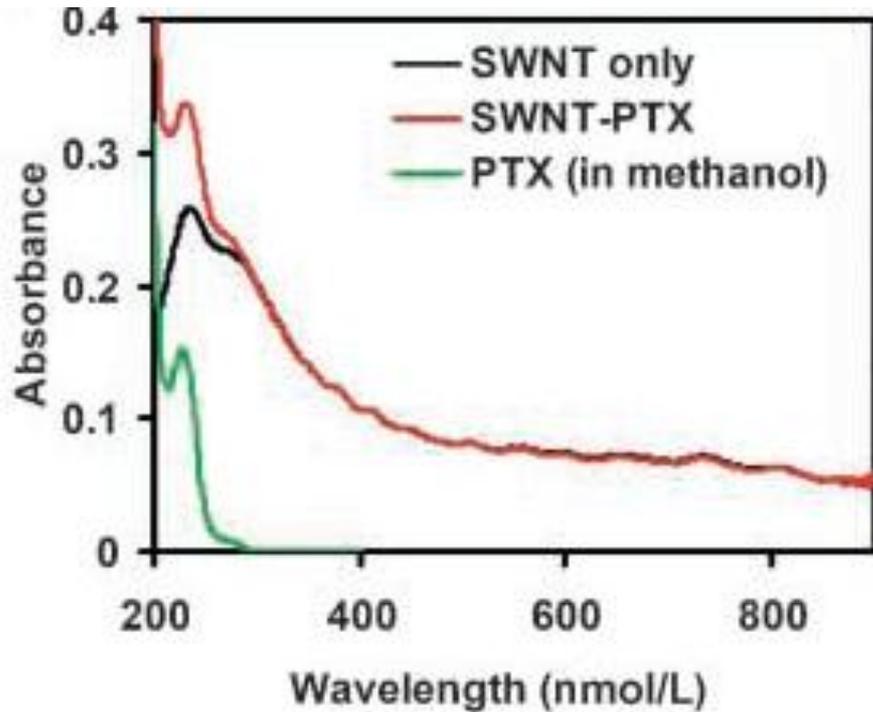


Dispersion in PBS

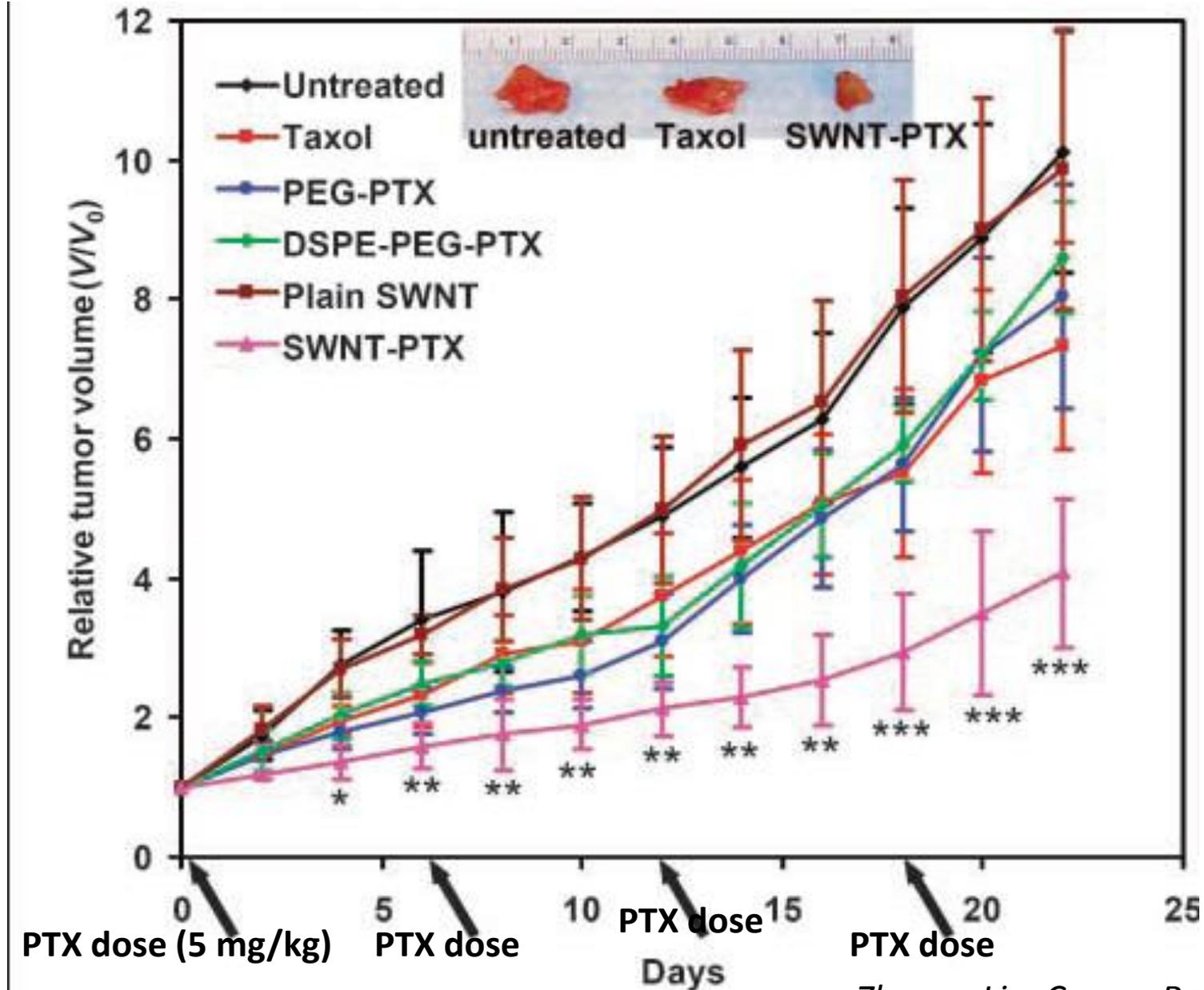
sonication of SWNTs in a water solution of phospholipid-PEG

230 nm PTXの光吸収
PTX量の計測に使う

細胞毒性、4T1細胞
50% Cell viability inhibition
(IC50)、3日



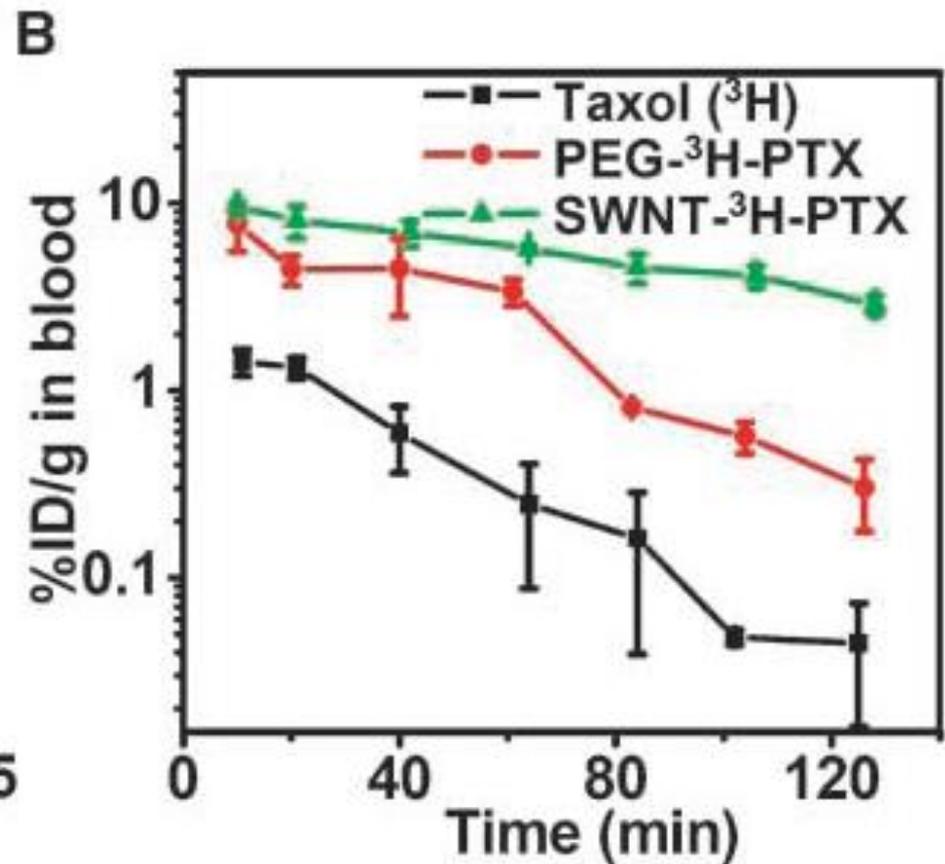
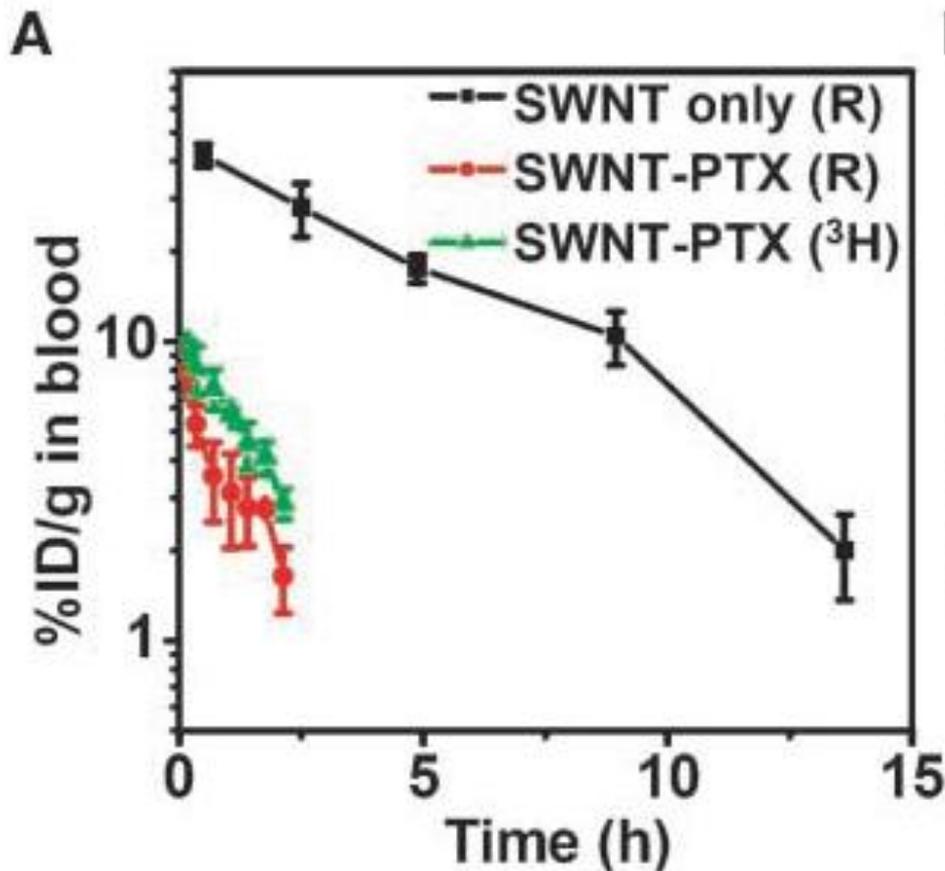
4T1 breast cancer mice model (皮下移植)、i.v.投与



Blood circulation of SWNTs and PTX.

SWNT – by Raman

PTX--- Scintillation counting of ^3H radioactivity in blood data

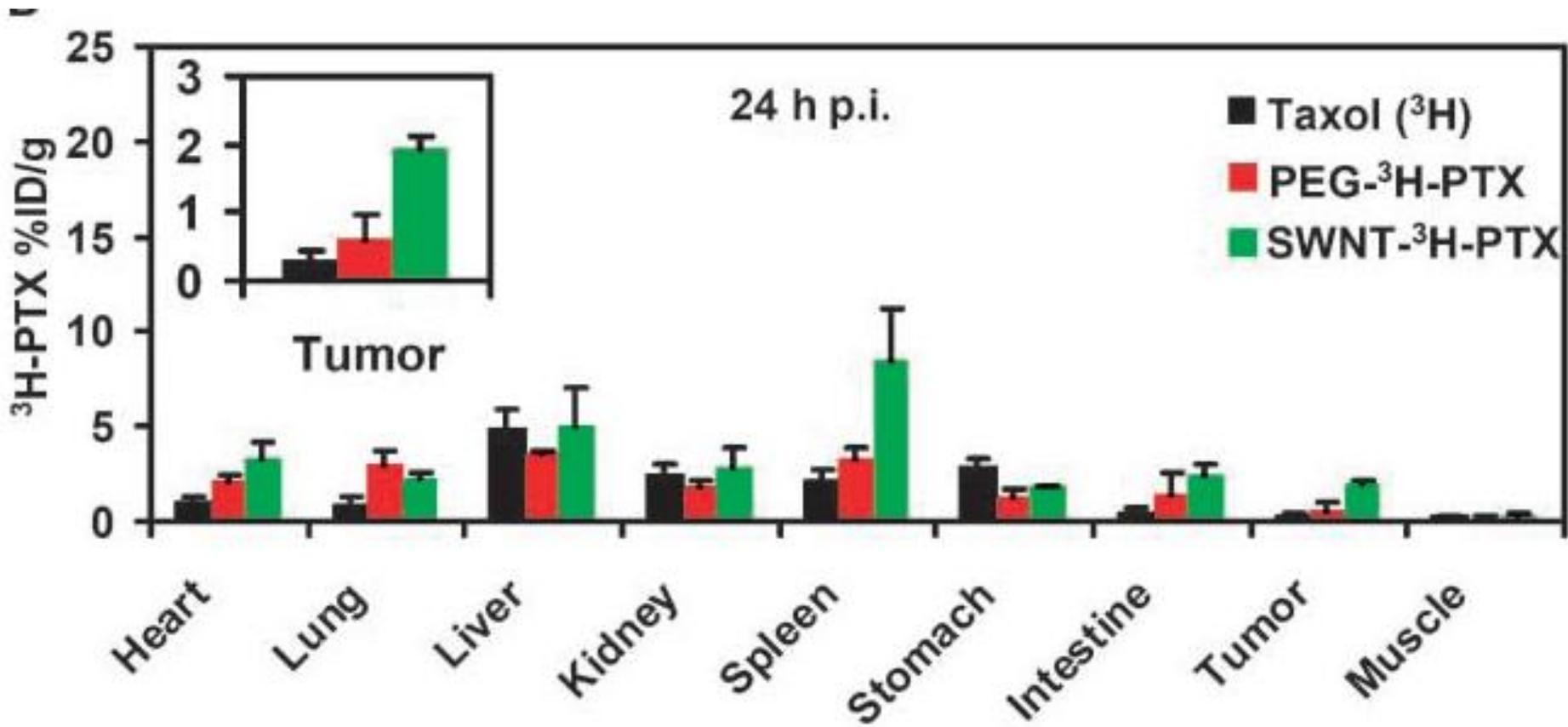


Zhuang Liu, Cancer Research 2008

タキソール:パクリタキセルの商品名

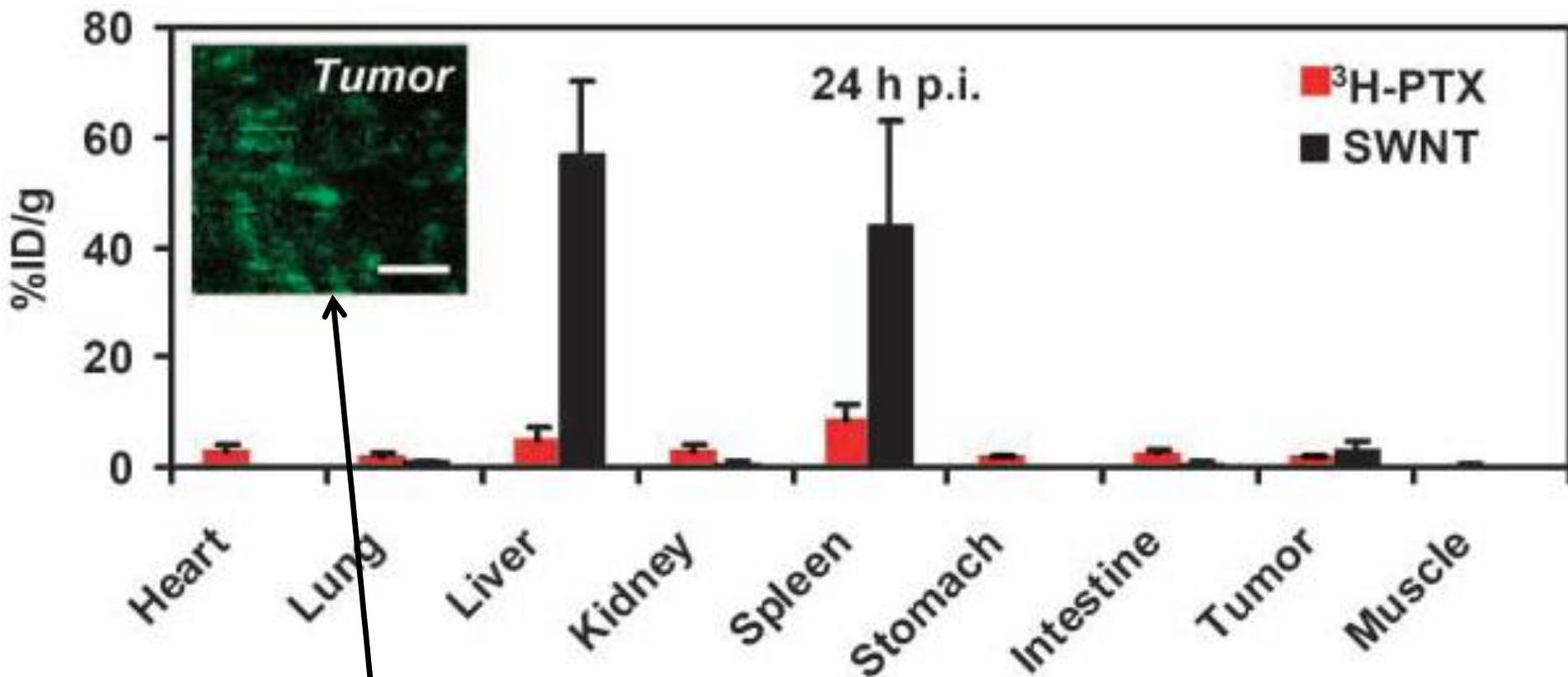
PTX biodistribution measured by scintillation counter.

TumorにDeliveryされるPTX量は、少ない。しかし、SWNTを使うとPTXの血中滞留時間は長くなる。



SWNT biodistribution measured by Raman spectroscopy

肝臓や脾臓にたまったSWNTからPTXが徐放されて、血中のPTX量が保たれている。これで、SWNT-PTXの高い抗腫瘍効果が出ているらしい。



Raman Gバンドのマッピング

Zhuang Liu, *Cancer Research* 2008