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Defect Engineering of ZnO Nanoparticles for Bio-imaging Applications

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Abstract

Many promising attributes of ZnO nanoparticles (nZnO) have led to their utilization in numerous electronic devices and biomedical technologies. nZnO fabrication methods can create a variety of intrinsic defects that modulate the properties of nZnO, which can be exploited for various purposes. Here we developed a new synthesis procedure that controls certain defects in pure nZnO that are theorized to contribute to the n-type conductivity of the material. Interestingly, this procedure created defects that reduced the nanoparticle band gap to ~3.1 eV and generated strong emissions in the violet to blue region while minimizing the defects responsible for the more commonly observed broad green emissions. Several characterization techniques including TGA, FT-IR, XPS, TEM, Raman, photoluminescence and ICP-MS were employed to verify the sample purity, assess how modifications in the synthesis procedure affect the various defects states and understand how these alterations impact the physical properties. Since the band gap significantly decreased and a relatively narrow visible emissions band were created by these defects, we investigated utilizing these new nZnO for bio-imaging applications using traditional fluorescent

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Author Contributions

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ASSOCIATED CONTENT

Supporting Information. XRD patterns and TEM images of the nZnO before and after annealing, DLS size distribution, XPS scans of the nZnO post annealing, energy level diagram for the nZnO, high magnification confocal images of the new nZnO, a table of ICP-MS results, absorbance and emission spectra for quinine sulfate/nZnO, table of results for quantum yield determination, and caption for the associated NP-time series video.

microscopy techniques. Whereas most nZnO generally require UV excitation sources to produce emissions, we demonstrate that reducing the band gap allows for a 405 nm laser to sufficiently excite the nanoparticles to detect their emissions during live-cell imaging experiments using a confocal microscope. This work lays the foundation for the use of these new nZnO in various bio-imaging applications and enables researcher to investigate the interactions of pure nZnO with cells through fluorescence-based imaging techniques.

Graphical Abstract

Keywords

ZnO Nanoparticles; Bio-imaging; Defects; Fluorescence; Photoluminescence; Raman; Cancer; Toxicity

INTRODUCTION

Nanomaterials composed of ZnO are highly produced and extensively studied due to their promising attributes in numerous fields. ZnO nanomaterials (NMs) are considered for various potential cancer treatment options, water treatment applications and electronic devices.^{1–3} The flexible nature of NMs, specifically the ability to control physical properties such as size, surface chemistry, catalytic activity and defect structure, makes them suitable for many novel devices. Researchers often focus on how these properties relate to ZnO nanoparticle (nZnO) toxicity; especially as they appear to be selectively toxic towards cancer cells when compared to their normal non-transformed counter-parts.⁴ Although there has been a wealth of data collected about nZnO properties and toxicity, the mechanism of toxicity and cell selectivity remains controversial as it is not fully understood. The ability to fluorescently track nZnO within biological systems is an avenue that should be further explored as it would likely facilitate a better understanding of nZnO interactions with various cell types and cancer.

Numerous semiconducting nanoparticles (NPs) have generated great interest as biological labels due to their small size, photo-stability and relatively long fluorescence lifetimes.^{5, 6} The most traditional systems use cadmium chalcogenides as their emission is tunable

through size modulation and generally have narrow emission spectra. Unfortunately these particles are toxic towards biological systems through the release of Cd^{2+} ions; reportedly highly toxic in the nanomolar range.⁷ In contrast, nZnO display relatively low toxicity, at an even higher micro-to-millimolar range.^{8, 9} As nZnO are comparatively less toxic, their potential use in bio-imaging has received considerable attention and yet only modest progress has been made to date.

Using nZnO as a biological labelling tool is limited by the need to use UV excitation sources coupled with corresponding emission spectra of nZnO that is generally in the UV range (correlating to near band emissions of ~ 3.37 eV or ~ 368 nm) and commonly a broad green emission (~ 500 – 700 nm). True UV excitation sources (100 nm– 380 nm) are generally absent in conventional fluorescence imaging instruments with most traditional fluorescence microscopy techniques using excitation wavelengths of 405 nm or longer, which is insufficient to excite nZnO as it is below the band gap energy of ZnO. Monitoring UV emissions with standard fluorescent imaging equipment is often not possible and UV light has a relatively low tissue depth penetration, which limits the ability to study nZnO in biological systems. Additionally, the broad green emission of nZnO is theorized to originate from surface defects, such as oxygen vacancies, that can be quickly quenched by transformations in solution such as hydroxide formations and conversion to zinc carbonate/phosphate species.^{10–12} An additional complication to targeting the broad green emission region for fluorescence microscopy techniques is that emissions in this region of the spectrum is broad enough to overlap with fluorophores commonly used in cell/tissue research and the autofluorescence generated by various biological structures. Therefore, targeting the broad green emission spectra of nZnO for imaging purposes may not be suitable for many applications.

Some of the aforementioned drawbacks have been overcome through modifications of nZnO, creating novel fluorescent particles through methods such as encapsulating dyes including fluorescein isothiocyanate (FITC), coating nanoparticles (NPs) with polymers, and doping elements into nZnO to modulate the band gap.^{13–16} However, these modifications may impact other properties of nZnO, potentially altering their toxicity mechanism and making them unsuitable for specific types of studies. While these avenues may shed some light into understanding the interplay of nZnO with various cell types, conclusions drawn from these approaches do not necessarily reflect how unmodified nZnO interacts with cells.

In addition to biological applications, nZnO are well suited for optoelectronic devices. Modulating the absorbance, emission spectra and charge carriers in nZnO has been a goal for optoelectronic device applications. To these ends, one approach has been to dope various elements in the crystal lattice of ZnO,^{17, 18} while other researchers have sought to improve ZnO performance by producing highly oriented and defect-free ZnO nanomaterials. Producing ZnO materials free of defects is hard to achieve, especially at the nanoscale; some have focused their investigations on ways to reduce the defects produced when synthesizing nZnO, while others have actively pursued ways to utilize the various defects states that are inherent to ZnO.

Control of ZnO NM defect states is achieved through various means such as growth on substrates, annealing in various atmospheric conditions and via numerous deposition techniques.^{19–22} For nZnO specifically, achieving control of various defects in wet chemical methods can be challenging. Many reports have focused on the green luminescent broad band produced through differing methods and often attribute this emission to oxygen related defects, but the precise defect states or surface species responsible are still controversial, making precise control difficult due to lack of a known mechanism.^{23–25}

In this report, we pursued controlling defects of highly pure nZnO that affect the emission spectra in the violet to blue region, theoretically creating shallow donors such as zinc interstitials. By controlling these defects, the emission spectra were red-shifted and broadened, creating strong emissions up to ~430 nm. Additionally, the optical band gap was shifted down to ~3.1 eV allowing excitation sources, such as a 405 nm laser used in confocal microscopy, to produce sufficient emissions to image the particles without any other modifications. Through variations in the wet chemical synthesis procedure, we demonstrate that the described defect states can be systematically controlled. To our knowledge, this is the first report to demonstrate the ability to fluorescently image pure nZnO using a 405 nm excitation source due to defects altering the physical properties of the nZnO without any further modifications to the material.

MATERIALS AND METHODS

ZnO Nanoparticle Synthesis

The new synthesis procedure for producing nZnO was adapted from previous procedures with various modifications.²⁶ Zinc acetate dihydrate was used as the zinc source and diethylene glycol (DEG) as the solvent. To control the defects in the wet chemical synthesis, three parameters were varied; the amount of polyvinylpyrrolidone (PVP), water, and annealing temperature. The solution was created first by the simultaneous addition of PVP (molecular weight 58k) and zinc acetate, followed by the addition of DEG and initially heated to 80° C. Various amounts of water were then added, the temperature brought up to 150° C, and then held for 75 minutes. Once the solution cooled to room temperature, the samples were centrifuged at 41,410 X g to remove the DEG and subsequently washed with absolute ethanol. The pellet was dried overnight at 60° C and then annealed in air at the various temperatures indicated.

Characterization.—The nZnO samples were characterized using several techniques. X-ray diffraction (XRD) spectra were collected using a Rigaku Miniflex 600 X-ray diffractometer and used to determine the crystal phase, lattice parameters and to estimate the average crystal size using Rigaku PDXL software version 1.8.0.3. Thermal gravimetric analysis (TGA) was performed using a Netzsch STA449F1 TGA-DSC in order to define the ideal temperature and time to remove retained chemical species from the synthesis procedure. Transmission electron microscopy (TEM) was utilized to obtain both average crystal size distributions and determine the NP morphology using a JEOL JEM-2100 HR analytical transmission electron microscope. To obtain atomic concentrations and evaluate sample purity, both X-ray photoelectron spectroscopy (XPS) and Fourier transform infrared

spectroscopy (FTIR) were utilized. A Physical Electronics Versaprobe system with a monochromated Al K α X-ray source was used to obtain the XPS spectra and a Bruker Tensor 27 spectrometer was used for FTIR spectra collection. FTIR pellets were prepared by grinding 1.5 mg of the NP sample with 0.200 g of spectroscopic grade KBr and subsequently pressed for 4 minutes with 8 tons of pressure. A Malvern Zetasizer NanoZS was used to collect dynamic light scattering (DLS) measurements by using a concentration of ZnO NPs of 0.5 mg/mL in nanopure water. For inductively coupled plasma mass spectrometry (ICP-MS) experiments, the nZnO were dissolved in 25 mL of highly pure 2% nitric acid solution and analyzed by a Thermo-electron X-series II quadrupole ICP-MS. The samples were analyzed for various metal contaminations such as iron, cobalt and nickel against the total zinc content. For Raman and photoluminescence (PL) experiments, the nZnO were mounted onto a silicon oxide wafer and the spectra were measured in backscattering geometry using a Jobin Yvon T64000 0.64m triple spectrometer equipped with a liquid nitrogen cooled multichannel CCD detector. Spectra were recorded at 10 K using a variable temperature closed cycle helium cryostat. Ultraviolet light (325 nm line of He-Cd laser) was used for excitation.

ZnO Nanoparticle and Live Cell Imaging.—Confocal microscopy was utilized to image both the nZnO and T47D cells treated with nZnO. Because this new method of nZnO synthesis achieves a lower band gap of ~ 3.1 eV, a 405 nm laser was able to be used as an excitation source and nZnO fluorescence monitored.

For live cell imaging experiments, T47D (ATCC HTB-133), a cell line derived from ductal breast carcinoma (ATCC; Manassas, VA) was maintained in log phase and cultured in RPMI 1640 media following ATCC recommendations. Prior to imaging experiments, the cells were washed and resuspended in a custom made RPMI 1640 media free of HEPES, phenol red and phosphate (PPH free RPMI 1640; Thermo Fisher Scientific; Grand Island, NY) to prevent increased NP dissolution, remove fluorescent background signal and reduce the chemical transformation of the NPs, respectively.^{10, 27} The cells were then transferred to Nunc Lab-Tek II Chambered cell culture slides (ThermoFisher; Waltham, MA). Following an overnight incubation to ensure cell adhesion, nZnO were added at a final concentration of 30 $\mu\text{g/mL}$ and further incubated to allow for nZnO-cell interactions. Control (nZnO-free) cells were cultured in the same manner to assess auto-fluorescence generated using the 405 nm laser to excite the nZnO. Prior to imaging, live cells were stained with the plasma membrane stain Cell Mask Orange (Invitrogen; Carlsbad, CA). In brief, stain was added to culture media for at a final concentration of 5 $\mu\text{g/mL}$. All confocal images were acquired with a Zeiss 510 LSM system with the Zeiss Axiovert Observer Z1 inverted microscope and ZEN 2009 Imaging software (Carl Zeiss, Inc.; Thornwood, NY) utilizing Diode (405 nm) and HeNe (543) lasers as excitation sources; a Plan-Apochromat 20x/NA 0.8, Fluar 40x/NA 1.3 oil, or α -Plan Fluar 100x/NA 1.44 oil objective; and band-pass BP filters of 420–480 nm and 550–647 nm to image the nZnO particles and lipid layer, respectively. The pinhole was set to 0.95 airy units correlating to a 0.6 μm section.

Viability.—Jurkat T cells (ATCC TIB-152) were cultured in log phase using RPMI 1640 media supplemented with 10% FBS (fetal bovine serum), 1% penicillin/streptomycin and 2

mM L-glutamine per ATCC (American Type Culture Collection) recommendations. For viability assessments, the cells were first washed with PPH free RPMI 1640 and then seeded at a concentration of 2.5×10^5 cells/mL using the same media in a 96 well plate. A stock of nZnO was prepared at 4.07 mg/mL (50 mM) and sonicated for 10 minutes. The stock solution was added to fresh cellular media to achieve a final nZnO concentration of 1.63 mg/mL (20 mM) and sonicated for an additional 10 minutes. The cells were then treated at the indicated concentrations and incubated at 37° C and 5% CO₂ for 48 hours. Cell viability was assessed using the Alamar Blue metabolic assay where Alamar blue was added to the wells at a final concentration of 10% 44 hours post treatment and incubated for an additional 4 hours. The fluorescence intensity measurements were performed using a Biotek Synergy MX plate reader using an excitation/emission of 530/590 nm.

RESULTS AND DISCUSSION

ZnO Nanoparticle Synthesis and Characterization

Polyvinylpyrrolidone (PVP) is known to modify the structure and morphology of many different NMs, including metal oxide NPs.²⁸ The inclusion of PVP in synthesis procedures has been shown to act as a surfactant, stabilizing agent, dispersant, binding agent and a capping agent to obtain better dispersions of hydrophobic materials.²⁸ In this report, it was determined that the PVP content, annealing temperature and the ratio of water to zinc acetate (ZnAc) were the three key factors that affect the defect states of nZnO. The as-prepared nZnO, to which various amounts of PVP were added pre-synthesis, produced nZnO with an average crystal sizes near 10 nm in all cases (Figure S1A–C). Similar methods, though lacking PVP, have demonstrated the ability to control the average crystal size of the nZnO by varying the amount of water added during synthesis to produce nZnO with a broad range of sizes - from ~4 nm to ~50 nm or greater. The addition of PVP into the solution with the zinc precursor (2:1 PVP:ZnAc; w/w) removed the ability to control the size of the particles, regardless of the ratio of water to zinc acetate. For this reason, any changes in the physical properties of these newly synthesized nZnO are not size related effects. It is possible that PVP is coordinating the growth of the NPs in the cavities of the polymer and/or capping the particles which restrict their growth, as theorized in reports for other types of NPs.²⁸

In our procedure described here, the nZnO produced have a similar morphology to that in other reports; nearly spherical ZnO crystals that form spherical aggregates (Figure S1). One consequence of including PVP in the synthesis is that the as-prepared NPs retained some of the polymer and other species from the precursors and solvents. Annealing was performed to remove residual polymer and precursor species to obtain a highly pure nZnO sample. The Thermogravimetric Analysis (TGA) plot of nZnO synthesized with PVP (Figure 1A) demonstrates that after only 10 minutes of annealing at 450° C, no additional mass loss is evident, even when increasing the temperature to 575° C. To confirm the complete loss of retained chemical species, FTIR was utilized to evaluate the removal of the retained chemical species in the sample when annealed at various temperatures for 10 minutes. As seen in Figure 1B, after reaching 450° C, all the bands associated with the precursors and PVP have effectively been removed and even the band associated with O-H stretching group at $\sim 3400\text{ cm}^{-1}$ is almost entirely eliminated. XPS confirmed the results of the post annealed

samples, with only zinc, oxygen, and carbon atoms (associated with carbon dioxide) detected in the spectra (see SI figure S2). Since all the surface coatings and retained species from the synthesis method were annealed away after 10 minutes at 450° C, all subsequent experiments annealed the samples at 500° C for 10 minutes to ensure complete removal of impurity species unless otherwise noted.

As annealing can induce crystal growth, XRD analysis was performed and it was determined that the average crystal size was ~15 nm; an increase of only ~5 nm (See SI Figure S1D–F). The modest increase in nZnO size was confirmed with TEM. It was noteworthy that upon thermal treatment the color of the samples visibly changed from an off-white to light yellow. It was this observation that prompted further investigation into the physical and optical properties of these nZnO. Through these evaluations, we demonstrate that certain modifications to the synthesis procedure allow control of the relative number of defects present within these nZnO.

Raman spectroscopy was employed to investigate the potential changes in vibrational properties, verify the sample purity and assess defects induced during the synthesis and annealing procedure. Figure 2 shows the low temperature (10 K) resonant UV Raman spectra (325 nm excitation) for bulk ZnO and three samples of the nZnO produced with varying PVP to zinc acetate ratios. The E_1 longitudinal (LO) phonon modes are enhanced under resonant conditions. In the bulk ZnO sample, the first-order LO phonon peak appears at 584 cm^{-1} and the second-order (two-phonon) peak appears at 1164 cm^{-1} .^{29–31} Interestingly, a systematic downward shift of the LO phonon peaks was observed with increasing PVP content. The initial downward shift of the peak, when compared to the bulk nZnO sample (580 vs 584 cm^{-1}), is likely due to phonon confinement associated with the smaller particle sizes.^{32–34} However, as discussed above, all the nZnO samples produced were ~15 nm in crystal size, so the continued shift of the peak, down to 574 cm^{-1} for the sample with the highest PVP content, is not likely due to phonon confinement from decreasing crystal size. The systematic shift of the phonon peak correlates with the increasing intensity of the peak that appeared in the spectra at 470 cm^{-1} . The E^2 (High) phonon mode that should appear at 437 cm^{-1} was ruled out as being responsible for the peak found at 470 cm^{-1} , due to the large frequency difference, the fact that it was not observed in the bulk ZnO and is not commonly seen in resonant Raman spectra of ZnO.^{31, 35} As we noted, the intensity of this peak increased with increasing PVP content. We attribute this peak to defect(s) present in the nZnO because all the residual impurities retained from the synthesis procedure were removed during the annealing process. Increasing amounts of defects would also explain the continued shift of the LO phonon modes to lower wavenumbers. As the defect concentration increases, the phonon mean free path would decrease due to scattering by defects, therefore shortening the effective phonon confinement length and inducing the observed systematic shift of the peak,³⁶ similar to size related phonon confinement. To further investigate the defect(s) responsible for the origin of the peak found at 470 cm^{-1} , photoluminescence was employed.

Photoluminescence (PL) is often utilized to assess nZnO-associated defects as they produce various energy states and optical transitions that are within the band gap region. Numerous papers have reported on how the different emission peaks relate with various defects^{35, 37–42},

but many of the conclusions remain controversial. Oxygen related defects are often present in ZnO and are believed to be responsible for the broad green emission (~500–700 nm) seen in PL. In our attempt to determine the origin of the defects present in these samples, low temperature (10 K) PL was collected and associated with the energy transition modes commonly reported in the literature. Given the systematic shift of the LO phonon modes and the new peak that was observed in the Raman spectra, PL was first collected for samples that have various ratios of PVP to zinc acetate (PVP:ZnAc; w/w), while water content and annealing temperature were held constant. As seen in Figure 3A, varying the ratio of PVP systematically altered the PL emission spectra as it did in Raman spectroscopy. Modest changes were noted in the lower PVP:ZnAc ratios, but a significant broadening of the emission peak evolved as the ratio increased indicating an increased number of defects present that correlates to the PVP content used in the synthesis procedure. At the highest PVP ratios, the difference in PL emissions was negligible. As such, subsequent synthesis studies varying the annealing temperature and water content utilized the 2:1 PVP:ZnAc for the synthesis.

Utilizing the nZnO sample produced with a PVP to zinc acetate ratio of 2:1, additional PL spectra were obtained to evaluate how the changes in surface chemistry, from annealing at various temperatures, impacted the emissions of the sample. As seen in Figure 3B, the as-prepared sample's PL spectrum has a peak centered at ~365 nm, which is potentially influenced by the retained species from the precursors on the surface of the particles. However, even after annealing at 350° C, the temperature at which the majority of the impurities were removed (Figure 1), the PL emission has an apparent red-shift and the peaks are better defined. Once annealing temperature reaches 400° C, the PL emission peak starts to broaden and extends into the visible region indicating the emergence of defects, possibly by diffusion of zinc ions that were originally associated with the retained PVP. Since the samples annealed at or above 450° C are highly pure nZnO, the origin of the visible emission is most likely due to defects in the crystal structure and not from interactions between ZnO and retained chemical species from the precursors. At annealing temperatures above 500° C, only modest changes are observed in the violet/blue range of the PL spectra, but the broad defect-related green emission peak around 575 nm, often attributed to oxygen vacancies,⁴³ becomes evident. Since the focus of this report is primarily to obtain a highly pure nZnO, relatively free of defects related to the broad green emission (oxygen vacancies), all subsequent samples were made with a 2:1 PVP:ZnAc ratio and annealed at 500° C.

The final component varied in the synthesis procedure was the volume of water added to the solution (Figure 3C). According to published reports on a similar method, the precise control of the nZnO crystal size is dependent upon the ratio of water to zinc acetate,²⁶ as water is a source for the oxidation of the zinc ions during the Ostwald ripening process. As discussed previously, there were no changes to the crystal size found by varying the amount of water added during this synthesis procedure and therefore we ruled out effects due to alterations in the crystal size. Surprisingly, it was observed that the ratio of water to zinc acetate had a more significant impact on the formation of defect states than the ratio of PVP to ZnAc or the annealing temperature (Figure 3C). Low ratios of water in the synthesis procedure create a zinc-rich environment, potentially allowing for the production of intrinsic defects such as interstitials and antisites, as well as large portions of the crystal surface terminating in zinc

atoms. A systematic change in the PL emission was demonstrated but it was noted that increasing amounts of water drastically reduced the strong emission near and above 400 nm. The single emission peak at 368 nm, which correlates with the commonly reported near-band-edge emission, is the only significant peak noted at higher water to ZnAc ratios. Its presence could be due to the reduction of zinc to oxygen ratios, creating a more balanced zinc to oxygen environment, therefore, allowing for better oxidation and formation of the crystals. From these results, we utilized the particles made with a 1:5 water to zinc acetate (w/w) ratio for fluorescence imaging studies.

The systematic control of PL emission spectra associated with defects present in our nZnO samples was achieved by varying the PVP content, annealing temperature and water ratios. Numerous batches of NP samples were produced by this method in order to ensure reproducibility of the desired material properties based on the synthesis conditions. To demonstrate the significant changes in the defect content utilizing this new method, the PL emission spectra of other ZnO NPs, synthesized using a variety of methods, were also plotted and compared with our nZnO sample found to contain the strongest emissions in the violet/blue region (Figure 3D). A good majority of papers published have focused on the defects related to the broad green emissions observed near 575 nm in nZnO, as seen in the spectra of ZnO NPs produced using ethanol as a solvent (Figure 3D). This emission is generally attributed to oxygen vacancies and several of these reports focus on the origin of this feature and ways to induce its formation. However, as the inclusion of PVP largely restricts the formation of the defects responsible for this broad peak in this synthesis procedure, we chose to further investigate the emission in the 365–450 nm range induced by this method.

To get reasonably well assigned peak positions, PL emission spectra with well-defined peaks were first deconvoluted (Figure 4A). These peak positions were then assigned to the spectra generated from the nZnO that contained a relatively large number of defects and broad peaks in the violet to blue region. As seen in Figure 4A, only four significant peaks were found that contribute to the emissions of the sample produced with a low number of defects. The main peak at 3.36 eV (369 nm) is related to the band gap of the material and the peak found at 3.32 eV (374 nm) is attributed to free excitons.³⁹ The peak found at 3.24 eV (383 nm) has been reported as a phonon replica of the exciton peak and the last peak found at 3.07 eV is often attributed to zinc vacancies. In contrast, the sample with a relatively high number of defects (Figure 4B) was determined to contain seven different peaks. The main contributors to the broadening of the emissions were found at positions that have been reported to be related to transitions involving zinc interstitials and zinc vacancies. The three new major peaks near 3.14 eV, 3.07 eV and 2.97 eV are often attributed to transitions from zinc interstitials to the valence band, the conduction band to zinc vacancies and the conduction band to oxygen interstitials respectively.³⁹ It is also possible that some of these emissions may arise from interface traps as reported for ZnO thin films⁴⁴, as the crystals are bound together in aggregates. There has been no clear consensus on which defects are responsible for the observed emission in this region. A report employed both PL and positron annihilation spectroscopy, which found both techniques to be well correlated when assessing defects states in ZnO.⁴⁵ This paper evaluated ZnO with a similar violet-emissions with the main peak centered at ~434 nm and related the peak to zinc interstitials and zinc vacancies.

Various experimental procedures have assigned specific peak positions to the various native defect states, whereas reports relying on theoretical calculations have demonstrated that some of these defects are unlikely to occur due to diffusion rates and the required formation energies.^{23–25} However, with the numerous reports relating PL spectra to the various defects, it is likely that zinc interstitials are the major contributor to the strong emissions observed near 400 nm. Regardless of the origin of the defects, many are regarded as donors in the n-type conductivity of ZnO. Since a relatively high number of these defects are present, we wanted to evaluate how these defects affect the band gap of our new samples.

UV-Vis diffuse reflectance spectra were recorded to determine the influence these defects have on the optical band gap of the material. For reference, nZnO produced using the most similar reported method was plotted against some of the new samples. As can be seen in Figure 5, the samples produced using different ratios of PVP and annealed at 500° C induce a significant shift of the band gap. The ZnO NP sample produced using a similar synthesis procedure, though lacking PVP and annealing, produced nZnO with a band gap estimated at ~3.31 eV, which is close to the commonly expected value of 3.37 eV. This sample was chosen for comparison as it utilizes a similar synthesis procedure, which produced NPs with nearly identical crystal and aggregate sizes, therefore ruling out band-bending effects due to changes in the particle size. However, the samples produced with the new method that have relatively high concentrations of defects, created a dramatic shift of the band gap to as low as 3.08 eV. This change of almost 0.3 eV from the commonly accepted value of the band gap is usually obtained through doping of other elements into the crystal lattice.

To verify the lack of metal impurities that may contribute to the observed shift of the band gap, ICP-MS measurements were performed. When compared to the ~2500 ppb of zinc ions from the nZnO, less than 1 ppb of metal contamination was detected (See SI Table S1). Since FTIR, XPS, and ICP-MS all demonstrate the lack of impurities, coupled with the evaluation using Raman and PL, we concluded that the alterations in the properties of these new nZnO are related to defects induced by this synthesis method and are not related to changes in the crystal size, retained chemical species or impurities.

While many have demonstrated band gap modulation through doping, others have also shown negative Burstein-Moss effects or band gap narrowing induced by oxygen related defects in nZnO.⁴⁶ With the lack of impurities found in the nZnO described in our report, the band gap narrowing (Figure 5) is attributed to a large number of defects present in the crystal structure, most likely zinc interstitials. The PL spectra in Figure 4b further strengthens this argument as the major peak is situated near 3.1 eV. Since a large number of defects are present, the additional energy transition modes that generated the observed violet-blue emissions changed the density of states for these nZnO and lowered the optical band gap. While these changes in the electronic band structure altered the optical band gap of the material, the higher energy transitions are still present, creating the broad emissions peak that extends from the UV region up to ~2.8 eV (~440 nm) observed in the PL experiments. Figure S4 depicts an energy level diagram of the various transition modes believed to be present in these nZnO. With the dramatic shift of the optical band gap from these defects and since a relatively narrow emission was created in the visible region, studies were performed utilizing these new nZnO for bio-imaging applications.

Fluorescence Imaging

Fluorescence microscopy was employed to assess whether the new nZnO could be imaged based on their changes in optical properties and the observed shift of the optical bandgap to ~3.1 eV (400 nm). Confocal microscopy is commonly used in biological imaging and the instrument is generally configured with a 405 nm laser to image fluorescent dyes such as DAPI or Hoechst that stain the nuclei of cells. A 405 nm excitation source is generally the highest energy of light available with most conventional fluorescent microscopes which limits the ability of nZnO to be fluorescently tracked as the band gap of ZnO is typically near 3.37 eV. Since the optical band gap of these new nZnO is near 400 nm, we attempted to use the 405 nm laser as an excitation source to fluorescently image these new nZnO. Figure 6A shows the emission spectra recorded using the confocal microscope. When compared to the broad green emission spectra typically seen in nZnO containing a large number of oxygen vacancies, a relatively narrow emission peak was recorded for this new nZnO and is easily distinguishable from other commonly used fluorescent stains. To demonstrate that the fluorescence stems from the NPs, fluorescence and bright field images were collected by individual detectors and overlaid (Figure 6B–D). Additional higher resolution and magnification images of the nZnO are available in the supporting information (Figure S5).

The toxicity of nZnO could be a potential obstacle for its use as a potential bio-imaging tool as ZnO has been shown to be toxic to a variety of cell types when produced at the nanoscale. With the changes to the physical properties of these new nZnO, toxicity evaluations were performed to assess concentration at which they become toxic to cells. As seen in Figure 7, concentration up to 100 μ M showed no apparent toxicity to Jurkat cells after 48 hours of treatment. This is in stark contrast to cadmium-based quantum dots which have been shown to be toxic towards cells in the nanomolar range.⁷ In addition to potential bio-label applications these new nZnO may allow for investigations into the controversial toxicity mechanisms of nZnO by tracking their interactions with cells and may help decipher the reason(s) behind their demonstrated cell type selective toxicity.

Another obstacle to the development of new imaging modalities is photobleaching. Photobleaching is the irreversible alteration of a dye or fluorophore leaving it unable to fluoresce and is a common issue in immunofluorescent imaging. The emergence of fluorescent quantum dots, such as CdSe, demonstrate that NMs hold certain advantages over other more traditionally used fluorescent stains, such as their narrow emission spectra, longer fluorescence decay time and their ability to resist photobleaching.⁵ In order to assess the ability of our new nZnO to resist photobleaching, their fluorescent signal was monitored over time during continuous exposure to laser light. Specifically, nZnO were excited with a 405 nm laser for twenty minutes and single plane images were acquired every minute. Time series were collected utilizing different objectives, and regardless of the magnification, no changes in fluorescence were detected (See Figure 8). Since the images might appear to be replicates, each time series was exported into a video format to further demonstrate the stability of the NPs over time (See SI Video). High magnification and resolution images (Figure S5) demonstrate that the large agglomerates seen are comprised of the smaller aggregates seen in the TEM images (Figure S1). Additionally, absorbance, fluorescence

emissions and quantum yield measurements were performed and are available in the supporting information (Figure S6 and Table S2).

Some of the problems associated with using nZnO in bio-imaging are due to the broad green emission peak associated with surface defects. When introduced into biological media, various components such as carbonates and phosphates react with the surface of nZnO^{10–12, 47, 48} and can potentially reduce or completely remove the fluorescence associated with surface defects. In our new nZnO, the defects in this system can persist throughout the crystal lattice, such as zinc interstitials, and perhaps stabilize their ability to fluoresce even in cellular media. Having a pure, unmodified and naturally fluorescent nZnO sample that is excitable with a laser source commonly available in fluorescent microscope systems can expand the use of nZnO in fluorescence imaging studies. In addition, a stably fluorescent nZnO will also facilitate research focused on understanding nZnO interactions with living systems.

To demonstrate the ability to image nZnO in cellular media conditions, live-cell imaging was performed using T47D breast cancer cells. A stock solution of nZnO was prepared by sonicating 4.07 mg/mL of nZnO in nanopure water for 20 minutes. The cells were treated with 30 µg/mL of nZnO and incubated for either 2 or 24 hours. Cells were then stained with CellMask Orange Plasma Membrane for 30 minutes prior to imaging. For a control, untreated T47D cells were stained with CellMask Orange only and imaged using the same configuration and laser settings in order to rule out any autofluorescence generated by the cells in the nZnO channel. As seen in Figure 9, the untreated cells have little to no apparent fluorescence in the nZnO channel. After 2 hours of incubation, a few nZnO are seen in the same plane as the cells and appear to have begun the agglomeration and sedimentation process commonly seen with NPs,^{49–51} causing association with the lipid membrane on the surface of the cells. No apparent changes in cellular morphology were noted at this time point. Surprisingly, after 24 hours of nZnO exposure, no particles were found near the exterior of the cells, and in stark contrast to the fluorescence observed at 2 hours, the only fluorescence detected appears to be from nZnO that have been internalized within the plasma membrane. It is unlikely that every NP was internalized by the cells, yet no particles could be found in the exterior. It is possible that the non-internalized nZnO underwent dissolution or transformed into zinc carbonate or hydroxide species and were no longer fluorescent. However, many NPs were still detectable in the interior of the cells after 24 hours and may resist degradation or transformation upon cellular internalization. Another noteworthy feature from this experiment was that the morphology of the T47D cells changed dramatically. The cells rounded up and appear to be detaching from the culture plate. Furthermore, cells showed evidence of blebbing; the bulging of the cell membrane, characteristic of cytoskeletal breakdown that accompanies apoptosis.

Despite the number of studies that investigate nZnO, many questions remain regarding toxicity mechanisms and their inherent selectivity towards cancer cell destruction. Some of the more commonly cited mechanisms of nZnO toxicity has been attributed to ROS generation, particle dissolution, and to a lesser extent, membrane damage.^{1, 52–55} Since there is still controversy on the primary source of toxicity, the unmodified fluorescent nZnO described here may help elucidate the various toxicity mechanism(s) and give insight into

why reports demonstrate cell type selective toxicity. Even after 24 hours post treatment, a nZnO fluorescence signal was still detectable in the interior of the cells and may suggest NP internalization is key to asserting the NPs toxic effects. However, even though these interesting observations were made, the source(s) of the changes in cellular morphology were not deciphered and is the goal of future projects. Utilization of zinc ion specific dyes may allow for simultaneous tracking of both nZnO and the release of zinc ions from nZnO dissolution to help elucidate the complex nature of these interactions. Other specific labels, such as fluorophores specific to various endocytic processes and ROS sensitive dyes, would be keys to understanding the interactions of the nZnO with cells but is beyond the scope of this paper. Other future work will investigate reducing the size of the aggregates through additional modifications to the synthesis procedure and through mechanical processes such as utilizing a ball mill, stabilizing the particles in solution and conjugating antibodies to the surface of the NPs to facilitate their use as a potential bio-label for targeting specific cellular compartments.

CONCLUSION

Achieving systematic control of native defects in ZnO NPs can often be challenging, especially in wet chemical methods. This work demonstrates the ability to control specific defect-states of nZnO through modifications to the synthesis that modulate key properties of the material. Other reports have also targeted the defect related blue emission in nZnO and have demonstrated the ability to produce blue emitting nanoparticle NPs when excited.^{56–59} While these particles have utility in a variety of applications, most of these reports show that impurities are still present, mainly from the precursors or capping agents. To our knowledge, none have demonstrated a reduction in the band gap allowing for non-UV excitation sources to produce sufficient emissions to fluorescently image the NPs. A recent review article surveyed numerous papers about the luminescent properties and biological applications of nZnO.⁶⁰ Multiple novel approaches to creating luminescent particles, including defect related emissions have been reported. However, many of these particles required modification through different capping agents, doping and conjugations. Notably, many of them still required the use of UV excitation to produce emissions.

The combination of the changes to the physical properties that arises from the relatively high number of defects, likely zinc interstitials, allows these new nZnO to be used in ways not previously described. The reduction of the band gap to ~3.1 eV and the shift of the emission spectra to the visible region allowed for imaging of the NPs using traditional fluorescence microscopy techniques without any other modification such as surface coatings, doping or labeling with fluorescent stains. The ability to perform live cell imaging with essentially pure nZnO may help in deciphering their complex interactions with various cells types and understanding their cancer cell selective toxicity. Many of these interactions have relied upon end-point effects and imaging pure nZnO is normally done through TEM based experiments. This opens the door for new methodologies to track nZnO interactions with live cells in real-time.

As a potential bio-label, the relatively low toxicity of nZnO compared to other quantum dots such as cadmium chalcogenides may also make them more suitable for fluorescence

imaging with live cells. However, similar to other quantum dots, further research will be needed to effectively use them as a bio-label such as increasing their stability in aqueous solution and surface conjugation to effectively target them to specific cellular structures. Since the defects responsible for the observed changes to the NP properties are believed to be related to zinc interstitials and not necessarily surface defects, including a passivation layer on the surface may further increase the stability of these new nZnO in cellular media conditions without interfering with the species responsible for their fluorescent emissions. Additionally, the alterations to the physical properties discussed in this report may also be exploited in a variety of other applications such as optoelectronic devices. Further research is needed to fully investigate their utilization in these various fields but, based on these studies, there is promise for them to be used for numerous applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

NP	nanoparticles
NM	nanomaterials
nZnO	ZnO nanoparticles
TGA	thermogravimetric analysis
XRD	X-ray powder diffraction
XPS	X-ray photoelectron spectroscopy
ICP-MS	inductively coupled plasma mass spectrometry
TEM	transmission electron microscopy
FITC	fluorescein isothiocyanate
DEG	diethylene glycol
PVP	polyvinylpyrrolidone
FTIR	Fourier transform infrared spectroscopy
PPH free RPMI 1640	HEPES, phenol red and phosphate free RPMI 1640

ZnAc	zinc acetate
LO	longitudinal
PL	photoluminescence

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Figure 1.

(a) Thermogravimetric Analysis plot of nZnO synthesized with Polyvinylpyrrolidone (PVP) demonstrating the mass loss is complete after 10 minutes of annealing at 450° C. (b) FTIR spectra of pure PVP, the as-prepared (non-annealed) nZnO and nZnO annealed at various temperatures for 10 minutes. FTIR data confirms that after 10 minutes of annealing at 450° C that no other chemical species from the precursors are retained with the NPs.

Figure 2.

(a) Low temperature (10 K) resonant Raman spectra (325 nm laser) of bulk ZnO and the nZnO synthesized with various amount of PVP. Spectra shows a systematic shift of the LO phonon modes as the defect related peak centered at 470 cm^{-1} increases in area. (b) Zoomed in spectra of the defect related peak and the 1 LO phonon mode to highlight the systematic shift of the peak as the defect related peak increases in area. This systematic shift is likely due to phonon confinement due to increases in defects present in the ZnO NPs.

Figure 3.

Low temperature Photoluminescence (PL) spectra of the nZnO synthesized by varying different parameters in the synthesis procedure. (a) PL spectra of the nZnO synthesized with various amounts of PVP while all other parameters are kept constant. At low PVP:Zinc Acetate (ZnAc) ratios, little change in the PL spectra is noted. As the amount increases, the well-defined peaks broaden and extend into the visible spectrum. (b) PL spectra of the nZnO annealed at various temperatures. The unannealed sample spectra extends further into the UV range, likely due to the presence of various retained species from the synthesis procedure. As the annealing temperature increase, the main peak in the spectra red-shifts and broadens out to ~450 nm. At the highest temperature used, the broad green emission spectra, commonly reported on nZnO, becomes apparent. (c) PL spectra of the nZnO with various amounts of water added in the synthesis. At relatively high additions of water, the main broad peak narrows likely due to changes from a zinc rich environment to a more balanced oxygen to zinc environment. (d) PL spectra of ZnO NPs synthesized using various methods (ethanol as the solvent, Flame spray pyrolysis (FSP), diethylene glycol as the solvent (DEG) and micron sized (Bulk) ZnO) to demonstrate the extreme difference of the new nZnO compared to other commonly used methods.

Figure 4.

Deconvolution of the low temperature photoluminescence spectra of nZnO synthesized with (a) no PVP and (b) a 2:1 (w/w) PVP to zinc acetate ratio. (a) The NPs with low defect states and well-defined peaks were first deconvoluted to assign positions and acquire reasonable peak widths for the near band edge related emissions. (b) The nZnO sample with a relatively high number of defects shows multiple peaks related to various defects formed during the new synthesis procedure.

Figure 5.

UV-Vis spectra of the new nZnO synthesized with various amounts of PVP compared to the most similar reported method using DEG as a solvent. The UV-Vis spectra were converted into a Tauc Plot (inset) to determine the optical band gap of each sample. The DEG method produced nZnO with an optical band gap of ~3.31 eV whereas the new method shifted the optical band gap to below 3.1 eV.

Figure 6.

(a) The emission spectra of the nZnO recorded with a confocal microscope using a 405 nm laser as an excitation source. Using a 100x objective, the fluorescence image (b) of the nZnO and bright field (c) images were collected and overlaid (d) to demonstrate that the fluorescence is from the novel nZnO.

Figure 7.

Viability profile of Jurkat cells after treatment with nZnO for 48 hours at various concentrations. No apparent toxicity is present for concentrations up to 100 μ M.

Figure 8.

Times series fluorescent images of the nZnO over a 20-minute period. The NPs were subjected to 20 minutes of laser exposure and no apparent decrease in fluorescence intensity was noted, demonstrating their resistance to photobleaching. Like quantum dots, the fluorescence stems from the physical properties of the NPs and is proposed to stem from transitions between energy levels due to the defect present in the samples.

Figure 9.

Confocal images (optical plane thickness= 0.6 μm) of T47D (breast cancer) cells stained with CellMask Orange. The top row (a-c) depicts untreated T47D cells to assess any auto-fluorescence generated using the same laser settings in the 405 nm laser channel. The second row (d-f) depicts the T47D cells after 2 hours of nZnO treatment. Little to no change in cell morphology is noted. The bottom row (g-i) depicts T47D cells after 24 hours nZnO treatment. The cellular morphology is drastically changed, many blebs are noted, and essentially all NPs that are detectable appear to have been internalized.