Comprehensive bioimaging with fluorinated nanoparticles using breathable liquids

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Fluorocarbons are lipophobic and non-polar molecules that exhibit remarkable biocompatibility, with applications in liquid ventilation and synthetic blood. The unique properties of these compounds have also enabled mass spectrometry imaging of tissues where the fluorocarbons act as a Teflon-like coating for nanostructured surfaces to assist in desorption/ionization. Here we report fluorinated gold nanoparticles (f-AuNPs) designed to facilitate nanostructure imaging mass spectrometry. Irradiation of f-AuNPs results in the release of the fluorocarbon ligands providing a driving force for analyte desorption. The f-AuNPs allow for the mass spectrometry analysis of both lipophilic and polar (central carbon) metabolites. An important property of AuNPs is that they also act as contrast agents for X-ray microtomography and electron microscopy, a feature we have exploited by infusing f-AuNPs into tissue via fluorocarbon liquids to facilitate multimodal (molecular and anatomical) imaging.
Fluorocarbons have remarkable chemical and physical characteristics that allow unique surface properties, high stability, low reactivity and an ability to solubilize gases. The biocompatibility of fluorocarbons has been demonstrated and has inspired researchers to utilize these compounds for a wide range of biomedical and research applications. For example, these properties have enabled fluorocarbons to be applied in areas as diverse as surface coatings, drug delivery, liquid ventilation, synthetic blood and several imaging modalities including mass spectrometry imaging.

Mass spectrometry imaging facilitates the spatially resolved mass analysis of metabolites in situ where investigators have a choice of multiple technologies each with their inherent advantages and disadvantages. These techniques include matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), secondary ion mass spectrometry, desorption electrospray ionization, MALDESI, and others. While there have been some successful developments in low energy and low background matrices for MALDI-MS, the most widely used of these technologies, these matrices do not facilitate multiple modes imaging.

Gold nanoparticles (AuNPs) are well known as contrast agents for X-ray imaging technologies and electron microscopy. For example, the attenuation property of X-rays by AuNPs has led to their use as contrast agents in computed tomography (CT) imaging. AuNP have also been exploited for mass spectrometry molecular imaging, thus presenting a single platform capable of supporting comprehensive bioimaging. However, AuNPs’ ability to facilitate molecular imaging via mass spectrometry is limited due to the required high laser energies, resulting in significant fragmentation and generally poor sensitivity. While AuNPs are attractive biomedical imaging agents, they have not been explored as agents to correlate structure with molecular information given their limitations with mass spectrometry.

In a representative experiment, we have demonstrated the utility of these f-AuNPs to carry out MS analysis of pure standards and human plasma using gold-thiol chemistry. Here we used two separate instruments designed for MALDI, an AB SCIEX TOF/TOF 5800 and a Waters Synapt G2. AuNPs also offer a straightforward platform for functionalization using gold-thiol chemistry. Here we used two separate commercially available thiolos, 1H,1H,2H,2H-perfluorodecanethiol (FD10) and 1H,1H,2H,2H-perfluorooctanethiol (FO8). However, there is also the opportunity to design new ligands for the AuNPs that could enhance ionization, for example, amine fluorocarbon ligands could facilitate desorption/ionization of negatively charged metabolites since the ligand’s amine would act as a proton acceptor.

Soft ionization with fluorinated AuNPs. To illustrate the laser-induced thermal energy generated at f-AuNPs, 4-nm f-AuNPs were measured with transmission electron microscopy (TEM) before and after laser irradiation (Fig. 2). The size distribution in the top panel of Fig. 2 shows an increase in size (to ~6 nm) indicating that the particles melt and anneal following exposure to the laser pulses. The accompanying TEM images in Fig. 2b offer further evidence that laser radiation has melted and fused the f-AuNPs. The thermal energy responsible for melting the
particles can be used to desorb the fluorocarbon ligand, thus indirectly desorbing analyte molecules into the gas phase for mass spectrometry analysis. Thermal desorption, however, typically requires high energy, which leads to significant fragmentation, including the formation of ions that are primarily cation adducts\(^3\) and the formation of gold cluster ions\(^7\). Therefore, our next set of experiments explored the performance of the f-AuNPs and whether thermal release of the fluorocarbon ligand was beneficial to the mass spectrometry analyses. To do this, the f-AuNPs were compared with MALDI and AuNPs functionalized with the non-fluorous ligand octanethiol (C8-AuNPs) by tracking the survival yield of the thermally labile molecule methoxybenzyl pyridinium with respect to laser energy (Fig. 2). Survival yield was calculated as the ratio of \([M]^{+} (m/z = 200)\) to the major fragment at \(m/z = 121\). Desorption of methoxybenzyl pyridinium was initiated at energies above 0.78 \(\mu\)J; when using MALDI with \(\alpha\)-cyano-4-hydroxycinnamic acid, (C8-AuNP) initiated desorption at energies above 0.50 \(\mu\)J, while the f-AuNP initiated desorption at energies above 0.25 \(\mu\)J for the 8-carbon fluorous ligand (F8-AuNP) and 0.34 \(\mu\)J for the 10-carbon fluorous ligand (F10-AuNP). The low-energy behaviour of f-AuNPs allows NiMS experiments to be carried out at energies that are below the threshold for gold cluster formation (Supplementary Fig. 3), a significant advantage since these ion clusters can complicate the mass spectrum in the low mass region. In addition, the low energies used tend to create minimal background signal compared with typical MALDI matrices (Supplementary Figs 4–15).

The survival yield plot in Fig. 2 shows that the f-AuNPs performed exceptionally well in terms of fragmentation as compared with the C8-AuNPs. We observed that fragmentation begins immediately at the threshold energy required for desorption and levels out at \(\sim 20\%\) survival for C8-AuNP, whereas the F8-AuNP maintained 100\% survival over 0.25–0.50 \(\mu\)J and reached a steady state of 60\% at 1.25 \(\mu\)J. These results suggest that the fluorous-thiol functionality effectively insulates the sample from the potentially damaging thermal energy from the irradiated AuNPs. This is further evidenced by the observation that the longer fluorinated alkane thiol functionalized F10-AuNPs maintain 100\% survival over a longer range (0.34–0.80 \(\mu\)J) as presumably the longer chain better insulates the analyte from the heat generated at the particle surface. It is also interesting to note that fragmentation begins at energies far below the threshold for desorption of bare nanoparticles, showing the inherent harshness of nanoparticle-based thermal desorption. The comparison to be made with MALDI-MS is more nuanced; at very low energies, desorption does not occur in MALDI-MS, while it does occur using f-AuNP with essentially no damage to the molecular ion. As the energy approaches the threshold for MALDI, the survival yield for F-AuNPs begins to decrease and continues to decrease at a slower rate than MALDI until both desorption methods reach a steady state of 60\% at 1.25 \(\mu\)J.

Generally, we observed that the f-AuNPs outperform MALDI at low energy and perform comparably at higher energies. The signal-to-noise ratios plotted in Fig. 2 show that the particles perform better than both MALDI and the C8-AuNP in terms of the generation of \([M]^{+}\) signal. This result is again most likely due to the low energy requirements for this type of desorption/ionization. In addition, the longer chain appears to perform slightly better than the shorter chain. The length of the chain appears to shift and extend the optimal energy range for this experiment; however, in both cases, it should be noted that the maximum signal-to-noise occurs at energies above 100\% survival. Furthermore, it could be the case that the trend would continue with the use of a perfluorododecanethiol (F12); however, this ligand was not readily available.

**Figure 2 | Soft ionization with f-AuNPs.** (a) The diameter of AuNPs increases from 3.5 ± 0.5 nm to 6.0 ± 1.4 nm after laser irradiation. (b) TEM images of AuNPs before and after laser irradiation. Scale bar, 10 nm. (c) Survival yield versus laser energy plot of methoxybenzyl pyridinium for F8-AuNP, F10-AuNP, C8-AuNP and MALDI (error bars are s.d.). (d) Signal-to-noise versus laser energy plot for \([M]^{+} (m/z = 200)\) for F8-AuNP, F10-AuNP, C8-AuNP and MALDI (error bars are s.d.).
In addition to the low-energy requirements of the f-AuNP observed in the survival yield experiments, scanning electron microscopy (SEM) data indicate that the desorption process is particularly soft. Unlike the typical ablation ‘craters’ observed in MALDI-MS analysis of the tissue (Supplementary Fig. 16), we observed plateau-like structures in the f-AuNP experiments. Figure 3 shows a slice of lung tissue that was spotted with the f-AuNP solution and irradiated with a Nd:YAG laser. In the treated area, the laser pulses create raised protrusions. Outside of this f-AuNP-coated region, no protrusions were formed. Interestingly, the laser pulses that occurred at the interface create partial plateau structures. We suspect that the protrusion of the surface following laser radiation is a side product brought about by the heating of the f-AuNPs. We speculate that the heat of the f-AuNPs vaporizes the more volatile components in the tissue, creating the plateau-like effect, however this phenomenon requires further investigation.

NIMS and SEM imaging of biofilms. The overarching goals of this technology are to increase both the accessibility and the functionality of nanostructure-based imaging. Using f-AuNPs as a platform accomplishes these goals by adding multiple methods of imaging capabilities. To this point, another advantage of f-AuNPs is their solubility in biologically inert fluorocarbon solvents that can be applied to the top of a tissue sample. This contrasts to classic silicon NIMS experiments, which require that the sample be thinly sliced and placed on top of a nanostructured surface. We tested the versatility of the f-AuNPs on a biofilm sample that would not be immediately accessible to silicon nanostructure-based techniques due to the thickness of the sample28. To analyze these samples, the aclar substrates that the sample be thinly sliced and placed on top of a nanostructured contrasts to classic silicon NIMS experiments, which require that the areas lacking nanoparticle signal did contain cancerous information and its distribution in this tissue (Fig. 5). To confirm one experiment. Here we chose to map a phosphatidylcholine (Supplementary Fig. 20) at m/z 756 to demonstrate molecular and characterized the airway and porosity of the lung. The blood vessels appear as dark areas and the regions of the lungs carrying metastases revealed dark spots consistent with the exclusion of nanoparticle from the metastatic lesions (Supplementary Fig. 19).

To obtain molecular information from liquid ventilated metastatic lung tissue, OCT-embedded lungs were sectioned (20 µm) and analysed. Since the f-AuNPs solution had been passed through the trachea into the lung, no additional f-AuNPs were applied to the tissue. A mass spectrometry image of the f-AuNP-ventilated left lobe of the lung is shown in Fig. 5. In general, mass spectrometry analysis is a multiplexed analysis where multiple molecular ions can be simultaneously detected in one experiment. Here we chose to map a phosphatidylcholine (Supplementary Fig. 20) at m/z 756 to demonstrate molecular information and its distribution in this tissue (Fig. 5). To confirm that the areas lacking nanoparticle signal did contain cancerous tissue, metastases in an intact liquid ventilated mouse lung were identified by haematoxylin and eosin staining and compared with an adjacent NIMS image (Supplementary Fig. 19). To better access these structures, it might be possible to approach the metastases from the blood stream by perfusing the animal with a synthetic blood form of the f-AuNP or to coat the tissue following sectioning (Supplementary Fig. 21).

To enable NIMS and X-ray μCT imaging of blood vessels in situ, we took advantage of fluorocarbons utility as a blood substitute20. The f-AuNPs were dissolved in the fluorocarbon...
perfluordecalin, which was emulsified to approximate the only
the Food and Drug Administration-approved fluorocarbon-based
blood substitute. The f-AuNP emulsion was perfused into the
circulatory system to demonstrate the ability to obtain structural
and chemical information from the circulatory system associated
with the lung (Supplementary Fig. 22) as well the liver
(Supplementary Fig. 23) of the mouse. In addition, we also
applied these imaging approaches to investigate the structural and
chemical effects of hyperoxia on the lung tissue of juvenile mice
(Supplementary Figs 24 and 25).

NIMS for analysis of central carbon metabolites. The majority of
central carbon metabolites are anionic and thus are better
suited for analysis in negative ionization mode imaging mass spectrometry. One successful approach used for the \textit{in situ} ionization
and imaging of central carbon metabolites employs amine-
functionalized initiators and MALDI matrices. Presumably,
these adjuvants work well because the basic amine group acts as a
proton acceptor to promote \( M - H^- \) production. Thus, to enable
the application of this imaging platform to the analysis of central
carbon metabolites in negative ionization mode, we have to
enrich the fluorocarbon solvent with the basic initiator
\( 1H,1H,2H,2H,3H,3H\text{-perfluoroundecylamine (PFUA)} \).

As it was not clear that the f-AuNPs would transfer the
requisite energy to the initiator molecule needed to induce
desorption/ionization, we tested the enriched f-AuNPs using a
standard mixture of nine metabolites in negative mode. A mass
spectrum generated from this experiment is shown in Fig. 6; in
addition to the peaks for each individual metabolite, we also
observe peaks for \( Au^- \) and \( PO_4H_2^- \). We attribute the
emergence of these ions to the higher energy used in this
experiment. The spectra in Supplementary Fig. 3 show that
under the standard operation energy of 1.4 \( \text{mJ} \) are
formed; the addition of PFUA, however, increased the energy
requirement to about 2 \( \text{mJ} \). Just as the energy threshold increases
with chain length (Fig. 2), the incorporation of the PFUA shifted
this threshold even further by ostensibly adding a second barrier
between the AuNP and the analyte. However, even at this
increased energy, the enriched f-AuNPs performed well in
negative ion mode.

The enriched f-AuNPs were then used to demonstrate central
carbon metabolite imaging in tissue using negative ionization
mode. The enriched particles were applied to the top of a sagittal
slice from one half of a bifurcated mouse brain after desiccation.
In Fig. 6, we have mapped the ions corresponding to lactate,
glutamate and docosahexenoic acid. The presence of each
mapped metabolite was then confirmed by LC-MS/MS analysis
of the second half of the brain. Here we have demonstrated that
this platform like Si-based NIMS has the ability to use
specialized initiators to expand the number of metabolites that
are amicable to mass spectrometry imaging.
Discussion
Fluorinated AuNPs (f-AuNPs) were designed to facilitate NIMS as well as act as contrast agents for EM and μCT. An important feature of the f-AuNPs is that they promote a gentle laser desorption/ionization mechanism facilitating intact molecular ion formation with little background interference or molecular ion fragmentation. The utility of AuNPs for structural μCT measurements is well established but the primary advance of employing fluorinated ligands to these structures has allowed for structural data (μCT) to be combined for the first time with mass spectrometry information (f-AuNPs NIMS). This multimodal imaging approach is particularly useful for performing mass spectometry-based metabolite imaging, where correlating metabolite concentrations with anatomical structure is key to data interpretation. The fluorocarbon liquid component of the method affords versatility in the delivery of the agent, which can be carried out via direct coating, liquid ventilation or perfusion allowing analysis of morphologically diverse biological samples.

Methods
Chemicals. Octanethiol-functionalized AuNPs (2–4 nm DLS, 2% w/v in toluene) and fluorinated ligands 1H,1H,2H,2H-perfluorodecanethiol (fC10) and 1H,1H,2H,2H-perfluoroctanethiol (fC8), methoxybenzyl pyridinium chloride and perfluorodecalin were purchased from Sigma-Aldrich (St Louis, MO, USA) and 10% Gibco Pluronic F-68 was from Life Technologies (Grand Island, NY)
**NATURE COMMUNICATIONS**

**Title:** Nanoparticle synthesis. Octanethiol-functionalized AuNPs were in totole with a concentration of 20 mg ml⁻¹. One ml of octanethiol-functionalized AuNPs (20 mg) was added to ~20 ml of ethanol to precipitate the AuNPs with centrifugation at 4,000 rpm for 15 min. The precipitated AuNP were then washed with acetone, briefly sonicated and centrifuged again. The cleaning procedure was repeated three times before being dissolved in 1 ml of hexane. The AuNP solution (1 ml) was then analysed using LDI-MS to test for the presence of tetractyl ammonium bromide by monitoring the tetractyl ammonium ion at m/z 466, which is commonly used as a phase transfer additive in nanoparticle synthesis. More acetone washes were conducted until tetractyl ammonium ion was not detected. An additional 1 ml of hexane and 400 μl of 1H,1H,2H,2H-perfluoroundecylamine (PFH) or 1H,1H,2H,2H-perfluorodecanecacid (FC10) was added and the vial was purged with nitrogen gas to remove air. The solution was stirred for one to three days until AuNPs precipitated. The solvent was evaporated with nitrogen flow and nanoparticles were washed three times with hexane, three times with acetone and two times with 1:1:1 methanol/acetone/water, respectively, to remove excess ligand impurities. Following the washing, the AuNP was dried in the oven (~80°C) for several minutes and stored as a solid. Before use, the AuNP was dissolved in tetraoctylfluorohexane (PFH) at 3 mg ml⁻¹ for mass spectrometry and electron microscopy analyses and 10 mg ml⁻¹ in perfluorodecalin for mouse lung perfusion experiments.

**Electron microscopy characterization.** For TEM imaging, fluorinated AuNPs were deposited on 400 mesh copper grids and characterized by a Philips CM100 electron microscope (FEI, Hillsborough, OR) at 80 kV. Images were collected using a Megaview III CCD camera (Olympus Soft Imaging Solutions, Münster, Germany). Fluorinated AuNPs deposited on copper grids were also irradiated with (355 nm, laser energy 6.6 mJ), 2,000 shots) and then measured with TEM. For SEM analysis, 10 μl of 3 mg ml⁻¹ fluorinated AuNPs in PFH were first deposited onto mouse brain or lung tissue which was then irradiated by a laser with a pre-defined 250-μm step size. Subsequently, the samples were imaged with a Hitachi S4800 SEM at 5 kV. SEM was done on biofilms from the same batch. Aclar slides with adhered biofilm were aldehyde fixed (2.5% w/v glutaraldehyde, 2.0% w/v paraformaldehyde, 0.05 M sodium cacodylate buffer pH 7.0) overnight. Samples were rinsed with double-distilled H₂O and ethanol dehydrated. Critical point drying was then carried on the samples using CO₂ and spot welded with f-AuNP solution (1 mg ml⁻¹).

**Bacterial strains and growth conditions.** DVH RCH1 was obtained from Dr Romy Chakraborty (Lawrence Berkeley National Lab) and was isolated from C. jejuni strain CIP 102707. It was grown in LS4D medium, which contains lactate as the carbon source and electron donor and sulfate as the electron acceptor. DVH RCH1 was grown in liquid LS4D medium, which contained lactate as the carbon source and electron donor and sulfate as the electron acceptor.

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Author contributions

Z.J.Z, M.E.K., J.L., M.E.S., P.I.O.B. and G.S. designed the experiments; Z.J.Z, A.M.S., M.E.K. and J.L. performed the MS experiments; M.R.W. performed the electron microscopy. H.I.O. performed the IM-MS experiments. K.L, J.M.D and A.G. collected the μCT data. B.H.F. and R.Q. collected the μCT data. A.M.S., G.J.P. and G.S. performed the bacterial experiments. M.E.K., J.L., Z.J.Z, A.M.S., G.J.P. and G.S. analysed the data and wrote the paper.

Additional information

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