Directly observing intracellular nanoparticle formation with nano-computed tomography

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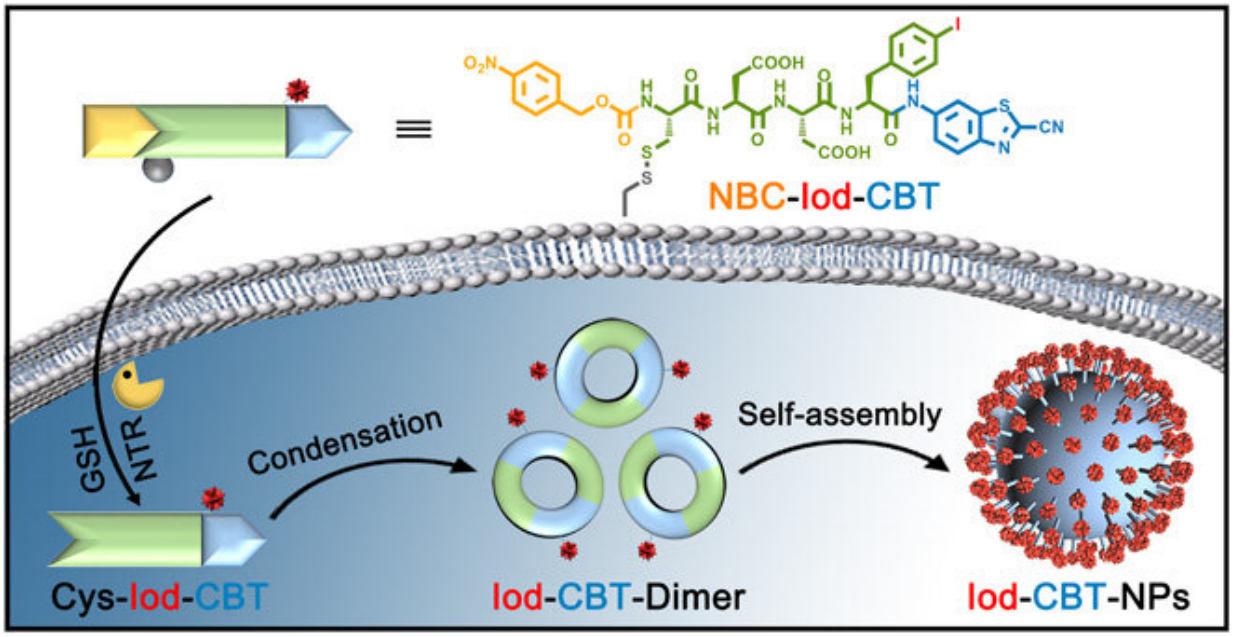


Schematic illustration of NTR-triggered self-assembly of NBC-Iod-CBT into Iod-CBT-NPs inside a cell. Under glutathione (GSH) reduction and nitroreductase (NTR) cleavage, small-molecule 4-nitrobenzyl carbamate–Cys(SEt)-Asp-Asp-Phe(iodine)–2-cyano-benzothiazole (NBC-Iod-CBT) undergoes an intracellular CBT-Cys click condensation reaction and self-assembles into iodinated nanoparticles (i.e., Iod-CBT-NPs). Credit: Science Advances, doi: 10.1126/sciadv.aba3190

It is currently challenging to directly observe the formation of

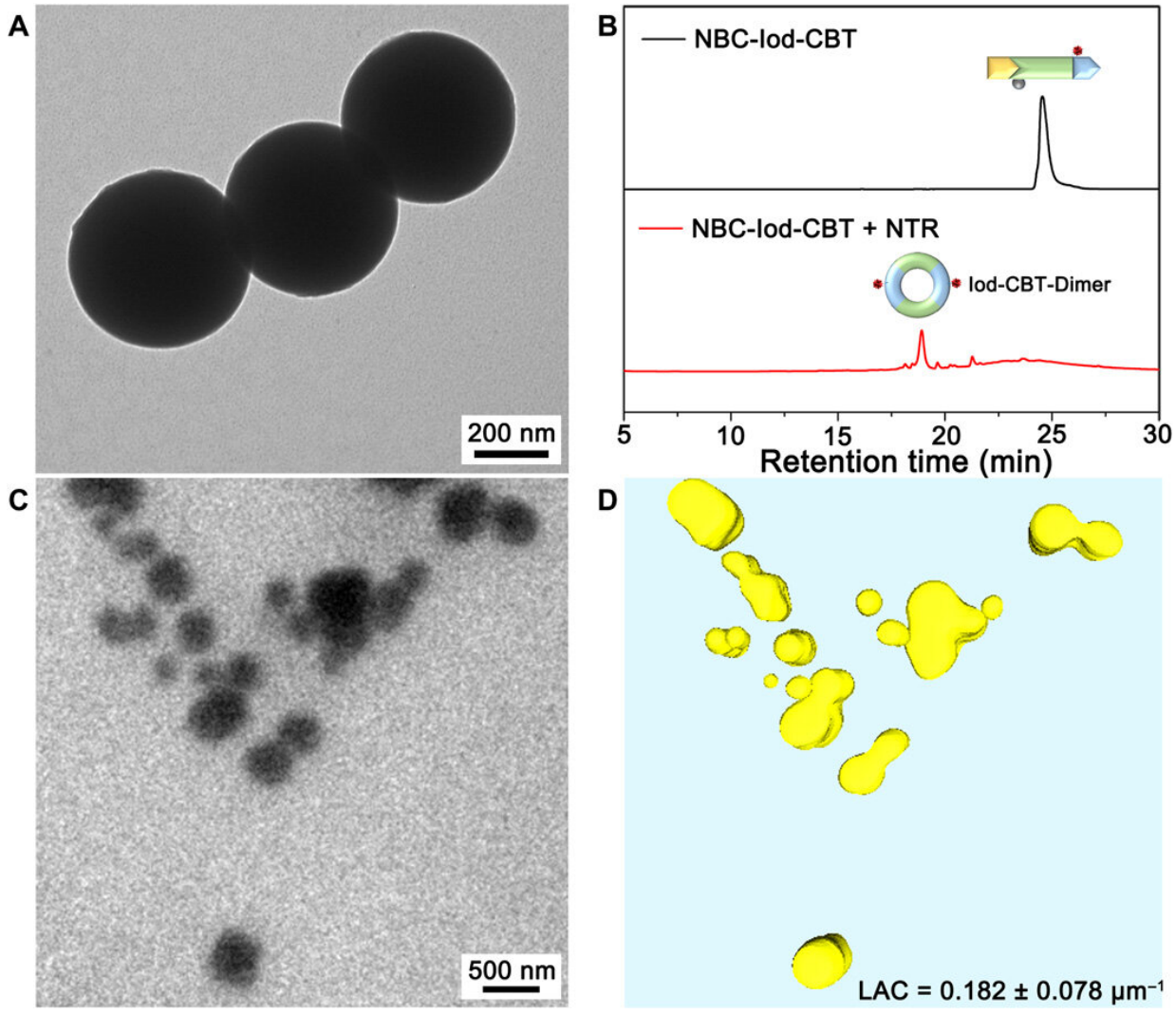
intracellular nanostructures in the lab. In a new report, Miaomiao Zhang and a research team in chemistry, life sciences, medical engineering and science and technology, in China, used a rationally designed small molecule abbreviated NBC-Iod-CBT (short for 4-nitrobenzyl carbamate–Cys(SEt)-Asp-Asp-Phe(iodine)–2-cyano-benzothiazole) and directly observed intracellular nanoparticle formation with nanocomputed tomography (nano-CT).

During the experiments, the glutathione (GSH) reduction and nitroreductase (NTR) cleavage mechanisms caused NBC-Iod-CBT molecules to undergo a click condensation reaction and self-assemble nanoparticles (NPs) as Iod-CBT-NPs. When the team conducted nano-CT imaging of NBC-Iod-CBT treated, nitroreductase-expressing HeLa cells in the lab, they showed the existence of self-assembled Iod-CBT-NPs in their cytoplasm. The new strategy is now published on *Science Advances* and will assist life scientists and bioengineers to understand the formation mechanisms of intracellular nanostructures.

A smart strategy for nanoassembly

Assembling nanostructures using small molecular precursors inside cells is an intelligent strategy with great advantages in molecular imaging and drug delivery. Small molecules can be taken up easily by cells, but they are also cleared out fast. In contrast, nanostructures with therapeutic agents have longer retention timeframes in cells with higher potency. Nevertheless, it is much more difficult for a cell to take up a nanostructure compared to a small molecule. Scientists therefore activate nanostructures for cellular uptake by modifying the cell surface with targeting 'warheads,' but such modifications can lower the reproducibility of the nanocomplex. As a result, a recently developed smart method aims to form intracellular nanoparticles, where cell cultures incubated with a small molecular precursor will have a nanostructure in them, for exciting applications in molecular imaging

and drug delivery. However, it is still difficult to differentiate between the artificially formed nanostructures from intrinsic cellular structures. To accomplish this, Zhang et al. first designed an iodine (iod)-containing small molecule precursor, they then subjected the compound to intracellular enzyme-instructed self-assembly to form the nanoparticles of interest and used nano-CT (nanocomputed tomography) to observe the intracellular nanoparticles.



In vitro characterizations of Iod-CBT-NPs. (A) TEM image of Iod-CBT-NPs. (B) HPLC traces of 500 μM NBC-Iod-CBT (black), 500 μM NBC-Iod-CBT

incubated with TCEP (2 mM) for 1 hour, and a further incubation with NADH (5 mM) and NTR (5 U/ml) for 2 hours in 10 mM PBS at 37°C (red). Wavelength for detection: 320 nm. (C) 2D projection image of Iod-CBT-NPs. (D) 3D rendering image of Iod-CBT-NPs (yellow). LAC, linear absorption coefficient. Credit: Science Advances, doi: 10.1126/sciadv.aba3190

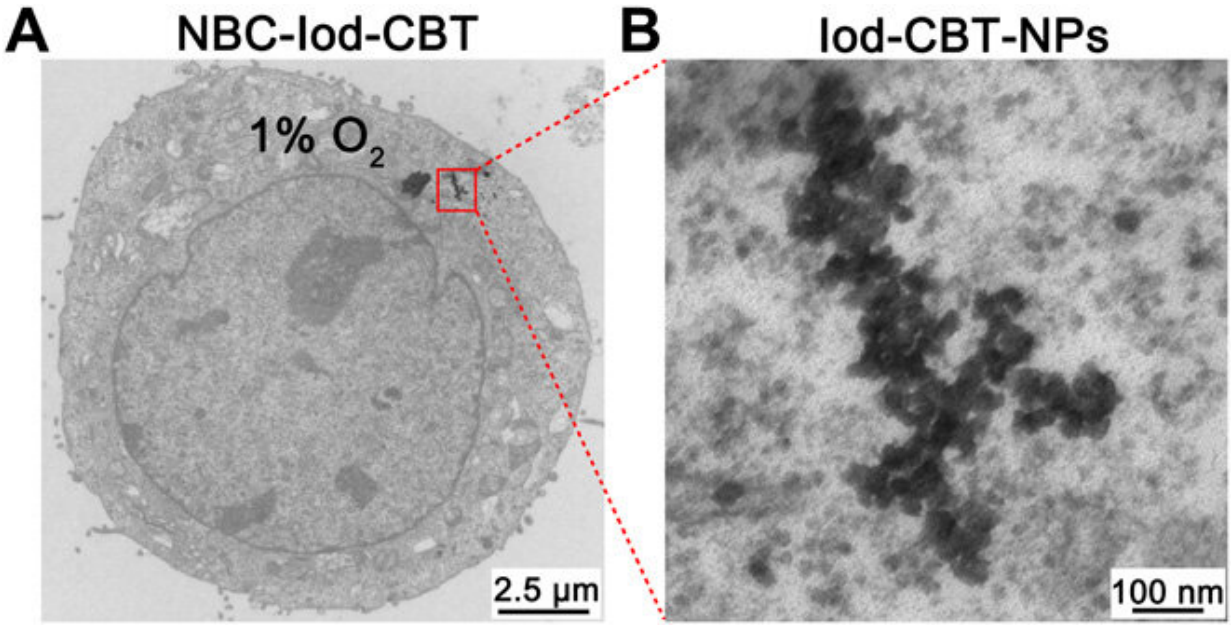
The experiment

The iodinated NBC-Iod-CBT small molecular structure had a rational design constituting of four parts, which included

1. A 4-nitrobenxyl carbamate (NBC) substrate to breakdown nitroreductase (NTR),
2. A latent cysteine (Cys) motif and 2-cyano-benzothiazole (CBT) structures for CBT-Cys click condensation reactions,
3. An iodinated region for computed tomography contrast enhancement, and
4. Two hydrophilic aspartic acid motifs for good water solubility under physiological condition

s.

When the compound entered nitroreductase (NTR)-overexpressing hypoxic (deprived of adequate levels of oxygen) cancer cells, they underwent self-assembly to form nanoparticles (NPs) known as Iod-CBT-NPs. To induce the nitroreductase-(NTR)-triggered nanoparticle formation in the lab, the scientists incubated the small molecule NBC-Iod-CBT with buffered saline solutions and added the nitroreductase solution for two hours to form nanostructures with visible absorbances between 500-700 nm.



TEM image of NBC-Iod-CBT–treated hypoxia HeLa cell. (A) Low-magnification TEM image of hypoxia HeLa cell incubated with 250 μM NBC-Iod-CBT for 4 hours. (B) High-magnification TEM image of the red square area in (A). Credit: Science Advances, doi: 10.1126/sciadv.aba3190

When Zhang et al. added an nitroreductase inhibitor known as dicoumarin to the solution, the visible absorbances of the mixtures decreased, confirming the formation of nanostructures in the presence of nitroreductase. Using transmission electron microscopy images, the team observed the appearance of nanoparticles and used high-performance liquid chromatography (HPLC) and high-resolution matrix-assisted laser desorption/ionization mass spectrometry to confirm the formation of Iod-CBT-NPs. Zhang et al. thereafter used three-dimensional (3-D) nano-CT images of the mixture with a soft X-ray microscopy nano-CT to ultimately reconstruct the 3-D nano-CT images, where different constituents of the compound displayed different X-ray absorption

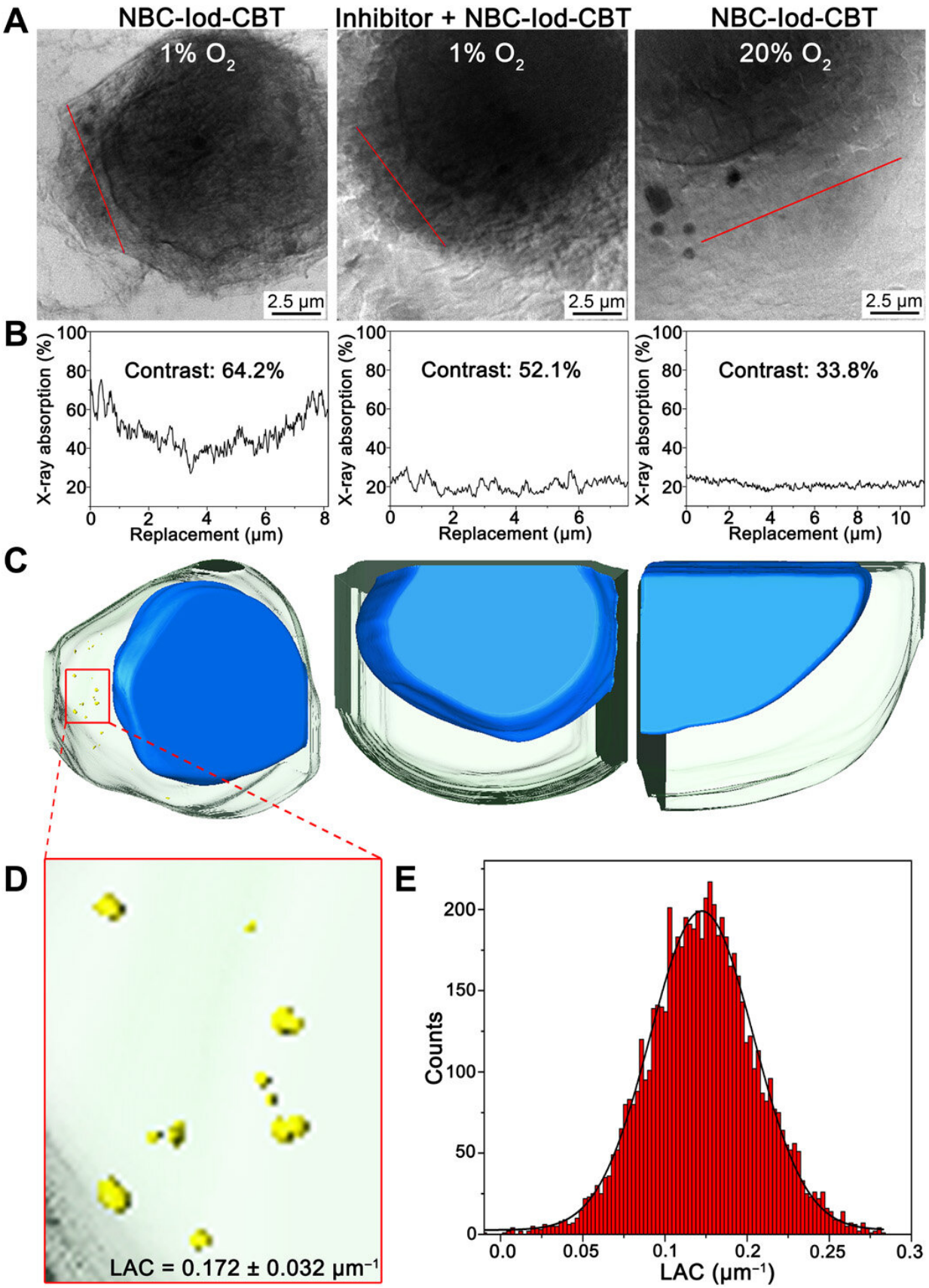
capabilities. In this way, the experiment facilitated the NBC-Iod-CBT compound of interest to undergo NTR-triggered self-assembly to form the expected nanoparticles (Iod-CBT-NPs) in the lab.

Intracellular formation of Iod-CBT-NPs and soft X-ray microscopy nano-CT imaging

Zhang et al. next investigated the same experimental process to induce nanoparticle self-assembly inside cells. The compound of interest (NBC-Iod-CBT) had higher selectivity toward nitroreductase, to thereby prevent any possible intracellular interferences in the presence of other intracellular constituents such as biothiols, oxidants and amino acids. The human cervical HeLa cancer cells typically overexpress nitroreductase (NTR) under hypoxic conditions (deprived of adequate levels of oxygen), reaching highest experimental levels within eight hours. When Zhang et al. incubated hypoxic HeLa cells with the small molecule NBC-Iod-CBT, they observed the eventual formation of nanoparticles within the hypoxic HeLa cells. Using electron microscopy images of the cells, they showed the existence of the nanoparticles as expected in the cell cytoplasm.

To directly observe the nanoparticles of interest (Iod-CBT-NP) inside the cells, the team experimentally treated the hypoxic HeLa cells and imaged them using soft X-ray microscopy nano-CT. They then used hypoxic HeLa cells pre-treated with dicoumarin or normoxia (normal levels of oxygen) as two positive controls and untreated hypoxia or normoxia HeLa cells as two negative controls. The results indicated the formation of the Iod-CBT-nanoparticles in the cytoplasm of hypoxic HeLa cells. When they subjected these cells to nitroreductase inhibitor treatment, the CT contrast of the cytoplasm decreased. The team reconstructed 2-D projections of the cells to obtain 3-D nanoCT images. Using the linear absorption coefficient (LAC) or linear attenuation

coefficient, Zhang et al. confirmed the feasibility of intracellular nanoparticle formation.


A
NBC-Iod-CBT
1% O2
Inhibitor + NBC-Iod-CBT
1% O2
NBC-Iod-CBT
20% O2
2.5 μm
2.5 μm
2.5 μm
B
X-ray absorption (%)
Contrast: 64.2%
Contrast: 52.1%
Contrast: 33.8%
Replacement (μm)
C
D
LAC = 0.172 ± 0.032 μm⁻¹
E
Counts
LAC (μm⁻¹)

Directly observed Iod-CBT-NPs with soft x-ray microscopy nano-CT imaging. (A) The 2D projection images of hypoxic HeLa cells treated with 250 μM NBC-Iod-CBT for 4 hours (left), hypoxic HeLa cells treated with 500 μM dicoumarin (an NTR inhibitor) and then treated with 250 μM NBC-Iod-CBT for 4 hours (middle), and normal HeLa cells treated with 250 μM NBC-Iod-CBT for 4 hours (right). (B) Corresponding absolute soft x-ray absorptions for the red lines in (A) part. (C) Corresponding 3D segmented HeLa cells in (A). In the segmented regions, the yellow structures are Iod-CBT-NPs, the green structures are cytoplasm, and the blue structures are nucleus. (D) Magnified view of the red rectangle area in (C) part. (E) LAC histogram of whole intracellular Iod-CBT-NPs [the yellow structures in the left image of (C)] and its corresponding Gaussian fitting curve (black). Credit: Science Advances, doi: 10.1126/sciadv.aba3190

Outlook

In this way, Miaomiao Zhang and colleagues rationally designed an iodinated small-molecule NBC-Iod-CBT construct to directly form and observe nanoparticles inside cells using nano-CT. After first-hand experiments conducted in vitro, the team conducted further studies in the cytoplasm of nitroreductase-expressing HeLa cells. Using analytical techniques, the team showed nanoparticle (Iod-CBT-NP) formation in the small molecule NBC-Iod-CBT-treated hypoxic HeLa cells. They verified their method using the linear absorption coefficient and confirmed the feasibility of intracellular nanoparticle formation. This work will assist researchers to gain deeper insights to the formation of intracellular nanostructures with significant applications in nanomedicine and bioengineering.

More information: Miaomiao Zhang et al. Directly observing intracellular nanoparticle formation with nanocomputed tomography,

Science Advances (2020). DOI: 10.1126/sciadv.aba3190

Hak Soo Choi et al. Design considerations for tumour-targeted nanoparticles, *Nature Nanotechnology* (2009). DOI: 10.1038/nnano.2009.314

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