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Relative quantitation of metal oxide nanoparticles in a cutaneous exposure model using enhanced darkfield microscopy and hyperspectral mapping

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ABSTRACT

The biological effects associated with the unique properties of engineered nanoparticles (ENPs) remain largely unknown. Animal models of exposure are valuable for assessing potential toxicity and adverse health effects. This study presents a method to determine relative quantitation of nanoparticle (NP) abundance in histological samples in an ex vivo model of cutaneous exposure to metal oxide NPs using enhanced darkfield microscopy (EDFM) with hyperspectral imaging (HSI) and mapping. Porcine skin tissue was topically exposed to alumina, ceria, and silica NPs using a modified Franz diffusion chamber and histologically prepared for imaging. EDFM allowed for rapid direct visualization of NPs, while hyperspectral mapping confirmed the composition of NPs throughout the tissue, based on positive matching of pixels to reference spectral libraries (RSLs). Relative quantitation of NPs was achieved based on calculating the percentage of mapped pixels per field of view (FOV). The greatest abundance of mapped NPs was found in the ceria-exposed group regardless of tissue layer, relative to the other groups. Fewer NPs were found in the dermis compared to the *stratum corneum* in both the alumina- and ceria-exposed groups. This study demonstrated EDFM-HSI as a valuable method to determine relative quantitation of NPs in histological samples. The methods put forth in this study could be adapted for application to other NP types as well as other biological or environmental matrices.

1. Introduction

The rapid incorporation of engineered nanoparticles (ENPs) into consumer products and manufacturing processes necessitates investment in safe development, as the potential health effects associated with the unique properties of ENPs remain largely unknown (European Academies Science Advisory Council (EASAC), 2011; U.S. CDC-NIOSH, 2009; Oberdörster et al., 2005). In response, environmental and human health and safety research for nanotechnology is gaining momentum, investigating toxicity and biological effects that ENPs may have on living organisms and on ecosystems (Nel et al., 2015). It is imperative to proactively investigate the potential human health effects of occupational and/or consumer exposure to ENPs in order to guide their safe use, handling, and disposal (Contado, 2015; Shepard and Brenner, 2014a; Brenner and Neu-Baker, 2014; Singh and Nanda, 2014; Kessler, 2011).

Occupational settings present scenarios for potential worker exposure to nanoparticles (NPs) via inhalation and/or skin contact. The nanotechnology workforce is growing, with an estimated total of 2 million workers in the U.S. by 2020, with 6 million workers globally (Roco et al., 2010). Metal oxide and metal-based ENPs are increasingly used for industrial purposes and in consumer products: Research and Markets estimates over 1.6 million tons of metal oxide NPs will be incorporated into industries and technologies by 2020 (Global Industry Analysts, 2010). As such, developing direct visualization methods to assess NP location and abundance in skin tissue, as well as other biological and environmental models, is critical. Such methods development for nanoscale materials will aid in and facilitate related research efforts, including toxicology and exposures science.

Routes of potential human exposure to ENPs include inhalation, ingestion, injection, and cutaneous (skin) exposure (Oberdörster et al., 2005). Researchers are working to assess biological effects resulting from ENP exposure, and numerous studies utilizing different models for toxicological assessment of diverse NPs are underway (Krishnaraj

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et al., 2016; Adamcakova-Dodd et al., 2014; da Silva et al., 2014; Fröhlich and Salar-Behzadi, 2014). Studies in the last decade have investigated the effects of cutaneous exposure to NPs (Schneider et al., 2009; Sykes et al., 2014; Baroli et al., 2007; Sonavane et al., 2008), primarily regarding exposure to zinc oxide (Zvyagin et al., 2008) and titanium dioxide (Adachi et al., 2013), which are commonly used in cosmetics and sunscreens. Other metal oxide NPs, such as aluminum oxide (Al_2O_3; alumina), cerium oxide (CeO_2; ceria), and silicon dioxide (SiO_2; silica), are commonly used by the semiconductor industry as abrasives during chemical mechanical planarization (CMP) polishing processes and may be accessible for worker inhalation or cutaneous exposure (Shepard and Brenner, 2014a, 2014b; Brenner and Neu-Baker, 2014; Brenner et al., 2016; Roth et al., 2015a).

In order to facilitate risk assessment for those who may be exposed to NPs, there is urgent need for faster, less expensive analytical methods that can identify, characterize, and quantify NPs in biological samples, while preserving the sample itself. ENPs in biological samples are commonly characterized and quantified through conventional methods, such as electron microscopy (EM) and spectrometry. These methods, while capable of providing valuable information in terms of identification, characterization, and quantitation, are typically costly, time-consuming, and destructive to the sample (Roth et al., 2015b; Vanhecke et al., 2014). The limitations of EM for analysis of NPs in biological samples, such as it being expensive and time consuming, have been discussed by Sosa Peña et al., (2016). While various spectrometry methods provide valuable quantitative information, other questions remain unanswered, such as the distribution of NPs within a specimen and the biological responses to NPs, which are more easily visually assessed in a histological sample. In contrast to spectrometry methods, enhanced darkfield microscopy with hyperspectral imaging (EDFM-HSI) can address these questions simultaneously, without requiring special markers or destruction of the sample.

EDFM with hyperspectral mapping has emerged as a method with high utility for rapidly identifying metal oxide and other NPs in complex matrices, including biological and environmental sample types (Sosa Peña et al., 2016; Roth et al., 2015c; Mortimer et al., 2014; Grabinski et al., 2013; England et al., 2015; Badireddy et al., 2012). While EDFM allows for simple and rapid direct visualization of high contrast structures in biological samples, the spectral angle mapper (SAM) feature of the HSI software (ENVI 4.8) offers automated spectral identification of the NPs of interest by mapping against a reference spectral library (RSL) created from a positive control sample. A spectral signature from the material of interest is created and used to identify the same material in other (experimental) samples. Once identification of the NPs is obtained through hyperspectral mapping, relative quantitative analysis is possible by calculating the pixels per area of interest or per field of view (FOV) that is occupied by the NPs of interest (Mortimer et al., 2014).

HSI has been widely utilized for clinical applications: for example, it has been used to discern between different types of carcinomas and other pathological conditions using histological samples obtained through biopsy (Darwiche et al., 2013). In these cases, the first step is to confirm the diagnosis through rigorous visual assessment by a pathologist in order to create an RSL to then map to other similar samples. The difference with NP-exposed tissues is that, up to now, there is no consensus on whether tissues present changes that are characteristic of a certain exposure; thus, the "pathologist-based diagnosis method" would not be a viable option. Moreover, and probably more importantly, when dealing with NPs, unless those NPs have a characteristic shape (e.g., multi-walled carbon nanotubes) or are an obvious addition to the sample (e.g., clear presence of NPs within the cell membrane limits and that are not present in an unexposed sample), their presence in a determined sample should be confirmed by an additional method in order to confidently create an RSL to further assess other samples (Sosa Peña et al., 2016). The case of metal oxide NP-exposed tissues is a particular one and its analysis challenges should be considered; for example, in histological samples, spherical metal oxide NPs have no uniform or distinctive shape (and may be present in aggregates/agglomerates, clusters, or dispersed as single NPs), and are often indistinguishable from common sample artifacts (Sosa Peña et al., 2016; Husain et al., 2015), thereby making the method based on a "visual determination" of NPs for the creation of RSLs an unsuitable one for this type of specimen. While the importance of utilizing a third method to confirm the presence of the material of interest for an accurate and reliable RSL for histological samples has been recently emphasized by Sosa Peña et al. (2016), this method is still rarely utilized by the HSI scientific community dealing with uncharacteristically shaped NPs in histological tissues.

Moreover, HSI has been utilized as both a semi-quantitative (Mortimer et al., 2014; Badireddy et al., 2012) and a quantitative tool (Klein et al., 2008; Aalderink et al., 2009) in both biological samples at a single cell level (e.g., protozoa) and in environmental samples (e.g., wastewater), as well as in non-biological samples (e.g., physical documents, such as 17th-century historical maps), respectively. While EDFM was utilized by Mercer et al., (2013) to quantify multi-walled carbon nanotubes (MWCNTs) in histological samples, their study neither utilized nor required HSI or mapping to achieve reliable results, since the characteristic shape of the MWCNTs permitted direct visualization and subsequent counting of structure; this, for instance, differs greatly from the uncharacteristic shape of metal oxide NPs that impedes the utilization of the same quantitation method based on visualization alone. Thus, when working with morphologically indistinct NPs, it is imperative to use both EDFM and hyperspectral mapping for identification and quantitation purposes. While "semi-quantitation" of spherical or morphologically indistinct NPs utilizing EDFM-HSI has been achieved at the cellular level as reported by Mortimer et al., (2014) where the NPs can be easily identified as dispersed high-contrast particles found enclosed within the cellular membrane, a similar quantitation of NP abundance in histological samples has not yet been achieved, as challenges arise when the NPs are found either in clusters or dispersed form, and when they do not respect cellular limits. Similarly, the methods used for complex waters (Badireddy et al., 2012), where the dispersed nature of the NPs makes it easier to identify their size and number, fail to be useful for histological samples for the reasons mentioned above. In contrast, the term "relative quantitation" is used to describe the methods presented in this study, as we compare higher or lower abundance between different groups.

For the analysis of histological samples exposed to metal oxide NPs with EDFM-HSI, we propose a method for HSI visualization, mapping, and relative quantitation of NPs that combines several approaches in an effort to strive for consistency and accuracy in sample analysis that could potentially become a much-needed standard for NP-containing histological tissue analysis. The combined approaches and techniques: 1) are similar to the traditional methods used in pathology for histological sample assessment, where the load of particles in multiple frames within a sample is averaged; 2) utilize the known methods for visual NP identification with EDFM where the properties of metal oxide NPs provide high contrast allowing for easier identification, without the need for markers or tissue destruction; 3) include confirmation of EDFM findings by assessing the presence of the NPs of interest by a third method (in this study, Raman spectroscopy and energy-dispersive X-ray spectroscopy) to create an accurate RSL (a step typically absent in other studies); 4) combine HSI and mapping of multiple samples based on an accurate and confirmed RSL; and 5) establish a method for relative quantitation of NPs in tissue samples based on mapped pixels per FOV.

While EDFM-HSI may not be in a position at present to usurp EM in terms of resolution, or spectrometry in terms of quantitation, it is capable – as a single tool – of providing a wealth of information from an intact biological sample, which could then be used for additional investigation by other methods. Additionally, it allows for faster image