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A Scanning Transmission Electron Microscopy (STEM) Approach to Analyzing Large Volumes of Tissue to Detect Nanoparticles

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Abstract

The use of nanoparticles for the diagnosis and treatment of cancer requires the complete characterization of their toxicity, including accurately locating them within biological tissues. Owing to their size, traditional light microscopy techniques are unable to resolve them. Transmission electron microscopy provides the necessary spatial resolution to image individual nanoparticles in tissue but is severely limited by the very small analysis volume, usually on the order of tens of cubic microns. In this work we developed a scanning transmission electron microscopy (STEM) approach to analyze large volumes of tissue for the presence of polyethylene glycol coated Raman-active-silica-gold-nanoparticles (PEG-R-Si-Au-NPs). This approach utilizes the simultaneous bright and dark field imaging capabilities of STEM along with careful control of the image contrast settings to readily identify PEG-R-Si-Au-NPs in mouse liver tissue without the need for additional time consuming analytical characterization. We utilized this technique to analyze 243,000 μm^3 of mouse liver tissue for the presence of PEG-R-Si-Au-NPs. Nanoparticles injected into the mice intravenously via the tail-vein accumulated in the liver while those injected intrarectally did not, indicating that they remain in the colon and do not pass through the colon wall into the systemic circulation.

Introduction

Over the past few years nanotechnology has become a powerful tool in the development of novel techniques to diagnose and to treat cancer (Farrell et al., 2010; Ptak et al., 2010). These technologies have encompassed a wide variety of materials and approaches including iron oxide (Devaraj et al., 2009) and gadolinium (Liang et al., 2011) nanoparticles as MRI contrast agents, carbon nanotubes for surface enhanced Raman spectroscopy (SERS) imaging (Liu et al., 2010), gold nanoparticles for SERS imaging (Keren et al., 2008; Kircher et al., 2012; Kneipp et al., 2009; Thakor et al., 2011a, 2011b; von Maltzahn et al., 2009; Zavaleta et al., 2009), liposomes for drug delivery (Ferrari, 2005), and gold nanoshells for

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photothermal ablation therapy (Loo et al., 2005). While these approaches vary greatly they have two important aspects in common. First, they each utilize nanoscale materials and second, each is designed to be used in-vivo. The small size of these nanoparticles, which gives rise to the unique properties for which they were selected, raises concerns that they may interact negatively with the surrounding biological matter (Chang et al., 2006; Panessa-Warren et al., 2006). Before these novel technologies can be safely utilized to diagnose or treat cancer it is necessary to accurately determine accumulation within the body and their potential toxicity effects.

Raman-active-silica-gold-nanoparticles (R-Si-Au-NP) are very promising for use in SERS imaging to diagnose and locate cancer (Thakor et al., 2011a). They consist of a Raman organic molecule adsorbed onto the surface of a 60 nm diameter gold core providing a surface enhancement effect that can result in Raman signal intensities many orders of magnitude higher than those obtained for the Raman molecule alone (Mulvaney et al., 2003). This core is then encapsulated in a 30 nm thick silica shell to prevent desorption of the Raman molecule as well as providing a surface that can be functionalized. The active component of the nanoparticle is the 60 nm gold core with the adsorbed Raman molecule. R-Si-Au-NPs, figure 1, are currently demonstrating great promise as a diagnostic tool for colorectal cancer. To this end, an extensive study was undertaken by Thakor et al to determine if R-Si-Au-NPs coated with polyethylene glycol (PEG-R-Si-Au-NP) injected intrarectally into mice caused toxicity in the colon and whether the PEG-R-Si-Au-NPs passed through the colon wall and entered the blood stream (Thakor et al., 2011a). To study the latter, a second experiment was performed whereby the PEG-R-Si-Au-NPs were injected intravenously via the tail-vein (Thakor et al., 2011a). Toxicity and biodistribution were determined by analyzing a wide range of parameters over a two week period including clinical observation, cardiovascular measurements, detailed post-mortem histological evaluation, inductively coupled plasma mass spectrometry, gene expression and scanning transmission electron microscopy (STEM) (Thakor et al., 2011a, 2011b, 2011c).

Traditional toxicity studies provide a depth of information about the interactions of the nanoparticles with the surrounding tissue but lack the spatial resolution to study individual nanoparticles and their interactions within a biological system. Owing to its high spatial resolution electron microscopy has proven to be a useful tool to characterize not only such nanoparticles but their interactions with the system as well (Koh et al., 2008). Other spectroscopic and imaging techniques can provide evidence as to the presence of nanoparticles but only through electron microscopy can we definitively analyze their accumulation and spatial distribution within tissue. Electron microscopy analysis of tissue samples for nanoparticle localization can provide detailed information that in conjunction with standard cytotoxicological testing creates a clear and accurate picture of the toxicity and distribution of nanoparticles. As a component of this large toxicity study, in which all tissues from the mouse were examined, electron microscopy was employed to analyze the accumulation and distribution of PEG-R-Si-Au-NPs in the liver of intrarectally or tail-vein injected mice. The liver was selected for detailed analysis because the PEG-R-Si-Au-NPs circulating in the blood were expected to naturally accumulate in macrophages lining the liver sinusoids, as this is their primary route of excretion (Thakor et al., 2011a) making this the best option for determining whether PEG-R-Si-Au-NPs had passed into the systemic circulation after intrarectal injection.

The analysis volume for transmission electron microscopy (TEM) is historically low, with estimates of only 0.6 mm³ of material being examined since the advent of TEM in the 1930's (Williams & Carter, 2009). Typically, the analysis volume for a single TEM session is between 1 and 10 μm³. This limitation, combined with the possibility of low nanoparticle concentrations in-vivo, requires a new technique capable of imaging relatively "large"

volumes of tissue quickly and efficiently. Scanning transmission electron microscopy (STEM) was selected for this approach because it offers a number of advantages over traditional TEM. It is possible to collect both bright and dark field images simultaneously and to easily control the dynamic range of the detectors during sample analysis in STEM. Both of these features simplify the process of identifying PEG-R-Si-Au-NPs and are not available in traditional TEM analysis. In this work we developed a new scanning transmission electron microscopy (STEM) approach that allows for the fast examination of large tissue sections for the presence of PEG-R-Si-Au- NPs.

Materials and Methods

Mouse and Tissue Preparation

Five week old Friend virus B-type mice (N=120), half male and half female were selected for this toxicity study because of their fully competent immune systems (Thakor et al., 2011a). To determine if PEG-R-Si-Au-NPs pass through the colon wall, 30 mice were injected intrarectally with a 200 μ l solution containing 9.6×10^{10} PEG-R-Si-Au-NPs. The R-Si-Au-NPs, batch S440, were obtained from Oxonica Materials Inc. (now owned by Cabot Corporation) at a stock concentration of 0.8 nM. The nanoparticles were then coated with PEG and prepared for injection as described by Thakor et al (Thakor et al., 2011a). To study the effects of systemic circulation of the nanoparticles, 30 mice were tail-vein injected with the same dose of PEG-R-Si-Au-NPs. Thirty mice were set aside as control groups for both the intrarectally and tail-vein injected mice. These control groups were injected with 200 μ l doses of saline solution to mimic any injection specific reactions. At specific time points post injection; 5 minutes, 2 hours, 24 hours, 1 week and 2 weeks, six mice were euthanized via carbon dioxide asphyxiation from each mouse group injected with PEG-R-Si-Au-NPs as well as the control mouse groups (Thakor et al., 2011a).

During pathologic examination small samples, $\sim 1 \text{ mm}^3$, of liver, spleen, colon, small intestine, kidney, lung, brain and gonad were collected and immediately fixed in a solution of 2.5% glutaraldehyde 2% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4 (EMSdiasum) for 1 hour at room temperature. Samples were then stored at 4°C until further processing could be performed, up to two weeks. A total of 900 tissue samples were obtained from the 120 mice and fixed for further sample preparation, though only a small subset of these were selected for imaging using STEM.

STEM sample preparation

All 900 samples extracted from the mice were preserved and prepared following the procedure described below to ensure that any observations made during pathological examination could be further analyzed using STEM. Tissue samples were trimmed to less than 1 mm^3 with a razor blade and placed in a solution of 1% osmium tetroxide in water for two hours at 4°C (EMSdiasum). Samples were then rinsed twice in cold doubly de-ionized water and suspended in a solution of 1% uranyl acetate in water at 4°C overnight (EMSdiasum).

Samples were rinsed twice more in doubly de-ionized water to remove any residual heavy metal stains and dehydrated in increasing concentrations of ethanol; 50, 75 and 95% at 4°C in 15 minute intervals. Following the last treatment, samples were allowed to come to room temperature where they were further dehydrated twice in 100% ethanol. The samples were then suspended three times at 10 minute intervals in propylene oxide (EMSdiasum) to complete the dehydration and ensure the removal of any residual water.

After dehydration the samplers were embedded in Embed 812 (EMSdiasum), a standard electron microscopy embedding resin. The samples were suspended in a 1:1 solution of

embed 812 and propylene oxide for 1 hour followed by re-suspension in a 2:1 solution of embed 812 and propylene oxide overnight. The following morning the samples were incubated in pure embed 812 resin for 1 hour before being placed in block molds set in an oven at 60°C to cure overnight.

Owing to the time involved in analyzing samples in the STEM, a small subset of the 900 original samples was selected for further preparation and analysis. Liver tissue from a male and female mouse at each time point for both the intrarectally and tail-vein injected subgroups was selected for further analysis totaling 20 samples. The liver was selected because the PEG-R-Si-Au-NPs circulating in the blood were expected to naturally accumulate in macrophages lining the liver sinusoids, for the reasons cited earlier (Thakor et al., 2011a). The liver sample blocks were trimmed using a razor blade to expose a small flat surface of the tissue. 150 nm thick sections were cut from the block face using an ultramicrotome (Leica Ultracut S) equipped with a glass knife. This thickness was selected to minimize the risk of tearing out the 120 nm PEG-RSi-Au-NPs during sectioning. These sections were then placed on a 200 mesh bare copper grid (Ted Pella) with a hole width of 90 μm .

Scanning Transmission Electron Microscopy Setup

The samples were imaged using a Tecnai F20 X-Twin TEM operated in scanning transmission electron microscopy mode at 120 kV with an electron probe size and current of 1 nm and 1 nA respectively. The probe size and current were selected to obtain the necessary resolution and signal to noise to successfully image the nanoparticles. The dynamic range for the bright field STEM detector was calibrated at the beginning of each session using the copper cross bars of the TEM grid. The range was set such that white corresponded to the electron intensity after the beam passed through 150 nm of tissue and black corresponded to no electron intensity, caused by the electron opaque copper cross bar. Tissue sections were scanned using a dwell time of 0.8 μs per pixel resulting in a very fast refresh rate of approximately 0.2 s per frame, typical scan rates are 10–20 μs per pixel. Samples were then scanned rapidly with these settings to look for PEG-R-Si-Au-NPs. When PEG-R-Si-Au-NPs were located, annular bright and dark field image pairs were recorded for later analysis. The bright field and dark field detectors had collection angles of 21.5 mrad and from 21.5 to 61.3 mrad respectively, lower than the 50 mrad minimum threshold for high angle annular dark field imaging (Williams & Carter, 2009). As a result, these were not “Z-contrast images”. Ten grid openings were selected randomly and analyzed for each sample resulting in a total of 12150 μm^3 of tissue analyzed per sample and a total of 243,000 μm^3 for the whole set. This is three to four orders of magnitude larger than typical volumes analyzed in the TEM. The analysis usually took between three and four hours per sample to complete.

Results

STEM characterization of PEG-R-Si-Au-NPs in Mouse Liver Tissue

For the liver samples from the mice injected intrarectally no PEG-R-Si-Au-NPs were found anywhere in the analyzed volume of tissue. The total volume for an average mouse liver is approximately 1500 μl (Inderbitzin et al., 2004) or $1.5 \times 10^{12} \mu\text{m}^3$. From this 12150 μm^3 or $8.1 \times 10^{-5}\%$ of the tissue was examined using STEM for each mouse. This is a very small fraction of the total liver volume, but was necessary due to time constraints. Representative bright field images of the liver tissue taken at each time point are shown in figure 2 indicating no sign of the PEG-R-Si-Au-NPs.

As expected, PEG-R-Si-Au-NPs were located in the liver of each of the tail-vein injected mice. As the PEG-R-Si-Au-NPs pass through the sinusoids of the liver, macrophages remove the nanoparticles from the blood causing accumulation of the PEG-R-Si-Au-NPs. Table 1 shows the number of nanoparticles found at each time point (N=2) as well as their concentration. Assuming the average liver volume above, the initial PEG-R-Si-Au-NPs dose corresponds to a concentration of 0.064 nanoparticles per μm^3 in the liver. Using this value the percentage of administered nanoparticle dose in the liver at each time point was determined in Table 1, being as high as 51% of the original dose after 2 hours. This is a rough estimate due to variations in liver volume between mice.

Five minutes post tail-vein injection the PEG-R-Si-Au-NPs were largely located within the sinusoids of the liver with a few nanoparticles located within vesicles within the macrophages, figure 3. After two hours the majority of the nanoparticles had been taken up by macrophages and resided in vesicles within the liver, as shown by the representative image in figure 4(b). This trend continued at the 24 hour, 1 week and 2 week time points with the PEG-R-Si-Au-NPs still located largely within vesicles in the macrophages of the liver, figure 4(c–e). The nanoparticle concentration decreased over time starting at two hours as shown in table 1 and figure 5. These results are limited however because there were only two data points collected at each time point and may not be taken as statistically significant.

PEG-R-Si-Au-NPs were generally found in clusters as seen in figures 3 and 4. These clusters occurred at every time point and ranged from 1 to over 70 nanoparticles. The average number of nanoparticles per cluster varied from 9 ± 11 to 18 ± 17 PEG-R-Si-Au-NPs for the samples at 5 minutes and 1 week respectively as shown in table 1.

Scanning Transmission Electron Microscopy Technique

The PEG-R-Si-Au-NPs were readily identifiable even with the relatively short pixel dwell time of 0.8 μs . A standard search mode dwell time is between 10 and 20 μs per pixel. Individual samples were generally analyzed in 3 – 4 hours. Analysis of all 20 samples took approximately 70 hours of microscope time. The gold core of the nanoparticles appear very dark in the bright field image and dark with a white halo surrounding it in the dark field image as shown in figure 6. This very specific imaging pair allows for ready identification of the PEG-R-Si-Au-NPs without the use of any analytical technique (although the energy dispersive x-ray spectrum was checked on occasional samples). In addition to the gold core, the silica shell surrounding the nanoparticle is visible in each image. This is not universally the case for TEM imaging of these nanoparticles as shown in figure 7, where the silica shell is not at all visible in the representative bright field TEM image.

One important artifact needs to be carefully protected against while performing STEM on these samples. When an image is acquired, and the beam is not properly blanked, burn-in spots can on occasion form on the image as shown in figure 8(b). Figure 8 shows an annular dark field image of a burn-in spot that formed after the beam was allowed to dwell on one spot for just 10 seconds. This spot very closely resembles the nearby PEG-R-Si-Au-NPs and it was necessary to ensure that the spots do not form by immediately blanking the beam after image acquisition.

Discussion

STEM characterization of PEG-R-Si-Au-NPs in Mouse Liver Tissue

The lack of PEG-R-Si-Au-NPs in the liver of the intrarectally injected mice indicates that the nanoparticles did not cross through the colon wall and pass into the bloodstream (Thakor et al., 2011a). The liver as the primary filter for the circulatory system was selected for analysis because any PEG-R-Si-Au-NPs in the blood should arrive there and be filtered out,

as demonstrated by the high concentration of PEG-R-Si-Au-NPs in the tail-vein injected samples. The lack of any PEG-R-Si-Au-NPs in the liver after intrarectal administration indicates that the nanoparticles are not crossing the colon wall and can be utilized in a topical application inside the colon for colorectal cancer detection without likely concern for systemic toxicity. Similar results were also observed in the spleen, with nanoparticles being detected in the spleens of the tail-vein injected mice and not in the spleens of the intrarectally injected mice. It is important to observe that in the study performed by Thakor et al, trace amounts of gold were detected in blood of one intrarectally injected mouse at 5 minutes.

As noted in the results for the tail-vein injected mice, the concentration of PEG-R-Si-Au-NPs decreased over time starting after two hours. Owing to the limited data (N=2) any observable trends in the data have no statistical significance. A more complete and longer term study is needed to determine the fate of PEG-R-Si-Au-NPs that arrive in the liver. Clustering of the nanoparticles inside vesicles in the liver was noted in each tail-vein injected sample at each time point. This clustering was likely due to phagocytosis of the nanoparticles in the sinusoids, although no observations have been made to rule out aggregation prior to phagocytosis. Macrophages would phagocytose large numbers of these nanoparticles resulting in the formation of clusters.

Scanning Transmission Electron Technique

The STEM technique developed for this application allows for the rapid analysis of relatively large volumes of liver tissue for the presence of PEG-R-Si-Au-NPs. This was accomplished by carefully controlling both the samples and the microscope to optimize the conditions for locating the PEG-R-Si-Au-NPs in the tissue. STEM has a number of benefits for analyzing such large volumes. The very short dwell time allowed for rapid refresh rates, making it possible to very quickly scan through large areas of tissue for the presence of the PEG-R-Si-Au-NPs. While this technique made it possible to scan through large areas of tissue quickly, it is still not reasonable to analyze entire livers using STEM. Given the size of the liver at $1.5 \times 10^{12} \mu\text{m}^3$ and the rate of approximately 3.5 hours to analyze $12150 \mu\text{m}^3$ of tissue, it would take over four hundred million hours to analyze a total single liver.

The PEG-R-Si-Au-NPs are still visible at this short dwell time due to the high atomic number of the gold cores. The latter scatters or absorbs the electron beam very strongly resulting in very dark circles relative to the surrounding. By calibrating the dynamic range, it is possible to distinguish between nanoparticles and any salt crystals that form during the sample preparation process. Salt crystals scatter lightly and as a result appear as grey in the bright field image instead of black as is the appearance of the PEG-R-Si-Au-NPs.

The PEG-R-Si-Au-NPs are also readily identifiable in the dark field image by the bright halo surround a dark core as shown in figure 6. This halo effect is the result of the angle of collection for the annular dark field detector. Under traditional high angle annular dark field imaging using collection angles greater than 50 mrad, the gold core would appear as bright because the electron beam would be scattered to very high angles due to the high atomic number of the gold. Because we are only collecting up to 61.3 mrad here, many of these electrons are not collected and, as a result, at the thickest regions of the nanoparticle the electrons are scattered at too high an angle for detection, thus resulting in a dark core. At the edges of the nanoparticle the gold is much thinner and scatters to a lower angle resulting in a bright halo surrounding the core. This distinctive structure also allowed for easy identification and non-analytical confirmation that the dark regions in the bright field images are indeed PEG-R-Si-Au-NPs.

Under the STEM imaging conditions utilized in this experiment, the silica shell surrounding the gold core the nanoparticles is readily visible. This is due to the difference in structure between the stained tissue and the unstained amorphous silica resulting in slight variations in electron scattering. In traditional fixed beam bright field TEM imaging the silica shell is not always visible as shown in figure 7. This likely occurs because both materials are amorphous with similar densities making it difficult to distinguish between the two. This has led to numerous questions regarding the stability of the silica shell on the PEG-R-Si-Au-NPs. With the silica shell clearly visible in the STEM images these questions are readily answered and are no longer an issue.

The STEM controls, in particular the ability to change the raster direction and link this to the translate joystick, allows the TEM grid opening to be aligned with the left-right sample translations. This simple yet important feature enables more efficient analysis of the samples, speeding up and ensuring the completeness of the data acquisition.

Comparison with Other Techniques

There are a number of analytical techniques capable of quantifying the total amount of gold in a small tissue sample down to the pictogram level including inductively coupled plasma mass spectrometry and instrumental neutron activation analysis. These techniques however do not provide any information about the location and distribution of the nanoparticles within the tissue. The STEM technique developed in the work can provide detailed information about the inter-and intra-cellular location of the PEG-R-Si-Au-NPs. This approach is semi-quantitative and additional sample analysis is necessary to determine the minimum analysis volume needed to obtain accurate nanoparticle quantification and to validate the method for objectives beyond descriptive applications. Point counting would be one method to improve the statistical analysis, however due to the low volume fraction of nanoparticles in the liver it would require in excess of 10,000 points to achieve a standard error of 100% of the mean (Weibel, 1979). The STEM technique is complementary to bulk analytical techniques and by combining them it would be possible to obtain accurate quantification with information about nanoparticle location.

Conclusions

Scanning transmission electron microscopy (STEM) is a powerful tool for the analysis of large tissue samples, like liver tissue, for the presence of PEG-R-Si-Au-NPs. STEM allows for the fast and efficient examination of large volumes of tissue owing to the high mass contrast of gold nanoparticles obtained even at very fast scan rates. With this method over 243,000 μm^3 of liver tissue was investigated in the current study. Including other tissues analyzed with this technique, over 500,000 μm^3 of mouse tissue has been analyzed. The ability to obtain simultaneous bright and dark field images while setting the dynamic range of the detectors enabled us to distinguish between nanoparticles and artifact salt particles quickly and efficiently. These two parameters were essential to the success of this approach and our ability to readily locate and identify nanoparticles in the biological system. The STEM technique developed in this work provides an efficient approach towards analyzing large volumes of tissue for the presence of nanoparticles, and although not quantitative, is readily adaptable for other systems.

STEM analysis of liver tissue for the presence of PEG-R-Si-Au-NPs provides detailed information about the accumulation and uptake of the nanoparticles by the liver, improving upon the information obtained through standard toxicological studies. Mice tail-vein injected with PEG-R-Si-Au-NPs were found to have a high number of nanoparticles in the liver, starting in the sinusoids before being taken up in vesicles by macrophages. The PEG-R-Si-Au-NPs were largely found in clusters that vary greatly in number size from 1 to over 70

with an average of about 13 nanoparticles. Mice injected intrarectally did not have any PEG-R-Si-Au-NPs located in the liver at any time point. This shows that the PEG-R-Si-Au-NPs injected into the colon are not passing through the wall into the blood stream. This result is very promising for the use of these nanoparticles in a topical application in the colon. STEM analysis provides a level of detail regarding the localization of PEG-R-Si-Au-NPs that is not achievable through other analysis techniques.

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Figure 1. TEM bright field images of R-Si-Au-NPs. The 60 nm gold core appears dark due to high electron scattering and absorption and is surrounded by a 30 nm thick silica shell. Scale = 100 nm and 25 nm respectively

Figure 2. Bright field STEM images from liver samples of mice injected intrarectally at (A) 5 min, (B) 2 hours, (C) 24 hours, (D) 1 week and (E) 2 weeks respectively showing a lack of PEG-R-Si-Au-NPs at each time point. Cell nuclei are marked by single arrows and some vesicles are marked by double arrows. Scale = 1 μ m

Figure 3. STEM (A) bright and (B) dark field image pair of liver tissue from a male mouse 5 minutes post tail-vein injection showing PEG-R-Si-Au-NPs marked by single arrows. The bottom most cluster of nanoparticles is located within the sinusoid, the oval shaped feature in the bottom half of the image. Both the middle and upper most clusters of nanoparticles are located within vesicles marked by double arrows. Scale = 500 nm

Figure 4.

STEM bright field images of PEG-R-Si-Au-NPs in liver tissue from a male mouse at (A) 5 minutes, (B) 2 hours, (C) 24 hours, (D) 1 week and (E) 2 weeks post intravenous injection showing nanoparticles in vesicles, marked with double arrows, inside the cytoplasm of the macrophages. The material surrounding the vesicles in the images are largely composed of the cytoplasm. Scale = 200 nm

Figure 5.

Graph showing the concentration of nanoparticles in the liver tissue as a function of time post tail-vein injection. Due to the limited data (N=2) any observable trends in the data have limited statistical significance. No conclusions regarding the change in concentration over time are made from these limited data.

Figure 6.

(A) Bright field and (B) dark field images of PEG-R-Si-Au-NPs in liver tissue from a male mouse 24 hours after tail-vein injection. The gold core appears dark in the bright field image and dark with a bright halo surrounding it in the dark field image, indicative of the PEG-R-Si-Au-NPs. Also note the clearly visible silica shell surrounding the PEG-R-Si-Au-NPs in the dark field image. The PEG-R-Si-Au-NPs are located within a vesicle, indicated by the double arrow, surrounded by the cytoplasm within a macrophage in the liver. Scale = 200 nm

Figure 7. Bright field TEM image of PEG-R-Si-Au-NPs in liver tissue two hours post tail-vein injection where the silica shell is not clearly visible. The vesicle surrounding the nanoparticles is marked with a double arrow. Scale = 200 nm

Figure 8.
(A) Annular dark field image showing PEG-R-Si-Au-NPs in liver tissue from a male mouse two hours post tail-vein injection marked with a double arrow. After allowing the beam to dwell in a single spot for 10 seconds a burn in artifact (B) closely resembling a PEG-R-Si-Au-NP forms, marked with a single arrow. Scale = 300 nm

Table 1

Nanoparticle concentration

	5 min	2 hr	24 hr	1 wk	2 wk
# of PEG-R-Si-Au-NPs	755	788	444	438	358
PEG-R-Si-Au-NP Concentration (#/μm ³)	0.031	0.032	0.018	0.018	0.014
% of PEG-R-Si-Au-NP dose in liver	48.5%	50.7%	28.5%	28.1%	23.0%
Average # of PEG-R-Si-Au-NPs per cluster	9±11	15±16	14±14	18±17	17±17

The number of PEG-R-Si-Au-NPs found in 12,500 μm³ of liver from each of two animals (total volume analyzed = 24,300 μm³), the concentration of nanoparticles, the percentage of administered dose and the average number of PEG-R-Si-Au-NPs per cluster with standard deviation for tail-vein injected mice at each time point (N=2).