APPLICATION NOTE

Spectral signature analysis of surface functionalized nanoparticles

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Introduction

Nanotechnology is a rapidly developing field that has caught the interest of the scientific community due to its potential applications in biomedical research. Nanomaterials are typically less than 100 nm in diameter, making them small enough to penetrate mammalian cells. Nanomaterials can be synthesized in many shapes, such as rods, tubes, and particles, as well as in varying elemental compositions such as metals, metal oxides, and combinations of these. Their large surface area to volume ratio makes them suitable for surface functionalization, allowing for attachment of targeting or therapeutic molecules. When nanoparticles are delivered systemically, attached targeting molecules enable detection of certain cell populations, such as tumor cells, while attached therapeutic compounds can act on the targeted cells.

The material a nanoparticle is composed of has a specific band gap, or distance between the ground and excited states of its electrons. Generally, an electron exists in its ground state, or lowest energy state. Upon absorption of photons or light energy, the electron moves to its excited energy state. The distance between the ground state and the excited state is known as the band gap. The nanoparticle material absorbs one or more specific wavelengths, with some of the absorbed energy lost as vibrational energy and the remaining excess energy emitted as fluorescent light, returning the electrons to their ground state. A unique spectral signature can be obtained by plotting the relative fluorescence intensity across a range of different excitation and emission wavelengths.

Currently there are a limited number of techniques available to characterize nanoparticles and their molecular interactions. Here we propose spectral signature analysis as a method to confirm interactions between nanoparticles and surface coating molecules. Upon surface interaction with another molecule, the electronic properties of the nanoparticle material change, resulting in a shift in the peak fluorescence excitation and emission wavelengths, or spectral signature. Comparing the spectral signatures of surface coated nanoparticles and their uncoated counterparts can reveal a spectral signature shift indicative of electrostatic interaction.

Here we show how spectral signature analysis is performed using the SpectraMax® i3x Multi-Mode Microplate Reader and Spectral Optimization Wizard in SoftMax® Pro Software.

Benefits

- **Easily detect changes in spectral properties of uncoated vs. coated nanoparticles**
- **Monitor surface functionalization of nanoparticles**
- **Automatically analyze spectral signatures with the Spectral Optimization Wizard**

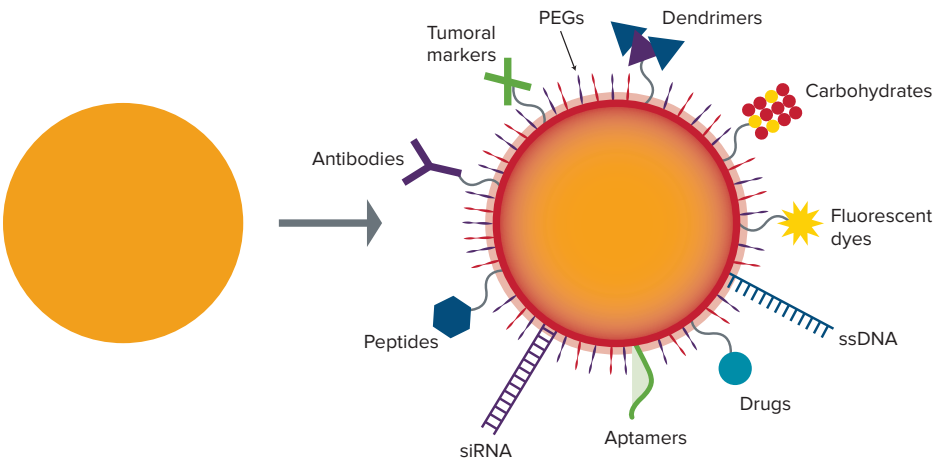

Figure 1. Diagram of surface functionalization of nanoparticles. Left: original nanoparticle. **Right:** nanoparticle with surface functionalization allowing the attachment of a variety of targeting molecules including drugs, antibodies, and nucleic acids.

The Spectral Optimization Wizard enables automatic scanning of a user-defined range of excitation and emission wavelength pairs. The resulting fluorescence values for each wavelength pair are plotted as a heat map with a 'hot spot' that indicates the wavelength pair producing the highest signal relative to a control. These hot spots can be compared for different samples to identify a spectral shift.

Materials

- Iron (III) oxide nanoparticles (PlasmaChem cat. #PL-FeO)
- Zinc oxide nanopowder, <100 nm size (Sigma Aldrich cat. #544906-10G)
- Methoxy poly(ethylene glycol) (mPEG 5000, Sigma Aldrich cat. #81323-250G)
- Ultra-pure water
- 96-well solid black microplate (Greiner cat. #655076)
- SpectraMax i3x Multi-Mode Microplate Reader

Methods

Nanoparticle preparation

Iron oxide nanoparticles (Fe_2O_3 NP) were weighed out on an analytical balance at 2 mg in a microcentrifuge tube and suspended in 1 mL of ultra-pure water to create a final stock concentration of 2 mg/mL. This stock concentration was vortexed to disperse the particles. To obtain a final sample concentration of 1 mg/mL, 100 µL of the stock was transferred to a new microcentrifuge tube, and 100 µL of ultra-pure water was added to make a nanoparticle control sample. This 200-µL sample was vortexed and transferred to one well of a black 96-well microplate.

Zinc oxide nanoparticles (ZnO NP) were weighed out on an analytical balance at 3.5 mg in a microcentrifuge tube and suspended in 1 mL of ultra-pure water to create a stock concentration of 3.5 mg/mL. This solution was vortexed and 57 µL of the stock solution was transferred to a new microcentrifuge tube. To this tube, 143 µL of ultra-pure water was added to bring the volume to 200 µL and the final sample concentration to 1 mg/mL. The sample was vortexed and transferred to one well of a black 96-well microplate as a nanoparticle control.

	Fe_2O_3 NP +/- mPEG	ZnO NP +/- mPEG
Optical configuration	Monochromator	Monochromator
Read mode	Fluorescence	Fluorescence
Read type	Spectrum	Spectrum
Wavelengths	Excitation: 260 nm Emission start: 295 nm Emission stop: 750 nm Step: 5 nm	Excitation: 350 nm Emission start: 375 nm Emission stop: 750 nm Step: 5 nm
Read height (mm)	1	1
Flashes/read	6	6
PMT & optics	Auto	Auto
Read area	Top	Top

Table 1. Settings for preliminary spectral scans in SoftMax Pro Software.

Optical configuration	Monochromator
Read mode	Fluorescence
Read type	Endpoint
Wavelengths	Unknown

Table 2. Settings for prompting initiation of the Spectral Optimization Wizard in SoftMax Pro Software.

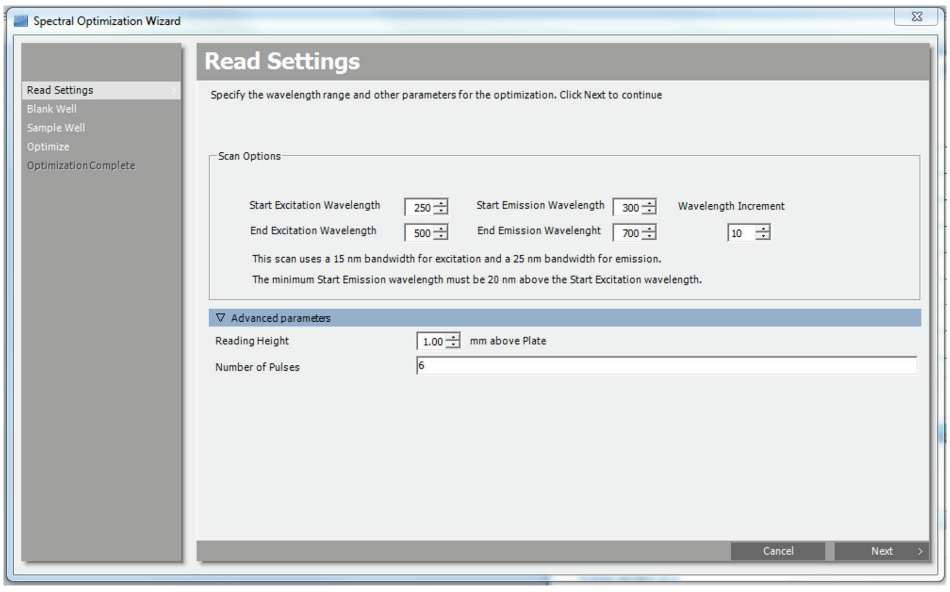

Figure 2. Spectral Optimization Wizard settings for nanoparticles. Settings used for both Fe_2O_3 and ZnO NP samples with and without mPEG. For the ZnO NP samples, a wavelength increment of 5 nm was used.

Nanoparticle surface functionalization

To two new microcentrifuge tubes, either 100 µL of Fe_2O_3 NP stock solution or 57 µL of ZnO NP stock solution was added. On an analytical balance, mPEG was weighed out to 4 mg and suspended in 1 mL of ultra-pure water for a final concentration of 4 mg/mL. This stock solution of mPEG was vortexed, and 50 µL was transferred to each microcentrifuge tube. The samples were brought up to a final volume of 200 µL by adding 50 µL of ultra-pure water to the Fe_2O_3 NP-mPEG sample and 93 µL of ultra-pure water to the ZnO NP-mPEG sample. The samples were then vortexed and transferred to a black 96-well microplate. Samples were incubated in the microplate for 30 minutes prior to reading to ensure surface coating.

Fluorescence detection

Fluorescence of samples in the 96-well microplate was detected on the SpectraMax i3x Multi-Mode Microplate Reader using the settings shown in Table 1. A preliminary fluorescence spectral scan was performed using an excitation wavelength of 260 nm for Fe_2O_3 NP and 350 nm for ZnO NP with and without mPEG. Emission was measured from 295 nm to 750 nm at 5 nm intervals for Fe_2O_3 NP samples, and from 375 nm to 750 nm at 5 nm intervals for ZnO NP samples (Table 1). The microplate was shaken for five seconds using the orbital setting at high speed prior to reads.

Following this initial validation, the Spectral Optimization Wizard (SOW) was used to perform a series of fluorescent reads with user-specified excitation and emission wavelength ranges, from which an optimal wavelength pair was identified for each sample. The SOW was initiated by selecting the settings shown in Table 2. When Read is selected, a new dialog box appears in which the user selects a range of excitation and emission wavelengths to test. The range of excitation wavelengths to scan was set to 250-500 nm, and the range of emission wavelengths to scan was set to 300-700 nm. For Fe_2O_3 NP samples, 10 nm steps were selected, and for ZnO NP samples 5 nm steps were used. For all samples, the default read height of 1 mm was used. The setup dialog box is shown in Figure 2.

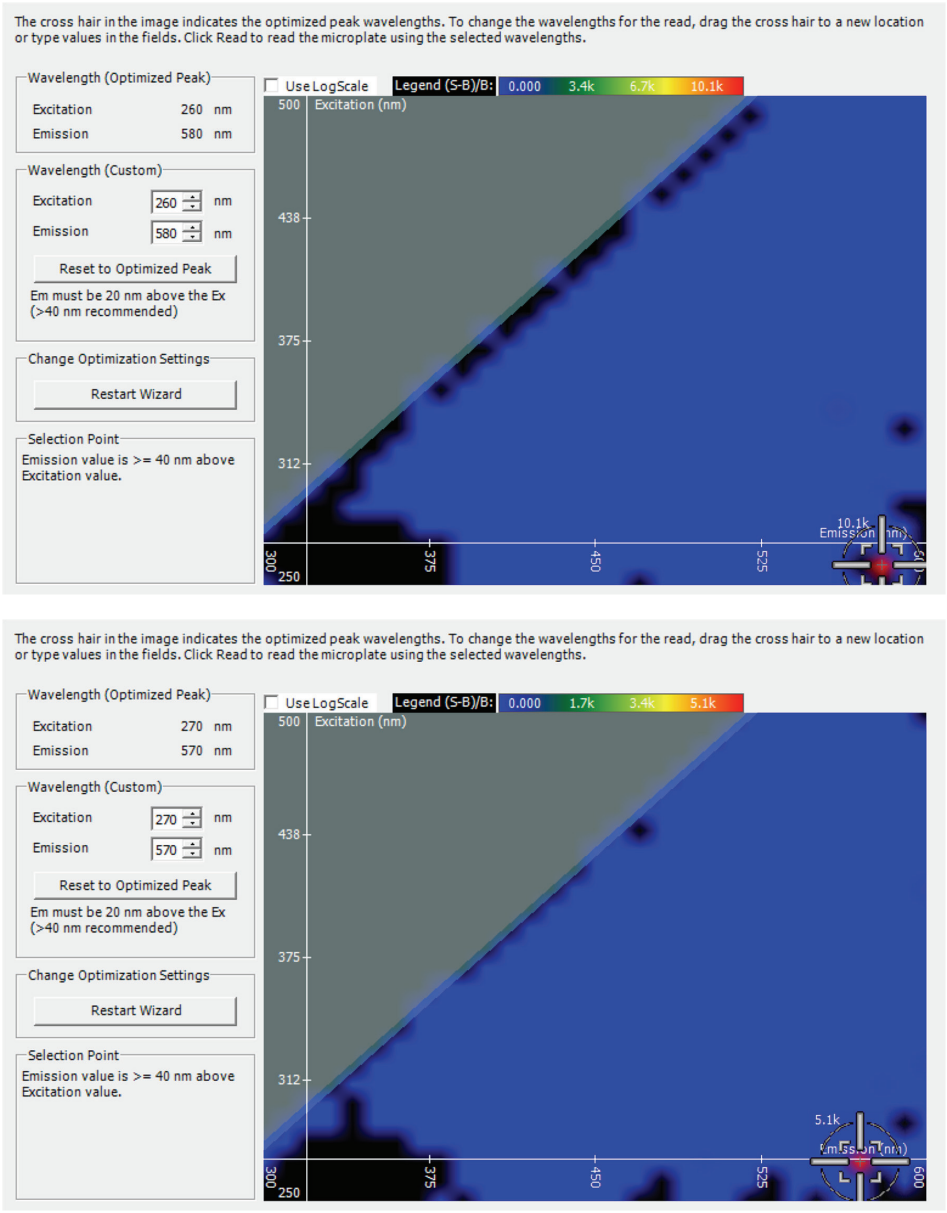

Figure 3. Spectral signature of Fe_2O_3 NP (top) and mPEG surface functionalized Fe_2O_3 NP (bottom). The optimal wavelength pair identified by the software appears as a red 'hot spot' on each heat map of excitation vs. emission wavelengths. The gray areas of the heat maps represent non-feasible wavelength combinations that are avoided by the software. Black areas represent readings where fluorescent signal was very close to background values.

Data acquisition

The Spectral Optimization Wizard was designed to identify a sample's optimal excitation and emission wavelengths and then use these wavelengths in a subsequent plate read. The software does not automatically save the heat map and its associated fluorescence values as data. However, while the heat map window is still open, this raw data can be copied and pasted by right-clicking on the heat map and selecting 'Copy Raw Data', then pasting the data into the desired software. If the data are pasted into a spreadsheet, the excitation and emission wavelengths used in the spectral optimization must be entered manually, as they are not exported automatically with the raw data values. The original heat map image produced by SoftMax Pro Software can be saved for reference by right clicking it and selecting 'Save Image As'.

Results

Fe_2O_3 NP displayed a spectral signature at an excitation of 260 nm, emission of 580 nm, and a fluorescence intensity of 10.1K relative light units (Figure 3, top). In the presence of mPEG, Fe_2O_3 NP exhibited a spectral signature with an excitation of 270 nm, emission of 570 nm, and a fluorescence intensity of 5.1K (Figure 3, bottom). Fe_2O_3 NP was used as a negative control, where a 10 nm shift in excitation and emission wavelengths was observed in the presence of mPEG. However, due to the fact that 10 nm steps were used to derive those spectral signatures, the shift is negligible and rather a result of measurement variation. However, the decrease in fluorescence intensity by approximately half suggests some minor interaction, even if it associates and dissociates at equilibrium, causing the fluorescence quenching to take place.

Comparatively, ZnO NP showed a spectral signature with an excitation wavelength of 390 nm, emission at 670 nm, and fluorescence intensity of 13K relative light units (Figure 4, top). In the presence of mPEG, ZnO NP gave a spectral signature with an excitation wavelength of 380 nm, emission at 695 nm, and a fluorescence intensity of 95.6K relative light units (Figure 4, bottom). ZnO NP displayed a 25 nm shift in the emission wavelength of the spectral signature in the presence of mPEG when 5 nm steps were used. This is indicative

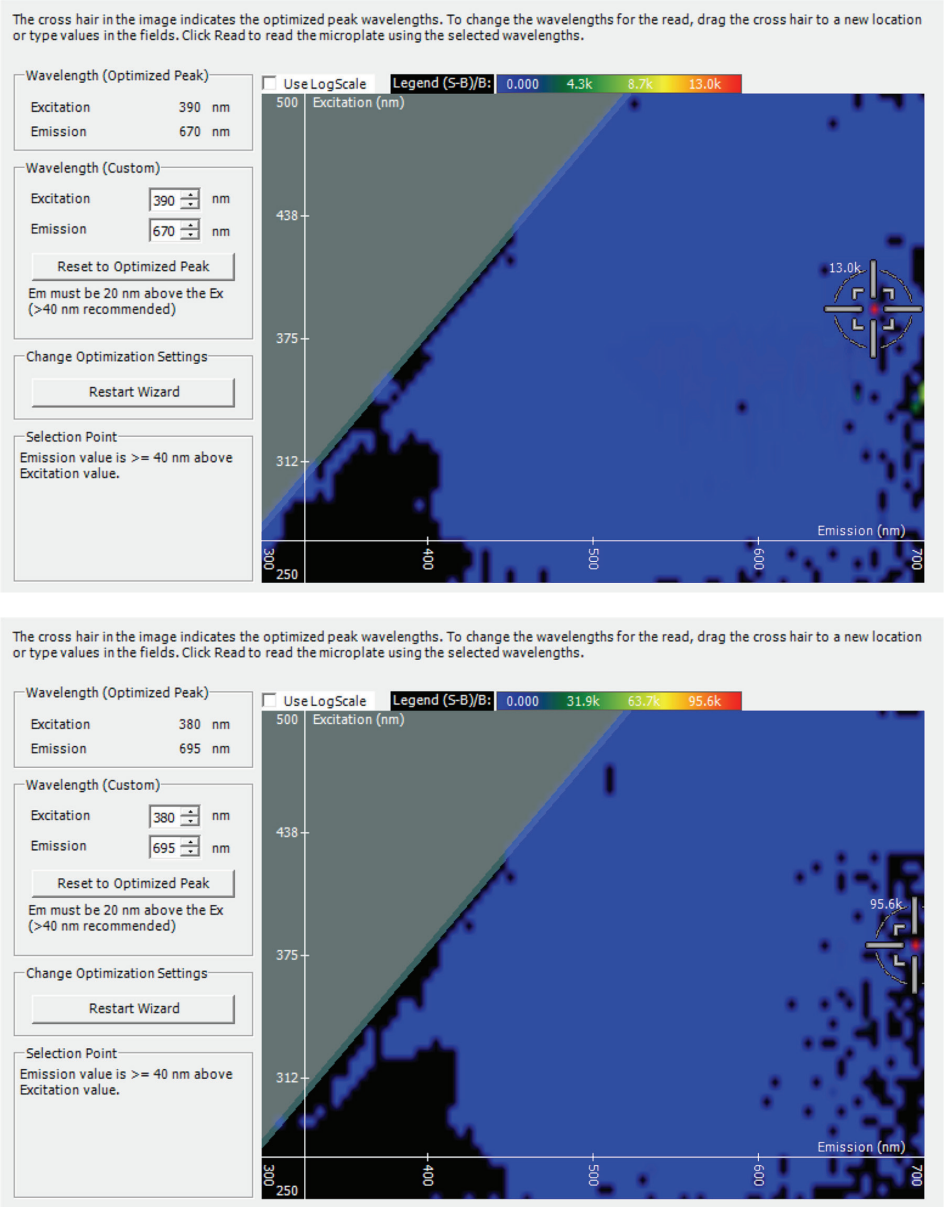

Figure 4. Spectral signature of ZnO NP (top) and mPEG surface functionalized ZnO NP (bottom). Here there was a significant shift in the optimized wavelength pair identified for the non-coated vs. coated nanoparticles.

Surface coating	Spectral signatures (Ex/Em)	
	FeO	ZnO
none	260 nm/580 nm	390 nm/670 nm
mPEG	270 nm/570 nm	380 nm/695 nm

Table 3. Spectral signatures of FeO and ZnO NP with and without mPEG. Optimized wavelength pairs obtained using the Spectral Optimization Wizard in SoftMax Pro Software are shown. With the addition of mPEG, FeO NP only have a small shift in spectral signature, while ZnO NP exhibit a more robust 25 nm shift with mPEG addition.

of interaction as the electronic properties of the surface atoms are altered due to binding in the presence of mPEG polymer. The enhanced fluorescence of the coated nanoparticles is indicative of interaction of the polymer with oxygen molecules within the material. Table 3 summarizes the spectral signatures obtained.

Conclusion

The 20 nm shift in excitation and emission wavelengths indicates electrostatic interaction and complexation between ZnO NP and mPEG polymer, which was not observable with Fe_2O_3 NP in the presence of mPEG. This characterization of nanoparticle and its surface interactions can be detected using the SpectraMax i3x Multi-Mode Microplate Reader and the Spectral Optimization Wizard in SoftMax Pro Software. This technique allows for quality control and assurance when performing surface functionalization of nanoparticles. Additionally, this technique can be extended to assure correct product formation during drug development utilizing nanoparticles as a delivery vehicle of targeting therapeutic compounds.

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