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14. ABSTRACT Targeted imaging agents use specific biomarkers that are present in tumor tissue to distinguish cancerous cells from their immediate benign environment. They are able to provide both structural and functional characteristics of the tumor such as shape, size, growth rate and expression level of cell-surface makers. The research aims to design an imaging platform using targeted gold (Au) nanoparticles (NP) to accurately determine the level of HER2 density in solid tumors. During the past year, I have focused my efforts on developing the imaging platform needed to optimize the visualization of the nanoparticles. The work has led me to better understand the mechanics of dual-energy x-ray imaging, and to look into the use of other metal nanoparticles such as silver. I have presented here my findings related to using dual-energy computed tomography to quantify the concentration of AuNP, as well as spectral optimization using a monoenergetic simulation. Additionally, I have included results from my attempt to attach the targeting ligand and fluorescent agent to the surface of the particles.					
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1. Introduction

Targeted imaging agents use specific biomarkers that are present in tumor tissue to distinguish cancerous cells from their immediate benign environment. They are able to provide both structural and functional characteristics of the tumor such as shape, size, growth rate, and expression level of cell-surface markers. Today, the most commonly used x-ray contrast agents are iodine-based compounds¹. However, the non-specific nature of these agents results in random vascular permeation, rapid renal clearance, and poor tumor-targeting potential.

The research aims to design an imaging platform using targeted gold (Au) nanoparticles (NP) to accurately determine the level of HER2 density in solid tumors. The nanoparticles will be synthesized in-house, and functionalized with an anti-HER2/neu affibody (for targeting) and a polyethylene glycol moiety (for stabilization). HER2/neu is a cell surface receptor protein that is overexpressed in roughly 25-30% of all breast cancers^{2,3}. The success of any therapeutic agent targeted to HER2 will depend greatly on the accurate assessment of the level of HER2 density in tumors. The long-term goal of this project is to develop a system that can identify those patients who will benefit the most from HER2-targeted therapies, and consequently reduce unnecessary side-effects, avoid false negatives, and ultimately improve patient survival rates.

2. Body

2.1. Research Overview

The initial research plan consisted of three major subsections:

- (i) Synthesize, functionalize and concentrate AuNP
- (ii) Characterize the structural and radiographic properties of the AuNP.
- (iii) Evaluate the *in vivo* effect of the nanoparticles: tumor-enhancement, biodistribution, and toxicity

In the first year from Feb '09 to Feb '10, I successfully synthesized spherical AuNP using a modified Turkevich method. The AuNP were then surface stabilized (s-AuNP) using a heterobifunctional polyethylene glycol (PEG) chain, shown to enhance stealth characteristics and improve the circulation of nanoparticles *in vivo*. The physical characteristics (diameter, size distribution, zeta potential) of the AuNP agent had been determined through transmission electron microscopy (TEM), dynamic light scattering (DLS), and UV/Vis spectroscopy.

In the second year of the grant (from March 2010 to March 2011), I refined the techniques needed to image and synthesize the gold nanoparticles for the final testing in animals. I developed a spectral simulation platform that allows for the simulation of mammographic spectra with any kVp and filter combination the user desires. Furthermore, initial testing of s-AuNP was conducted in immunocompromised mice. The particles showed no visible signs of toxicity and could be seen successfully by a clinical mammographic unit when injected into the stomach cavity of the mice.

In this final year (from March 2011 to March 2012), I focused my efforts on developing the imaging platform needed to optimize the visualization of the nanoparticles. The work has led me to better understand the mechanics of dual-energy x-ray imaging, and to look into the use of other metal nanoparticles such as silver. I have presented here my findings related to using dual-energy computed tomography to quantify the concentration of a gold nanoparticle agent, as well as simulations on monoenergetic optimization. Additionally, I have included results from my attempt to attach the targeting ligand and fluorescent agent to the surface of the nanoparticles.

I am now entering the final year of my PhD. I have completed all the necessary coursework, and have met with my committee to discuss my research progress. The research that I have completed for the duration of this grant will form the majority of my final thesis dissertation and has been instrumental in my training as a graduate student. I have presented my work at several conferences over the past year and I hope to publish my findings before I graduate.

Comparison between the spectra of the product and the starting compound shows that one of the peaks corresponding to a CH₂ group has shifted. The chemical shifts for the product are consistent with those for 3-azidopropionic acid present in the literature.

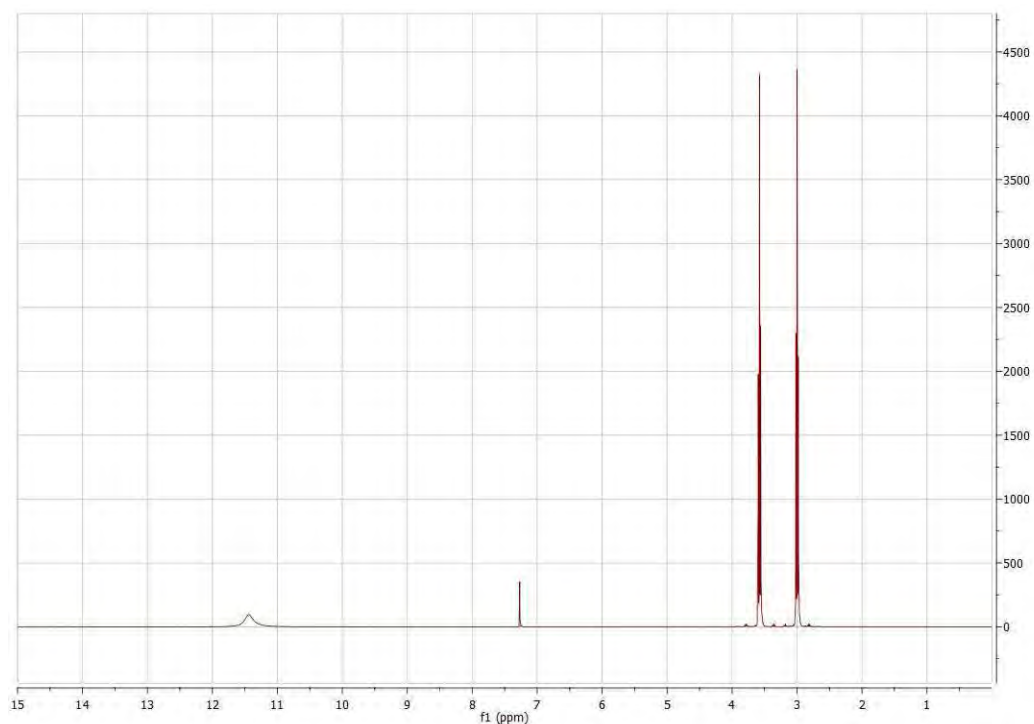


Figure 2. H-NMR spectrum for the starting material: 3-bromopropionic acid. The peaks obtained (2.59 – 2.63, 3.55 – 3.59) are consistent with the spectrum provided by the supplier.

The product was isolated by rotor evaporation. The solid was then dissolved in deionized (DI) water and purified using a Amico Ultra 3K Ultracell centrifugal filter (14,000 xg for 10 minutes, repeated 3 times) to remove any unwanted byproducts or unreacted materials of the reaction.

We are currently attempting to verify that the product is thiol-PEG-azide using matrix-assisted laser desorption/ionization (MALDI).

2.2.2. Contrast-enhanced imaging

Contrast-enhanced dual-energy (DE) x-ray imaging provides a technique to increase the contrast of radiographic imaging agents by suppressing the variation in signal between various tissue types. In the breast, this involves the suppression of the signal variation between admixtures of glandular and adipose tissue. By reducing the effect of this “anatomical noise”, it is then possible to more accurately segment and quantify the signal from the contrast agent. Dual-energy imaging utilizes two distinct energy windows (low- and high-) to quantify the variation in attenuation with energy. To achieve a suitable contrast between imaging agent and tissue, it is therefore necessary that their respective attenuation profiles do not follow the same general trend from low- to high- energy.

Initial Studies with Gold Nanoparticles using computed tomography

Initially, we explored the feasibility of using DE computed tomography (CT) to distinguish and quantify the concentration of AuNP agents. The dual-energy decomposition technique introduced by Kelcz *et al.*⁴ was used to estimate the concentration of gold at each pixel location using a linear combination of the low- and high-energy images.

$$\begin{aligned}
 CT^{low} &= \frac{1000}{\mu_w^{low}} \left[M_{cont}^{low} \cdot \rho_{cont} + M_{tissue}^{low} \cdot \rho_{tissue} \right] \\
 CT^{high} &= \frac{1000}{\mu_w^{high}} \left[M_{cont}^{high} \cdot \rho_{cont} + M_{tissue}^{high} \cdot \rho_{tissue} \right] \\
 \Gamma^j &= \mu_w^j \cdot CT^j / 1000 \\
 \rho_{cont} &= \Gamma^{low} - \Gamma^{high} \left(\frac{M_{tissue}^{low}}{M_{tissue}^{high}} \right) \bigg/ M_{cont}^{high} \cdot \left(\frac{M_{cont}^{low}}{M_{cont}^{high}} - \frac{M_{tissue}^{low}}{M_{tissue}^{high}} \right)
 \end{aligned}$$

Equation 1. Dual-energy computed tomography decomposition equations introduced by Kelcz *et al.*

Where CT is the CT number in Hounsfield units at a given pixel location, M is the mass attenuation coefficient (cm^2/g) of contrast (cont) or background material (tissue), the superscripts of *low* and *high* representing the two energy windows, ρ is the density (g/cm^3) of the material.

Imaging was performed on a Scanco Medical vivaCT 40 micro-CT system at two beam energies – 45 and 70 kVp. A calibration phantom was first used to determine the M coefficient of gold using these two

spectra. The phantom consisted of a 15 mL centrifuge tube that contained a solidified mixture of 4% gelatin in water. A micropipette tip was then inserted into the gelatin, and filled with a known concentration of gold (0.25 to 50 mg Au/mL).

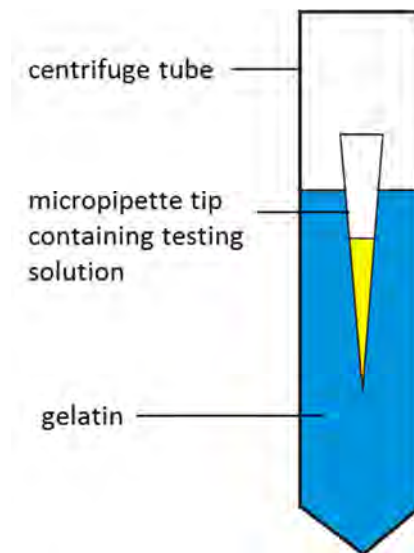


Figure 5. Calibration phantom used for DE gold imaging with CT.

The phantom was then imaged at the two energies, and the normalized (against water) linear attenuation coefficient (LAC) was plotted as a function of the concentration of gold. The slope of the linear fit connecting the data points represents the M coefficient for gold at that energy. Since we could not image the gelatin at various densities, a region of interest was chosen at each energy bin that encompassed the gelatin, and its mean normalized LAC value was assigned as the M coefficient. The final results are tabulated in

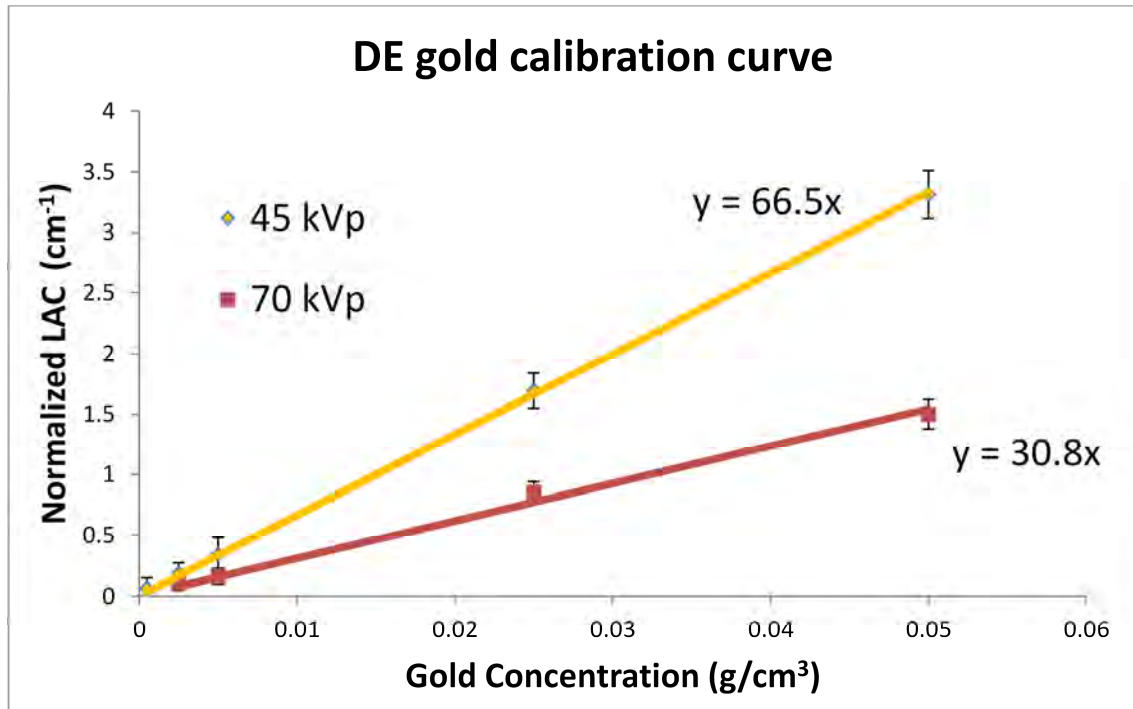


Figure 6. Gold calibration curve at two energy windows (45 and 70 kVp). Normalized LAC is plotted against the concentration of gold in g/cm^3 . The slope of the linear fit represents the M coefficient at that energy.

Table 2. M coefficients calculated at the low- and high- energy for gelatin and gold.

	M^{low}	M^{high}
Gel	0.72	0.54
Gold	66.5	30.8

The DE-CT decomposition method was testing using AuNP whose concentration was previously determined using ICP-MS (inductively-coupled plasma mass spectrometry). AuNP of two concentrations were placed in the micropipette tip of the testing phantom and imaged using 45- and 70- kVp beams. The concentration of gold in the nanoparticle solutions was then determined using the formulas in Equation 1. The final results are presented in Table 3 and a sample dual-energy image is shown in Figure 7. The DE-CT decomposition technique was able to accurately estimate the concentration of gold nanoparticles embedded in a test phantom to within 5%.

Table 3. Concentration of the AuNP (in mg Au/mL) as determined from ICP-MS and DE-CT decomposition.

AuNP Concentration (mg Au/mL)	
From ICP-MS	From DE-CT
17.9	18.9
5.9	5.5

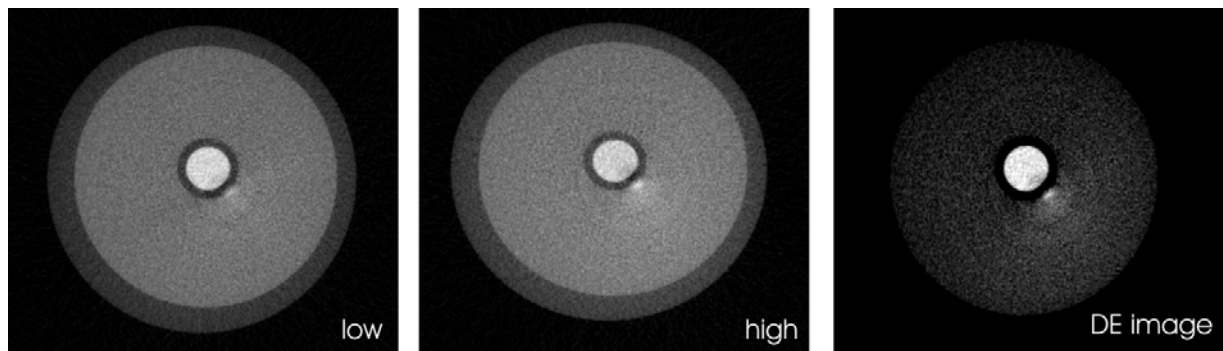


Figure 7. DE-CT decomposition of a solution of AuNP within the test phantom. The DE image in this case represents the concentration of gold at each pixel location.

These initial results support the hypothesis that we can use DE-CT to quantify the concentration of a gold nanoparticle agent injected into a living animal.

Technical characterization of a contrast-enhanced digital breast tomosynthesis system

A second generation contrast-enhanced (CE) digital breast tomosynthesis (DBT) unit was recently installed at the hospital at the University of Pennsylvania. The system, based on the Hologic Dimensions platform, acquires 22 images in approximately 7.3 seconds. During one sweep of the x-ray tube, low energy (LE) and high energy (HE) images are acquired alternately, resulting in a total of 11 HE and 11 LE images. The system alternates the applied voltage and the x-ray filter between two preset values, which are determined from the breast thickness.

My research involved providing a calibration tool which can be used to quantify the concentration of iodine in CE-DBT patient images. A 3D physical anthropomorphic breast phantom that was previously designed and built in our lab was used in this calibration. The phantom consists of several section consisting of adipose- and fibroglandular- like materials which can then be stacked to provide a breast-like imaging tool. One of the sections has been replicated to contain iodine-enhancing lesion compartments. There are 10 compartments in total, with areal concentrations of iodine ranging from 1.03 mg I/cm² to 4.83 mg I/cm².

The phantom was imaged using the CE-DBT Hologic machine at thicknesses ranging from 1 to 9 cm. For each concentration of iodine, a contrast in digital values (DV) was calculated as

$$Contrast = SI_{iodine} - SI_{background}$$

These data points were fit to a line, and plotted for each thickness. Representative results from the 1 and 9 cm thickness cases are shown in Figure 8. The slope of the linear relationship can be used as a calibration tool to quantify the areal density of iodine ($\text{mg I}/\text{cm}^2$) for that breast thicknesses (see . Figure 9)

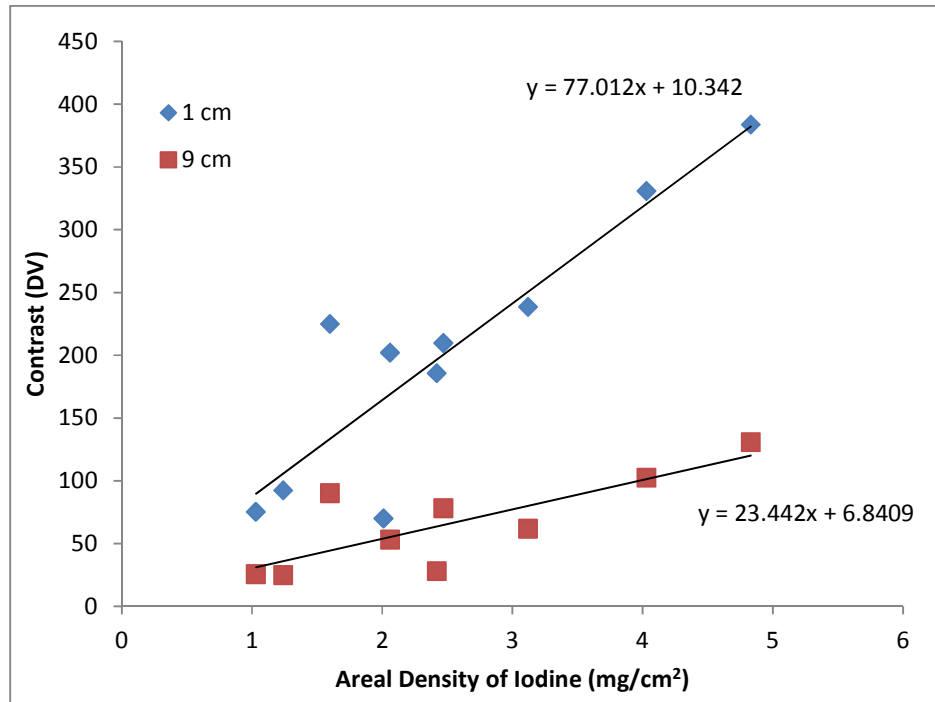


Figure 8. Contrast of iodine lesions plotted against the areal density of iodine. The slope of the linear fits can be used as a calibration tool to quantify the concentration of iodine in DE-DBT images.

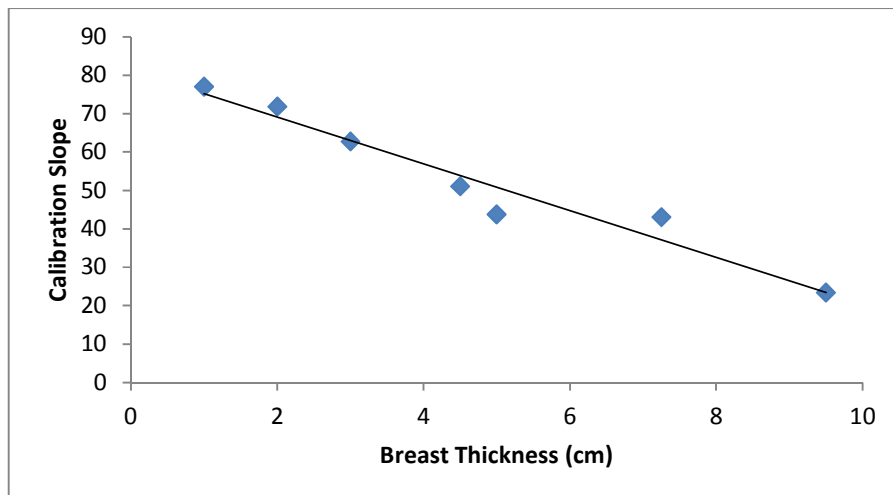


Figure 9. Calibration slope for various breast thicknesses.

This calibration tool was then used on DE-DBT patient images to obtain quantitative iodine concentration maps at the various time points.

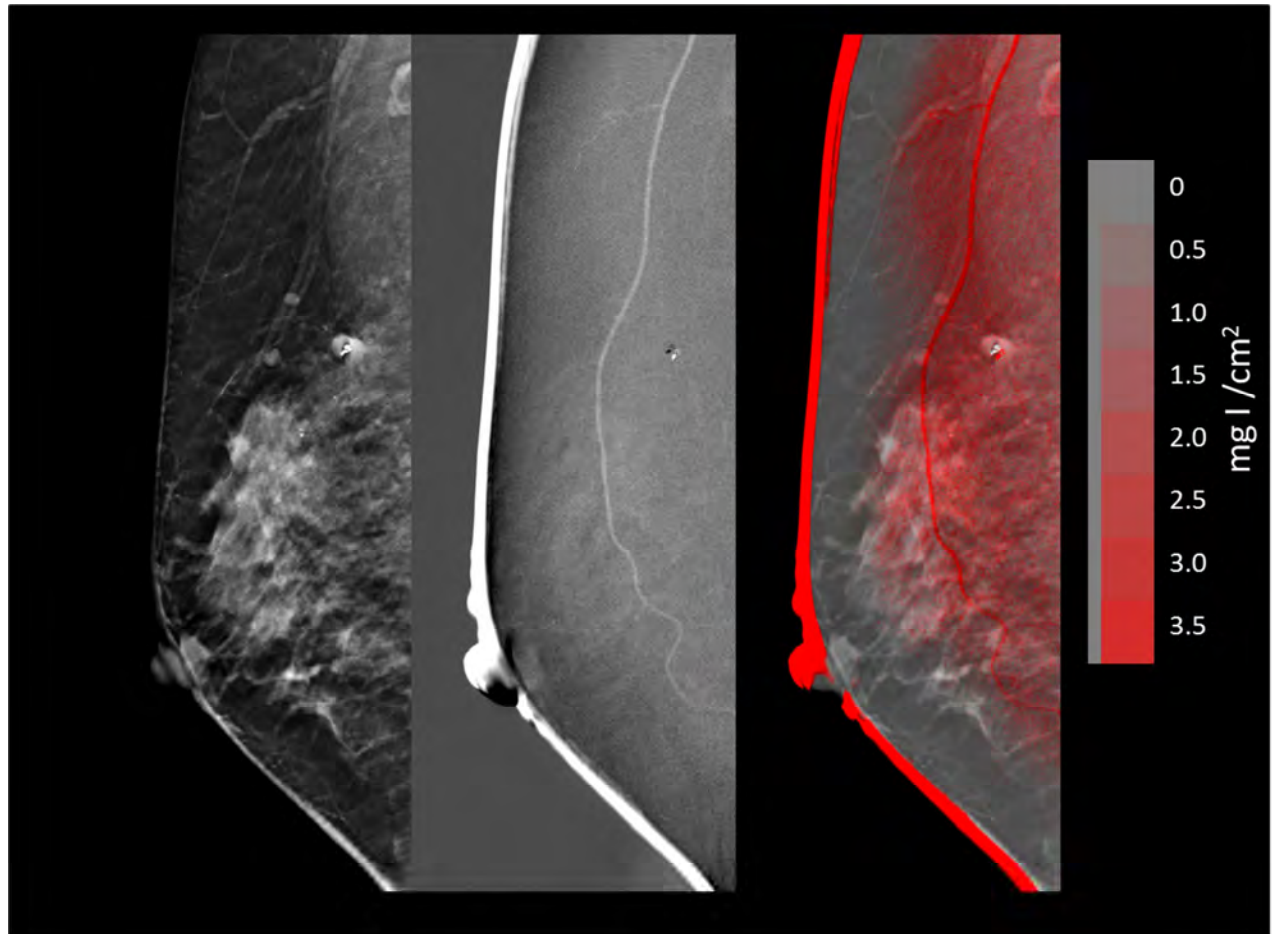


Figure 10. Iodine concentration maps of a DE-DBT patient image obtained after intravenous injection of a iodinated contrast agent. Shown are sample (left) low energy, (middle) dual-energy subtraction, (right) low-energy with iodine map overlay.

Please note that patient imaging was conducted as part of another IRB-approved grant not related to this predoctoral award. These images only serve to illustrate how the dual-energy calibration tool that I developed could be used clinically.

Attenuation coefficient analysis – Dual Energy

A monoenergetic analysis was performed to identify the combinations of low (LE) and high (HE) energies that would maximize the difference in linear attenuation coefficients (LAC) of tissue and gold. LAC were calculated for various admixtures of glandular and adipose tissues ranging from 0 to 100% glandular. Separately, the LAC was calculated for a 50% glandular, 50% adipose composite with increasing concentrations of gold. Mass attenuation coefficients needed for this calculation were obtained from the NIST XCOM online physics database⁵. Energy pairs ranging from 20 to 110 keV (in 1 keV intervals) were studied. For each energy pair, two-dimensional maps of linear attenuation coefficients for tissue

were calculated in terms of glandularity and concentration of gold. Linear relationships were observed for both variables. The metric R was defined as the angular separation between these two linear fits.

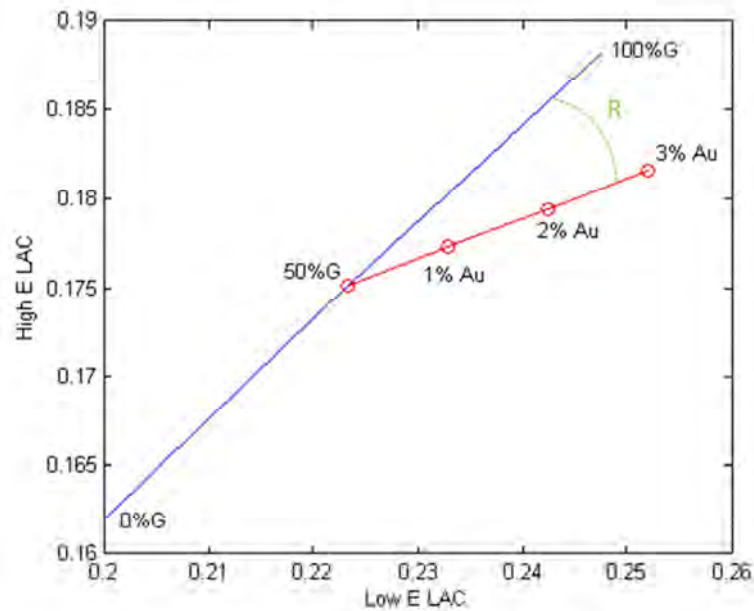


Figure 11. Two dimensional map of linear attenuation coefficient (LAC) for variations of glandularity (G) and concentration of gold (Au). The metric R was defined as the angle between the two linear fits.

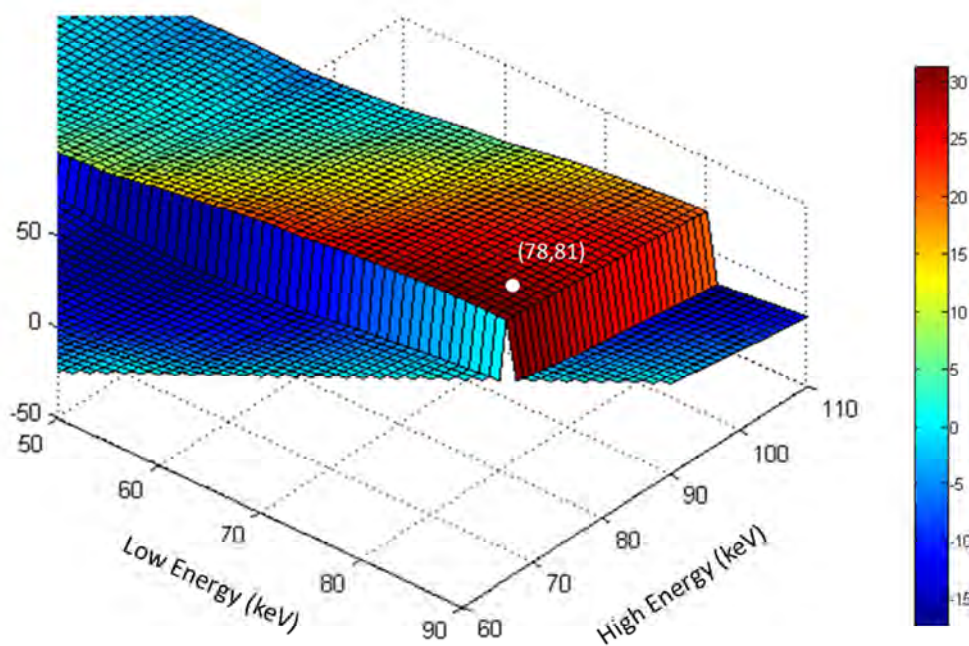


Figure 12. Surface plot of R for various combinations of low- and high- energy pairs. A maximum occurs at (78.81) keV with a value of 31°.

An energy pair of (78,81) keV was identified to maximize R using a gold contrast agent. However, the gradual decrease in R as the low energy decreases means that it may be possible to achieve suitable distinction between tissue and gold even with more clinically feasible energy pairs such as (60, 90) keV.

3. Key Research Accomplishments

Much of my work in the past year has been geared towards developing the imaging platform needed to optimize detection of contrast material in breast x-ray imaging. More specifically, it has involved detailed analysis into the mechanics of dual-energy x-ray imaging. I have shown over the past year, that dual-energy computed tomography can be used as a non-invasive imaging modality to quantify the concentration of a gold nanoparticle agent injected into a phantom. Additionally, I have developed a calibration tool that can allow for the quantification of an iodinated contrast agent in dual-energy digital breast tomosynthesis images. This tool has been used in patient images to create iodine concentration maps that provide an insight into the distribution kinetics of the imaging agent at various time points post-injection.

Monoenergetic analysis of the attenuation coefficients have led us to begin experimentation with new nanoparticle agents – such as silver (Ag). Ag represents an attractive contrast material for breast tomosynthesis and mammography, as its k-edge (26 keV) lies within clinically-used mammographic energies. Thus, the optimal energies that maximize R (discussed earlier) can be achieved more readily with these techniques. This work prompted us to pursue and eventually receive a DOD-funded Idea Award to investigate the use of silver as a dual-energy imaging agent for breast x-ray imaging.

4. Reportable Outcomes

- The quantification of a gold nanoparticle agent using dual-energy computed tomography was reported at the 2011 Annual World Molecular Imaging Conference (WMIC) in San Diego as a poster:

P570 - Quantification of a gold nanoparticle contrast agent using dual-energy computed tomography, Roshan Karunamuni, Andrew Maidment.

- The work related to the technical characterization of the contrast-enhanced digital breast tomosynthesis unit was reported as an oral presentation at the 2011 Radiological Society of North America (RSNA) meeting in Chicago.

SSC15-06 – Technical characterization of a contrast-enhanced digital breast tomosynthesis system; R Karunamuni, S C Gavanonis, B Ren, C Ruth, A.D. Maidment

- The monoenergetic spectral optimization analysis was translated to silver, and will be reported as an oral presentation at the 11th International Workshop on Breast Imaging in July 2012.

Examination of silver as a radiographic contrast agent in dual-energy x-ray breast imaging – R Karunamuni, A Al-Zaki, A Popov, J Delikatny, S Gavanonis, A Tsourkas, A Maidment

5. Conclusions

During the past three years funded by this predoctoral award, we have extensively studied the use of gold nanoparticles as imaging agents for use in breast x-ray imaging. The nanoparticles were synthesized

in-house and surface-stabilized to allow for biocompatibility. The nanoparticles were fully characterized in terms of their size, shape and surface properties. The x-ray attenuation properties of the particles was investigated in a variety of digital mammography systems and x-ray spectroscopy experiments. The nanoparticles were injected into immunocompromised mice and detected using a clinical digital mammography system. The particles showed no visible signs of toxicity in the animals.

Much of the recent trend in digital mammography (DM) and breast tomosynthesis (DBT) has been towards contrast-enhanced dual-energy imaging. The x-ray attenuation properties of gold lead it to be more beneficial to higher energy x-ray imaging modalities such as computed tomography where the mean energy of the x-ray beam falls closer to the k-edge of gold (80 keV). This has led us to begin investigation into other materials that share some of the same characteristics as gold, but whose attenuation properties better match the energies used in DM and DBT – such as silver. Silver lies within the same group as gold in the periodic table, and thus shares many of the properties of gold explored in this grant. The synthesis and stabilization steps reported here, can be directly transferred to a silver nanoparticle agent.

The experience, techniques and understanding of breast imaging that I have gained during this predoctoral award has been invaluable. The development of silver nanoparticles as a dual-energy breast x-ray imaging represents a natural progression of the work that I have completed. Our belief is that the development of dual-energy imaging agents will lead to gaining more useful functional information during a breast x-ray examination, which in turn will lead to more accurate patient diagnosis.

6. References

1. Rode, U. and R. Muller, Transformation of the ionic X-ray contrast agent diatrizoate and related triiodinated benzoates by *Trametes versicolor*. *Appl Environ Microbiol*, 1998. 64(8): p. 3114-7
2. Baselga, J., A.D. Seidman, P.P. Rosen, et al., HER2 overexpression and paclitaxel sensitivity in breast cancer: therapeutic implications. *Oncology (Williston Park)*, 1997. 11(3 Suppl 2): p. 43-8.
3. Baselga, J., D. Tripathy, J. Mendelsohn, et al., Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer.[see comment]. *Journal of Clinical Oncology*, 1996. 14(3): p. 737-44.
4. Kelcz *et al.* Noise considerations in dual energy CT scanning, *Med Phys*, 6(5): Sept 1979
5. National Institute of Standards and Technology (NIST) Physical Measurement Laboratory. *XCOM: Photon Cross Sections Database*. Retrieved December 10, 2011

7. Appendix

(none)