A Multimodal Nanocomposite for Biomedical Imaging

Aiguo Wu\textsuperscript{a,b}, Tatjana Paunesku\textsuperscript{a,c}, Zhuoli Zhang\textsuperscript{c}, Stefan Vogt\textsuperscript{d}, Barry Lai\textsuperscript{d}, Jörg Maser\textsuperscript{d}, Vahid Yaghmai\textsuperscript{c}, Debiao Li\textsuperscript{c}, Reed A. Omary\textsuperscript{c}, and Gayle E. Woloschak\textsuperscript{a,c}

\textsuperscript{a}Department of Radiation Oncology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611 USA

\textsuperscript{b}Division of Functional Materials and Nanodevices, Ningbo Institute of Materials Technology and Engineering, Chinese Academy of Sciences, No. 519 Zhuangshi Rd. Zhenhai District, Ningbo City, Zheijang Province, 315201 P.R. China

\textsuperscript{c}Department of Radiology, Feinberg School of Medicine, and Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL 60611 USA

\textsuperscript{d}X-Ray Operations and Research Division, Advanced Photon source, Argonne National Laboratory, Argonne, IL, 60439 USA

Abstract

A multimodal nanocomposite was designed, synthesized with super-paramagnetic core (CoFe\textsubscript{2}O\textsubscript{4}), noble metal corona (Au), and semiconductor shell (TiO\textsubscript{2}). The sizes of core, core-corona, and core-corona-shell particles were determined by TEM. This multimodal nanocrystal showed promise as a contrast agent for two of the most widely used biomedical imaging techniques: magnetic resonance imaging (MRI) and X-ray computed tomography (CT). Finally, these nanocomposites were coated with a peptide SN-50. This led to their ready uptake by the cultured cells and targeted the nanocomposites to the pores of nuclear membrane. Inside cells, this nanocomposite retained its integrity as shown by X-ray fluorescence microscopy (XFM). Inside cells imaged by XFM we found the complex elemental signature of nanoconjugates (Ti-Co-Fe-Au) always co-registered in the 2D elemental map of the cell.

Keywords

multimodal nanoconjugate; Magnetic Resonance Imaging; X-ray Computed Tomography; X-ray fluorescence microscopy

INTRODUCTION

The number of different nanocomposites used in fields as diverse as materials science, electronics, chemistry, physics, biology, and biomedicine has been rapidly increasing over the last several years. In the biomedical field, nanoparticles are most frequently used for magnetic resonance imaging. Superparamagnetic iron nanoparticles (SPIOs) and ultrasmall superparamagnetic iron nanoparticles (USPIOs) are currently in use in the clinical magnetic resonance imaging (MRI) practice, improving the image contrast in cancer and heart disease diagnostics procedures.\textsuperscript{1} Moreover, new applications for USPIOs are considered and developed through experimental MRI research. At the same time, the X-ray computed
tomography (CT) field of medical practice still relies exclusively on the natural contrast of tissues. Of late, MRI and CT techniques are frequently used as complementary imaging methods, especially in the field of interventional radiology. With the rapid increase of clinical applications of interventional radiology comes the idea that an integrated contrast agent suitable for both CT and MRI would be a great aid in biomedical imaging and minimally invasive transcatheter interventions. Since “single component” nanoparticles have already found their place in the clinic, a multicomponent nanomaterial could perhaps fulfill that role.

Nanocomposites can combine diverse chemical and physical properties—optical, superparamagnetic and electrical, etc. as determined by their chemical components, shapes, and sizes. Here, we discuss preparation and imaging properties of such a multimodal nanomaterial—a nanocomposite with superparamagnetic core (CoFe$_2$O$_4$), metal corona (Au), and optical semiconductor shell (TiO$_2$), and present cellular uptake of such nanoparticles functionalized by attachment of peptide molecules attached to the nanoparticle surface.

**EXPERIMENTAL SECTION**

**Preparation of Nanoconjugates**

**Preparation of CoFe$_2$O$_4$ Superparamagnetic Core Nanoparticles**—The procedure used was a modification of the methods employed previously by others. Sixty mL of 100mM aqueous solution of Co(NO$_3$)$_2$•6H$_2$O and 60 mL of 200 mM aqueous solution of Fe(NO$_3$)$_3$•9H$_2$O were mixed with 120 mL of water. 60 mL of 300 mM citric acid was quickly added into the as-mixed solution with vigorous stirring at room temperature. The stoichiometry needed for formation of the CoFe$_2$O$_4$ magnetic nanocrystals was maintained by keeping the ratio [Co(II)]/[Fe(III)]=1:2. After 1 hour incubation a burnt orange color solution was obtained. All chemicals came from Sigma.

**Preparation of CoFe$_2$O$_4$@Au Core-Shell Nanocomposites**—10 mM HAuCl$_4$·3H$_2$O was added to as-prepared CoFe$_2$O$_4$ nanoparticle solution volume to volume and diluted 50X with water with vigorous stirring. Stabilization of the Au corona was done by 30 mM citric acid injection. The ratio of the [HAuCl$_4$]/[Citric Acid] was 1:3. The color of this new solution color became wine red in the course of 20 min incubation at 60–75 °C.

**Preparation of CoFe$_2$O$_4$@Au@TiO$_2$ Core-Corona-Shell Nanocomposites**—As-prepared CoFe$_2$O$_4$@Au nanocomposites were diluted with water in ratio 3: 1 and mixed gradually (in ratio 50:1) with a 0.01M TiCl$_4$ solution in 20% HCl with vigorous stirring. Color of the final solution became transparent and clear. Prior to use with cells in culture these nanocomposites were dialyzed against 10mM sodium phosphate buffer of neutral pH.

**Preparation of Peptide Conjugated Nanocomposites**—100 µL of nanocomposites were mixed with 100 µL of SN-50 peptide (Calbiochem) dissolved in water at a concentration of 50ng/µL. This peptide [amino acid sequence H-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Ala-Pro-Val-Gln-Arg-Gln-Lys-Leu-Met-Pro-OH], is known to be taken up by the cells on its own, reaching the pores of the nuclear
membrane where it becomes permanently attached. The presence of hydroxyl groups on the peptide was sufficient to provide binding of peptides to the nanoparticle surface, since TiO$_2$ nanoparticulate surfaces have great affinity for binding hydroxyl groups.

**Cell Culture and Nanoparticle Treatment**

Human prostate cancer cell line PC3M (American Type Culture Collection) was grown in 5% CO$_2$ in RPMI1640 media supplemented with 10% fetal bovine serum, with addition of antibiotic and antimicotic. The cells were grown to 80% confluence and the complete growth cell medium was replaced with serum-free medium 16 hours prior to treatment with nanoparticles. Nanocomposites surface coated with the SN-50 peptide were added to the media for 1 hour. At the conclusion of experiment cells were washed with acidic glycine, harvested and applied onto TEM grids as described before.

**Transmission Electron Microscopy (TEM)**

A JEOL 1220 transmission electron microscope at Northwestern University Cell Imaging Facility was operated at 60 kV to collect the images. Alternatively, we used a JEOL JEM-2100F fast TEM: Analytical Scanning Transmission Atomic Resolution (A STAR) electron microscope, with a high-brightness Schottky FEG emitter operated at 200kV at NUANCE center at Northwestern University.

**Magnetic Resonance Imaging (MRI) and X-Ray Computed Tomography (CT)**

For MRI, 1ml each of pure water as a reference (negative control) or nanocomposites in aqueous solution were placed in Eppendorf tubes, and the tubes placed in a floating rack in a water containing box which served as a phantom. All MRI measurements were performed at Northwestern’s Center for Advanced Magnetic Resonance Imaging core facility using a 1.5T Siemens Sonata system. A four channel head coil was used for signal reception and body coil for excitation. T1 of each sample was measured using an inversion recovery sequence with repetition time (TR) = 4000 ms and inversion times of 50, 200, 500, 800, 1000, 1200, 1500, and 2000 ms. Other scan parameters were: field of view = 125 \times 200 mm$^2$; acquisition matrix = 115 \times 192; readout bandwidth = 965 Hz/pixel; slice thickness = 5 mm; all images were acquired in the coronal orientation.

For X-ray computed tomography, 1 ml aliquots of nanoconjugate colloids or water as a reference solution were placed into wells of a 24 well of tissue culture plate. The CT scanning was done with Siemens equipment at the CT center of Northwestern Memorial Hospital.

**X-Ray Fluorescence Microscopy (XFM)**

XFM studies were performed at the 2-ID-D beamline at the Advanced Photon Source (APS) at Argonne National Laboratory. An undulator source was used to generate hard x-rays with energy of 10 keV. A single bounce Si $<$111$> monochromator was used to monochromatize the X-rays; they are then focused to a beam spot of minimum 0.3\times0.2 \mu m using a Fresnel zone plate with focal length of 12.9 cm. Characteristic X-ray induced X-ray fluorescence was detected using an ultra-LEGe energy dispersive detector (Canberra, Meriden, CT). Elemental maps were acquired by raster-scanning the sample through the focal spot, and
acquiring a fluorescence spectra at each point. The fluorescence spectra were fitted with modified Gaussians corresponding to X-ray fluorescence lines. Quantification was done by comparison of normalized fluorescence counts from the sample to NBS thin film standards 1832 and 1833 (NIST, Gaithersburg, MD). Elemental quantification and co-localization of elemental signals was performed using MAPS program. 11

RESULTS AND DISCUSSION

Sizing of nanoparticles was done by transmission electron microscopy (TEM) (Figure 1). A droplet with 5 to 10 µL of each nanoparticle was deposited onto copper mesh grids with carbon film coating, dried and washed thoroughly before imaging. Using the approach described in the methods we created multicomponent nanocomposites with CoFe₂O₄ core of about 2 nm, covered with gold to create core-corona nanoparticles 16 nm in size and finally covered with TiO₂ shell leading to the final nanocomposite size of 20 nm.

The nanocomposite colloid was then investigated as a contrast agent for MRI (Figure 2a) and CT (Figure 2b). We anticipated that the CoFe₂O₄ core portion of the nanocomposite would serve as an MRI contrast agent. 12 T1 MRI imaging was chosen as the approach because it has been noted that USPIOs can be used both for T1 and T2 weighted imaging. 13 Images were acquired in the coronal orientation (Figure 2a). Significant signal lightening was noted only in those cases where nanocomposites were used. Water and a purely TiO₂ nanoparticle solution showed a dark signal, not significantly different than that of the water phantom.

Within the CoFe₂O₄@Au@TiO₂ nanocomposite the core has the potential to serve as an MRI contrast agent, while gold corona was expected to have high absorbance for X-ray CT beam. It is generally considered that the high density of gold atom electron cloud makes Au a potential CT contrast agent. 14 As shown in Figure 2b, water as a reference material had CT absorbance of about 40, while nanocomposite solutions had a CT signal of up to 230.

Dialyzed nanoconjugates were coated with the SN-50 peptide as described and used for treatment of PC3M prostate cancer cells in vitro. The N-terminal fibroblast growth factor motif of this peptide makes it cell-permeable, its nuclear localization sequence goes to but does not leave the pore complex of the nuclear membrane. 8 Therefore, dynamics of uptake of this peptide is rapid and nanoparticle treatment of cells lasted no more than 1 h. At that time cells were washed with glycine as described, 10 harvested by trypsinization and placed on formvar-coated gold mesh EM grids. 15,16 Fixed and dried cells were imaged first by visible light microscopy, then by X-ray fluorescence microscopy (XFM). Once coarse scans provided information on the cell position, high resolution scans were performed and elemental maps of cells recorded. The image of one such cell is shown in Figure 3a. In this case, raster scanning of the sample was done with 0.4 micrometer steps and complete elemental spectra were recorded for each pixel of the scan. XFM is a technique which allows simultaneous mapping and 2D quantification of elemental constituents of the sample. 15,16,17
In this instance, XFM provided a direct approach to map and quantify elemental content of both nanocomposites and cells that engulf them. Most abundant and biologically relevant cellular elements: P, S, Cl, K, Ca, Cu, Fe and Zn are detected by their Kα characteristic X-ray fluorescence. This is also true for cobalt, iron and titanium components of the nanocomposites, while the gold signal was derived from Mα1 shell characteristic X-ray fluorescence (Figure 3b). Since the energy level of gold Mα1 fluorescence partially overlaps with phosphorus Kα fluorescence, gold signal quantification was done only after data fitting with the standards using the MAPS program. Calculated ratios of different elements in the cell shown in Figure 3a were these, expressed in attograms/attograms Ti/P: 0.039569; Co/P: 0.000255; Fe/P: 0.023384; Co/Fe: 0.010925; Co/Ti: 0.006456; Co/Au: 0.000816; Ti/Au: 0.126407. These ratios correspond well with the stoichiometry of the nanocomposite and different thickness of core, corona and shell layers. The ratio expressed against the elemental content of phosphorus suggest avid nanocomposite uptake by PC3M cells. Finally, the fact that the elements comprising the nanocomposite in each case co-localized with each other (on a per pixel basis) suggests that the nanocomposite makeup inside cells remains the same as in solution. In other words, no dissolution of the nanocomposite components was detected by the XFM scan.

In conclusion, core-corona-shell CoFe2O4@Au@TiO2 nanocomposite has a stable intracellular composition. TiO2 shell layer can be modified by hydroxyl group carrying moieties (just like pure TiO2 nanoparticles), while the gold corona and superparamagnetic core make this nanocrystal a promising multimodal imaging agent, with potential applications not only in biomedical imaging but also in materials science, physics, chemistry, optics, and electronics.

Acknowledgments
This work was supported in part by the following NIH grants: CA107467, EB002100, P50 CA89018, U54CA119341 and by DOE FG02-04 ER 63920, the Special Support Program of President of Chinese Academy of Sciences (Grant No.:900424WP01), and Ningbo Natural Science Foundation of China (Grants No. 200901A6007019 and No. 2010A610159), Use of the Advanced Photon Source was supported by the U. S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38. We also thank both cell imaging facility center and NUANCE center in Northwestern University to provide TEM and AFM equipment and also thank Dr. Shuyou Li and Mr. Lennell Reynolds for TEM imaging help.

REFERENCES
1. Bernd H, De Kerviler E, Gaillard S, Bonnemain B. Invest Radiol. 2009; 44:336–342. [PubMed: 19661843]
2. Masotti A. Recent Pat Nanotechnol. 2010; 4:53–62. [PubMed: 20214655]
3. Yashchenok AM, Gorin DA, Badylevich M, Serdobintsev AA, Bedard M, Fedorenko YG, Khomutov GB, Grigoriev DO, Möhwald H. Phys Chem Chem Phys. 2010; 12:10469–10475. [PubMed: 20602000]
4. Song Q, Zhang ZJ. J. Am. Chem. Soc. 2004; 126:6164–6168. [PubMed: 15137781]
5. Cannas C, Falqui A, Musiniu A, Peddis D, Piccaluga G. J. Nanoparticle Res. 2006; 8:255–267.
6. Frens G. Nature. 1972; 241:20–22.
7. Kormann CD, Bahnemann W, Hoffmann MR. J. Phys. Chem. 1988; 92:5196–5201.
8. Lin YZ, Yao SY, Veach RA, Torgerson TR, Hawiger J. J Biol Chem. 1995; 270:14255–14258. [PubMed: 7782278]
9. Michelmore A, Gong WQ, Jenkins P, Ralston J. Phys. Chem. Chem. Phys. 2000; 2:2985–2992.
10. Thurn KT, Paunesku T, Wu A, Brown EM, Lai B, Vogt S, Maser J, Aslam M, Dravid V, Bergan R, Woloschak GE. Small. 2009; 5:1318–1325. [PubMed: 19242946]

11. Vogt S, Maser J, Jacobsen C. Journal De Physique IV. 2004; 104:617–622.

12. Lee J-H, Huh Y-M, Jun Y-W, Seo J-W, Jang J-T, Song H-T, Kim S, Cho E-J, Yoon H-G, Suh J-S, Cheon J. Nature Medicine. 2007; 13:95–99.

13. Tang TY, Patterson AJ, Miller SR, Graves MJ, Howarth SP, U-King-Im JM, Li ZY, Sadat U, Young VE, Walsh SR, Boyle JR, Gaunt ME, Gillard JH. Neuroradiology. 2009; 51:457–465. [PubMed: 19300987]

14. Hainfeld JF, Slatkin DN, Focella TM, Smilowitz HM. British Journal of Radiology. 2006; 79:248–253. [PubMed: 16498039]

15. Paunesku T, Rajh T, Wiederrecht G, Maser J, Vogt S, Stojicevic N, Protic M, Lai B, Oryhon J, Thurnauer MC, Woloschak GE. Nature Mater. 2003; 2:343–346. [PubMed: 12692534]

16. Paunesku T, Vogt S, Lai B, Maser J, Stojicevic N, Thurn KT, Osipo C, Liu H, Legnini D, Wang Z, Lee C, Woloschak G. Nano Letters. 2007; 7:596–601. [PubMed: 17274661]

17. Paunesku T, Vogt S, Maser J, Lai B, Woloschak G. J Cell Biochem. 2006; 99:489–502.
FIGURE 1.
TEM Characterization of core nanoparticles, core-corona nanoparticles and core-corona-shell nanocomposites. (a) TEM micrograph of the prepared CoFe$_2$O$_4$ nanoparticles shows that most of them have approximately 2 nm diameter. (b) TEM micrograph of the prepared core-shell of CoFe$_2$O$_4$@Au nanocrystals with 16 nm diameter. (c) TEM micrograph of the prepared core-corona-shell of CoFe$_2$O$_4$@Au@TiO$_2$ nanocrystals with 20 nm diameter. There were some unreacted core-shell nanocrystals or newly produced TiO$_2$ nanocrystals in the TEM images. Scale bar represents 50nm in each case.
Multimodal nanocomposites increase imaging contrast with biomedical scanning devices. (a) $T_1$-weighted MRI images of two batches of 20 nm of core-corona-shell CoFe$_2$O$_4$@Au@TiO$_2$ (spots 2 and 3). Pure water was used as a reference (spot 4). Additional negative controls were 6 nm of TiO$_2$ nanoparticles (spots 1 and 5). (b) CT images of nanocomposites. First well contained water as a reference, second and third wells contained two different batches of core-corona-shell CoFe$_2$O$_4$@Au@TiO$_2$ nanocomposites.
FIGURE 3.
X-ray fluorescence imaging of cells carrying multimodal nanocomposites. (a) XFM maps of PC3M cells transfected with the core-corona-shell nanocomposites made of CoFe$_2$O$_4$@Au@TiO$_2$ and coated with the peptide SN-50. Please note that the P signal “covers” the Au signal in the 2D map image. However, after fitting it has been found that the XFM signal ratio of Ti:Au:Fe:Co is 50:1000:70:1. (b) XFM spectra of the various elements of PC3M cells and nanocomposites. The elements detected by K alpha X-ray fluorescence are highlighted by the light blue arrows; Au signal coming from M shell fluorescence is labeled with a dark blue arrow.