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To achieve successful drug delivery via nanoparticles the interactions between the nanoparticle and the chemistry of the surrounding biological environment is of central importance. A thorough understanding of these interactions is necessary in order to better elucidate information regarding drug pathways and mechanisms of action in treatment protocols. As such, it is important to identify the location of the nanoparticle, the state of its functionalization, as well as any changes in the cellular environment. The use of cluster secondary ion mass spectrometry (SIMS) using C₆₀⁺ primary ions makes simultaneous acquisition of this information possible. Here, SIMS has been successfully used to chemically image gold nanoparticles (AuNPs) within a model, single cell system involving macrophage-like RAW 264.7 cells. The macrophage-like properties of this cell line make it extremely well-suited for cell-uptake studies. Both AuNPs and two pharmaceutical compounds, amiodarone and elacridar, were successfully imaged within a cellular system using cluster SIMS. To verify that SIMS can also be used to detect functionalization and nanoparticles simultaneously, fluorophore-functionalized AuNPs were studied as a model system. The fluorescent characteristics of these functionalized nanoparticles enabled the visual confirmation of the presence and location of the particles within the cell. © 2015 American Vacuum Society. [http://dx.doi.org/10.1116/1.4939463]

1. INTRODUCTION

The use of nanoparticles in medicine is a topic of great promise in current research. Much time has been dedicated to studying how nanoparticles can aid in solving a wide variety of biological problems, ranging from biomolecular detection, to medical diagnostics, to drug delivery, as well as many others. Targeted drug delivery systems, in particular, have become increasingly prevalent due to more successful treatment outcomes and reduced side effects when compared to traditional methods. The use of nanoparticles as a delivery platform for such therapies allows the treatment location to be changed through the tuning of properties of the carrier, rather than the active compound itself. The ability to control exactly where pharmaceutical compounds are released in the body allows for a reduction in the dose administered to the patient. This specificity in release results in a decrease in required dosage, which decreases side effects, increases patient compliance, causes greater success in treatment outcomes, and decreases cost of the treatment.

Gold nanoparticles (AuNPs) have shown great promise as platforms for targeted drug delivery, in part, to their low toxicity, excellent biocompatibility, ability to accumulate in areas of interest, and tunable stability. There are also many methods for manufacturing AuNPs that allow for control over the size, shape, surface functionalization, and state of aggregation, which provides researchers with a powerful tool in designing particles that contain the desired properties. Research efforts continue to be focused on developing materials which are able to target specific tissues and organs by incorporating a variety of biomolecules on the surface of AuNPs.

The success of these targeted drug delivery therapies relies on a thorough understanding of the nature of the interactions between nanoparticles, pharmaceutical compounds, and the surrounding biological environment, ensuring that the design and implementation of these compounds for therapeutic and diagnostic purposes is optimized. Traditional analysis methods for nanoparticles within biological systems, such as transmission electron microscopy and fluorescence microscopy, often require complicated sample preparation methods and have difficulty in offering chemical specificity. Obtaining information regarding the distribution of nanoparticle-bound pharmaceutical compounds in addition to the distribution of the compounds themselves after uptake into the biological system is challenging as a result of these shortcomings.

It is currently very difficult to simultaneously, and with one technique, analyze all components necessary to understand the nature of the interactions between AuNPs, pharmaceutical compounds (or other functionalization), and the surrounding environment. Cluster secondary ion mass spectrometry (SIMS) can provide valuable information to aid in this task due to its ability to provide both chemical specificity and high spatial resolution. The ability of SIMS to create chemical maps in both two and three dimensions is valuable in identifying the location of pharmaceutical compounds and AuNPs in a cellular system, chemically, as well as providing insight into the changes in the biological system itself. Monitoring in situ drug release to better understand drug kinetics, as well as identifying the state of the AuNPs and their functionalization within the cell is a possibility with...
these unique experimental attributes. One of the concerns involving AuNPs is how the AuNP, functionalized or not, changes within the cell. Cluster SIMS has the opportunity to make understanding these changes possible. It has been widely used in biological analysis and has already been successfully utilized to image nanoparticles of several varieties in mammalian cells, as well as within unicellular organisms. These studies focused on the location of the nanoparticles and their effects on the cellular system, mostly in reference to toxicological effects resulting from their uptake and potential future medicinal applications.

Herein is reported the first instance of the use of cluster SIMS in identifying AuNPs within a cellular system within the framework of target drug delivery applications. In this work, citrate-capped AuNPs, several model pharmaceutical compounds, and fluorophore-functionalized AuNPs were analyzed within RAW 264.7 cells in order to determine their integration into the cellular system. The RAW 264.7 cell line was chosen due to its macrophage-like characteristics, specifically the ease with which foreign compounds are engulfed and digested, making it well-suited for model studies interested in cellular uptake, as is the case here. Pharmaceutical compounds were chosen, which validated the detection of small medicinal molecules with SIMS, as these studies are designed to be model systems to be applied to further studies in the future.

As with all biological analyses using cluster SIMS, sample preparation is crucial to the success of the experiment, and so, chemically (formalin) and cryogenically (liquid nitrogen) fixed cells were studied to ensure results could be applied to a variety of conditions. Chemically fixed cellular samples were analyzed first, as they can be studied at room temperature. However, they add chemical information to the system in the form of the fixative and may result in the collapse of the cell itself. Cryogenically fixed cells offer an environment most similar to the native cellular state because they do not add exogenous compounds to the system. Such samples, however, require more complicated sample handling procedures and specific instrumental setups that not all systems are equipped with. While cryogenically fixed samples were the preferred form of analysis for this study, it was important to ensure that compounds could be successfully detected in both the chemically and cryogenically fixed states.

II. EXPERIMENT
A. Cell culture

RAW 264.7 cells, macrophage-like cells derived from tumors induced in mice by the Abelson murine leukemia virus, were maintained in Dulbecco’s modification of Eagle’s medium (DMEM 1×, Corning cellgro, USA) with 10% fetal bovine serum (Gemini Bioproducts, USA) at 37°C and 5% CO₂. After rinsing with Dulbecco’s phosphate-buffered saline (DPBS, Corning cellgro, USA), cells were detached from culture flasks using a cell scraper and cultured onto 5 × 5 mm silicon chips. These chips had been cleaned by sonication in chloroform, acetone, methanol, and purified water. Cells were allowed to grow for 24 h.

In cells exposed to 50 nm AuNPs (stabilized suspension in citrate buffer, Sigma Aldrich, USA) and amiodarone hydrochloride (Sigma Aldrich, USA), the desired concentration of compound was introduced at the time of cell seeding. Concentrations ranged from 1 to 150 μM for both compounds. Fluorescent AuNPs (cell uptake fluorescent spherical gold nanoparticles, 10 nm diameter, Nanopartz, Inc., USA) were treated the same as citrate buffered AuNPs for all sample preparation procedures. In cells exposed to elacridar (Novartis Pharmaceuticals, USA) cells that had been allowed to grow for 24 h were exposed to the desired concentration (1–150 μM) for 60 min prior to fixation or freezing.

B. Sample preparation

1. Formalin fixation

After the 24 h incubation period, the cell-covered silicon chips were removed from media. Cells were chemically fixed using formalin (1:10 dilution buffered) for 15 min. After fixation, cells were washed five times in DPBS to remove excess fixing solution, followed by rinsing in 0.015 M ammonium formate (pH 7.3) for 1 min. Ammonium formate was used to minimize variation from accepted cellular preparation methods which utilize this solution to remove residual salts. The samples were allowed to air dry prior to insertion into the SIMS instrument.

2. Frozen hydration

Frozen hydrated (cryogenically fixed) cells were prepared using the frozen hydrated cell preparation protocol as described by Piwowar et al. As in the formalin fixed cells, cells were allowed to grow on silicon chips for 24 h. They were then washed three times in DPBS and 0.015 M ammonium formate. After washing, excess liquid was aspirated, and the silicon chips were plunge-frozen in liquid ethane, followed by liquid nitrogen, and placed on a precooled sample block submerged in liquid nitrogen. Samples were then quickly inserted into the SIMS instrument, which had been cooled to −175°C.

C. Fluorescence microscopy

AuNPs (Nanopartz, Inc., USA) functionalized with a cell uptake peptide and CY3.5 fluorophore (ex/em: 591/604 nm) were used for nanoparticle visualization. An Olympus FV1000 laser scanning confocal microscope (Olympus America, Inc., USA) equipped with four single line lasers was used to capture fluorescent images from stained cell samples. For fluorescence studies of the fluorophore-functionalized AuNPs in the cells, a tetramethylrhodamine filter set was used for selection of appropriate emission wavelengths.
D. Cluster secondary ion mass spectrometry

Cluster SIMS characterization was performed using a J105 3D Chemical Imager (Ionoptika Ltd., UK) equipped with a 40-keV C\textsubscript{60}\textsuperscript{+} source, which has been described previously.\textsuperscript{26} Images were acquired with positive polarity using a beam with 1 \(\mu\)m diameter and 100 \(\times\) 100 \(\mu\)m field of view with an ion dose of 2.0 \(\times\) 10\textsuperscript{13} ions/cm\textsuperscript{2} per analysis cycle, defined here as “Layer.” Samples prepared using the frozen hydration method were analyzed at \(-175\ ^\circ\text{C}, while all others were analyzed at room temperature. In experiments utilizing depth profiles, “Layer 1” corresponds to the surface of the sample, while layers with subsequent values indicate additional acquisitions of the same area with the same experimental parameters. The acquisition of each layer took 25 min.

III. RESULTS AND DISCUSSION

A. Citrate-capped AuNPs

Prior to characterization within the cells themselves, the AuNPs were analyzed as a standard to determine a reference spectrum and to ensure that there was no interference with known biological signals. The spectrum of formalin fixed cells without exposure to any exogenous compounds can be seen in Fig. 1(a). The spectrum of similar cells which had been exposed to 100 \(\mu\)M AuNPs for 16 h prior to fixation can be seen in Fig. 1(b). The peak corresponding to atomic gold at \(m/z\) 196.96 is used as an identifying marker of the AuNPs, as there is no corresponding biological peak in untreated cells. This makes it possible to confidently assign this peak to the AuNPs, suggesting that the localization of this signal within the cell is indicative of the presence of the AuNPs on or in the cell itself.

Once it was determined that the signal attributed to atomic gold was unique, citrate buffered nanoparticles were characterized in cells in both the formalin fixed and frozen hydrated state. A variety of nanoparticle concentrations (1–150 \(\mu\)M) were examined to ensure that experimental conditions closely mimicked therapeutic levels that would exist in a medical application. The chemical images in Fig. 2 show cells exposed to 100 \(\mu\)M AuNP for 16 h prior to formalin fixation. The distribution of the phosphocholine (PC) head group (\(m/z\) 184.07), which indicates the region occupied by the cellular membrane, as well as the signal characteristic of atomic gold, can

![Fig. 1. Spectral comparison of layer 7 in positive mode of (a) formalin fixed cells and (b) formalin fixed cells exposed to 100 \(\mu\)M citrate-capped AuNPs for 16 h showing the unique nature of the gold peak at \(m/z\) 196.96.](image)

![Fig. 2. Formalin fixed RAW 264.7 cells exposed to 100 \(\mu\)M citrate-capped AuNPs for 16 h. The PC headgroup and atomic gold signal are shown on (a) the top layer, (b) the seventh layer, and (c) a 3D overlay with PC head group in blue and gold in magenta. All images are 100 \(\times\) 100 \(\mu\)m and were acquired in positive mode.](image)
be seen in Fig. 2(a). The lack of gold signal on the cell surface indicates that the washing procedure of the cells prior to SIMS characterization removes all residual AuNPs from the cell surface, and all observed gold signal appearing throughout the depth profile is indicative of the AuNPs taken up by the cells. The distribution of both the PC head group and the gold in the seventh layer (total ion dose, \(1.4 \times 10^{14}\) ions/cm\(^2\)) of the depth profile can be seen in Fig. 2(b). Here, the interior regions of the cell have been exposed and the nuclear region, evidenced by the void within the cellular membrane signal as indicated by the PC head group, can be distinctly seen. The nanoparticles introduced into the cells are too large at 50 nm to permeate into the nuclear region,\(^{27}\) meaning that they should be localized within the boundaries of the cellular membrane, but surrounding the nucleus. In layers below the cell surface where the appearance of the AuNPs would be expected, this colocalization between membrane and AuNPs is unmistakably observed. Both signals are concentrated in regions surrounding the nucleus, but are still within the bounding limits of the cell membrane. An overlay of the PC head group and gold signal, which further confirms the colocalization of these signals, is presented in Fig. 2(c).

This protocol was repeated using concentrations of 50 and 5 \(\mu\)M AuNPs in formalin fixed cells and at a 100 \(\mu\)M concentration in frozen hydrated cells (Fig. 3). It is evident that AuNPs are detected at concentrations which may be considered relevant to therapeutic applications (5 \(\mu\)M) and in the frozen hydrated state which constitutes a more native cellular environment.

B. Pharmaceutical compounds

With the goal of this investigation being to prove the feasibility of SIMS to aid in targeted drug therapy studies using nanoparticle platforms, several pharmaceutical compounds were tested to ensure that they could be visualized using this technique and sample preparation protocol. While SIMS sensitivity is dependent on the nature of the molecule, these compounds were chosen to validate the ability of SIMS to detect small pharmaceutical compounds within the cell. Amiodarone, a well-studied antiarrhythmic, and elacridar, an oral bioenhancer used in targeting multiple drug resistance in tumors, were chosen as test compounds. Both compounds are known to interact through the cell membrane and are expected to be located in that area. Amiodarone was chosen, as it had been shown to be identifiable in cellular systems using cluster SIMS,\(^{28}\) while elacridar yields a strong, characteristic SIMS signal that is unique and identifiable in the cellular system, as determined by a reference spectrum.

Just as in the cases involving AuNPs, the pharmaceutical compounds to be tested were first characterized as standards to determine reference peaks and to ensure that these reference peaks did not correspond to interfering biological signals. The identifying peak for amiodarone, identified as the [M+H]\(^+\) ion, is observed at \(m/z\) 646.03 and the characteristic peak for elacridar, identified as the [M+H]\(^+\) ion, appears at \(m/z\) 564.25.

Both compounds were tested in formalin fixed and frozen hydrated cells at high and therapeutically relevant concentrations. The localization of the unique secondary ion associated with amiodarone to the regions of the cell, as determined by

Fig. 3. Formalin fixed and frozen hydrated RAW 264.7 cells exposed to a range of concentrations of AuNPs for 16 h. The PC head group and atomic gold signal are shown for layer 7 of each sample. All images are 100 \(\times\) 100 \(\mu\)m and were acquired in positive mode.
the total ion signal, at therapeutically relevant concentrations (5 μM) can be seen in Fig. 4(a). The colocalization of elacridar (50 μM) in frozen hydrated RAW 264.7 for 16 h. All images are 100 × 100 μm and were acquired in positive mode with a total ion dose of 1.4 × 10^{14} ions/cm² applied.

C. Functionalized nanoparticles

After determining that AuNPs and several pharmaceutical compounds could be successfully identified in cellular systems, the same system was examined following exposure to functionalized AuNPs. In studies involving AuNPs as platforms for targeted drug delivery, functionalization in some form is necessary in order to tune the properties associated with the particle to accomplish the desired goals. As such, confirming the ability of SIMS to identify the AuNP in addition to its functionalization is of great importance. Fluorophore-functionalized AuNPs were chosen to allow for a visual confirmation of the localization of the AuNPs within the cellular system using fluorescence microscopy. The ability to visually confirm the presence of the functionalization of the AuNPs in the cell using a technique complementary to SIMS allows for greater confidence that the compound was internalized, as well as providing its location within the cell. Here, AuNPs are taken up by the cellular system where they accumulate in regions surrounding the nucleus (Fig. 5).

As in the cases involving AuNPs and pharmaceutical compounds, the fluorophore-functionalized AuNPs were first characterized as standards to determine unique reference peaks that do not correspond to interfering biological signals. The peak corresponding to atomic gold (m/z 196.96) is clearly identifiable in the reference spectrum, as are two peaks which correlate with functionalization on the AuNPs (m/z 557.35 and 571.85).

Results from SIMS studies using this system are seen in Fig. 6. The first layer [Fig. 6(a)] exhibits a strong signal characteristic of the PC head group (cell membrane). The signal which indicates the functionalization on the AuNPs is very weak and more distributed over the surface of the sample, with some localization to the cellular region. The localization which is present may indicate compound on the surface of the cell that had not been internalized or compound in which the gold nanoparticle was internalized with the functionalization remaining on the exterior of the cell. The first layer also shows a small, selectively located signal from the gold, which may be caused by partial collapsing of the cell due to the formalin fixed state in which it was analyzed.

After the acquisition of several layers (total dose, 8.6 × 10^{13} ions/cm²), the gold signal is strongly localized with the PC head group in regions surrounding the nucleus [Fig. 6(b)]. The 10 nm size of the AuNPs prevents permeation into the nucleus as in the previous studies, resulting in an accumulation of gold in organelles in the surrounding regions. The signal characteristic of the functionalization is lower in intensity than the gold, but still present and localized to cellular regions, mostly external to the nucleus. These results indicate the possibility that only a small portion of the fluorophore-functionalized AuNPs are actually taken up by the cell. This idea is highly plausible, as the bulky functionalization of the fluorophore on the nanoparticle can make cellular uptake difficult. In addition, the large signal corresponding to gold indicates that this decreased relative intensity of the functionalization signal may be due to differences
in ionization efficiency between these two compounds, something which may be expected when comparing metals and organics in a biological system. Fluorescence data confirm the presence of the fluorophore in the interior regions of the cell, indicating that the compound is being internalized. This, in conjunction with the presence of gold in the interior regions of the cell as indicated by the SIMS data, provides strong evidence for the belief that the fluorophore remains attached to the nanoparticle once internalized. Despite the lower intensities of the functionalization signal in the SIMS experiment, its presence can be confirmed to the regions of the cell, though fluorescence provides a much clearer confirmation of this. Nonetheless, the ability to successfully locate and identify a functionalized AuNPs within a cellular system using SIMS has been shown.

IV. SUMMARY AND CONCLUSIONS

These results serve as an important step in using cluster SIMS to aid in the understanding of targeted drug delivery using AuNPs. The ability to locate and identify not only AuNPs, but also model pharmaceutical compounds and functionalized AuNPs without compromising signal from other biologically relevant components of the cell, provides unique measurement results. The compounds of interest are not only identified within the cell itself, but are also localized to the areas they would be expected to concentrate in, biologically.

The protocols deemed successful in this experiment must be explored on further cellular systems to determine their implications in medical studies. Macrophages serve as a reliable model system due to their ability to engulf and consume exogenous compounds, but would not be the cell line chosen for medicinal research applications. Characterization of AuNPs functionalized with pharmaceutical compounds (e.g., elacridar and amiodarone) need to be explored in greater detail in an effort to provide a better understanding of specific biological problems, including cell–drug and cell–nanoparticle interactions. Compounds of medicinal significance in targeted drug delivery must also be explored and is the goal of future studies. The possibility of exploring the effects of nanoparticles of other varieties (i.e., nanocapsules, organic nanoparticles, liposomes, etc.) within cellular systems using chemically specific information can also be considered.

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